An essential function of sphingolipids in yeast cell division

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Summary

Ceramides are bioactive lipids and precursors to sphingolipids. They have been shown to take part in a wide variety of different physiological processes in eukaryotic organisms and are thought to be toxic at high concentrations. Ceramide is synthesized by condensation of the sphingoid base sphinganine and a fatty acyl CoA by ceramide synthases, a family of enzymes that differ in their specificity for the length of the acyl CoA substrate. We have engineered a yeast strain where the endogenous ceramide synthase has been replaced by one of the putative enzymes from cotton. As a result, the yeast strain produces C18 rather than C26 ceramides showing that the cotton protein is a bona fide ceramide synthase with specificity towards C18 acyl CoA. Strikingly, the accumulation of C18 ceramide is not toxic in Saccharomyces cerevisiae. This allows survival of the yeast after deletion of the normally essential AUR1 (inositol phosphorylceramide synthase) gene permitting us to address the essential roles of sphingolipids. Deletion of AUR1 allows cell growth, but leads to a defect in cytokinesis, which takes twice as long as in wild-type strains. Nuclear division and recruitment of septins is apparently not affected, but cytokinesis is delayed and cell separation is incomplete.

Introduction

Ceramides have been implicated in numerous processes in eukaryotic cells (Li et al., 2002; Koyanagi et al., 2003; Kitatani et al., 2008; Bartke and Hannun, 2009). The understanding of the synthesis of this lipid greatly improved with the discovery that two highly homologous genes, LAG1 and LAC1, in Saccharomyces cerevisiae (S. cerevisiae) are required for ceramide synthase activity (Guillas et al., 2001; Schorling et al., 2001). The double deletion led to reduced ceramide and sphingolipid synthesis, thicker cell walls and reduced transformability. Lag1p and Lac1p were then purified and shown to be dihydroceramide synthases (CerS) requiring another subunit, Lip1p, whose precise function is unknown (Vallee and Riezman, 2005). By sequence homology, six orthologues have been found in mammalian cells and at least one of them has been shown to be a bona fide ceramide synthase (Futerman and Riezman, 2005; Lahiri and Futerman, 2005). Moreover, each of the mammalian homologues has been shown to have a preference for specific fatty acid chain length, thus generating different ceramides (Venkataraman et al., 2002; Riebeling et al., 2003; Lahiri et al., 2007; Laviad et al., 2008). This specificity, at least to some extent, was reproduced when these genes were expressed in yeast (Guillas et al., 2003; Cerantola et al., 2007). Complementation of the double lac1Δ lag1Δ deletion has been used to show that at least two of three C. elegans Lag homologues are bona fide CerS (Menuz et al., 2009). In the same study it was proposed that differences in sphingolipid chain lengths could lead to either protection or hypersensitivity of worms to anoxia (Menuz et al., 2009). The different human homologues have also been implicated in different cancer pathways and their differential regulation can lead to apoptosis and cell death (Bose et al., 1995; Garzotto et al., 1999; Min et al., 2007; White-Gilbertson et al., 2009).

Ceramide has been suggested to be toxic in yeast because the use of aureobasidin A (AbA), a drug that prevents the synthesis of inositol phosphorylceramide (IPC) and its derivatives, leads to cell death that can be abrogated by an additional defect in lag1 and lac1 (Schorling et al., 2001; Vallee and Riezman, 2005; Cerantola et al., 2009). This situation has made it difficult to study the putative essential functions of sphingolipids because it has been impossible to inhibit their synthesis without causing cell death due to ceramide accumulation.

Here, we show that at least one of the LAG1 homologues found in cotton is a bona fide CerS, capable of rescuing the double lag1Δ lac1Δ deletion for growth and that the resulting strain produces mainly C18 sphingolipids, i.e. sphingolipids with an 18-carbon fatty acid in the ceramide moiety (for the sake of clarity, the word ceramide is
used here to represent all types of ceramide regardless of their hydroxylation or saturation status). Moreover, the accumulation of C18 ceramides does not promote cell death and does not appear to be toxic. This allows the depletion of sphingolipids from yeast cells, either by addition of AbA or by deletion of AUR1 in a GhLag1 background. The sphingolipid lacking strain is viable but shows a pronounced defect in cytokinesis. Even though abscission has not been achieved, cells continue to grow and divide through subsequent cell cycles forming a multinucleate, multicellular structure. Recruitment of septins is not affected, but they rest in place for longer than in wild-type (WT) cells and new buds stay connected to mother cells even after the separation of their cytoplasm. The strain lacking complex sphingolipids accumulates lipid bodies and the same effect is seen in WT cells treated with AbA. Proliferation of lipid bodies correlates with the accumulation of ceramides, but lipid bodies are not enriched in the major ceramide species produced.

Results

GhLag1p is a bona fide ceramide synthase

It has been shown that the double deletion of LAG1 and LAC1 in S. cerevisiae decreases the synthesis of ceramide and downstream sphingolipids and causes a severe growth defect or lethality. In our strain background the lag1Δ lac1Δ mutant grows very slowly and is thermosensitive (Fig. 1A). In order to test if one of the LAG1 homologues found in the cotton genome (GhLag1-1, GenBank JQ080174) is a bona fide CerS, we decided to introduce a codon optimized version of the coding sequence behind a TDH3 promoter into the genome of lag1Δ lac1Δ mutant. The growth of the resulting strain (which we named GhLag1) was normal at all temperatures tested (Figs 1A and 2C). This shows that the introduction of GhLag1p is able to rescue the growth defect of the lag1Δ lac1Δ mutant and its thermosensitivity.

GhLag1 strain produces mainly C18 sphingolipids

Knowing that the introduction of human LAG1 homologues into yeast changes the chain length of the sphingolipids produced according to the specificity of the heterologous CerS (Epstein et al., 2011; Jennemann et al., 2012), we decided to test which species of sphingolipids the GhLag1 strain produces. Using ESI-MS/MS we determined the relative amounts of ceramide,IPC and M(IP)2C produced by the WT and GhLag1 strains (Fig. 1B–D). As expected the WT strain produced mainly C26 sphingolipids. The GhLag1 strain however produced almost exclusively C18 sphingolipids. All ceramides, IPCs and M(IP)2Cs found in the GhLag1 strain were therefore 8 carbons shorter than the ones found in WT (an excel file with the ion counts and masses utilized for analysis can be found in Table S1). The amounts of sphingolipids, especially ceramides and to a lesser extent, IPCs, were substantially higher. Since no appropriate standards are commercially available to obtain absolute quantification of these ceramides and sphingolipids we sought another method to confirm that the GhLag1 accumulates more ceramides than wild type (WT). To this end we radiolabelled newly synthesized ceramides in WT and GhLag1 strains with 3H-sphinganine, extracted, analysed ceramides by TLC and visualized and quantified them using a phosphorimager (Fig. 1E). By this method the GhLag1 strain produced more radiolabelled ceramide than WT cells (2.9 ± 1.0-fold higher), confirming that there are more total ceramides in the engineered strain. Independent of the precise increase in total ceramide production, the GhLag1 strain provides us with a good tool to study the effects of shorter chain sphingolipids on various biological functions in yeast.

Production of C18 sphingolipids affects growth in the presence of different drugs, but does not affect endocytosis or protein localization

In order to uncover possible biological effects of different ceramide and sphingolipid chain length we tested our mutant under different conditions, some of which led to potentially interesting insights into the role of sphingolipid chain length. We first tested a series of inhibitors that affect the cell wall biosynthesis or the cell wall integrity response pathway. The addition of myriocin, a compound that inhibits the first enzyme in sphingolipid biosynthesis, serine palmitoyltransferase, and GPI-anchored protein transport (Horvath et al., 1994), strongly inhibited growth of the GhLag1 strain (Fig. S1). On plates with a low concentration of three compounds YW3548, a glycosylphosphatidylinositol synthesis inhibitor (Sutterlin et al., 1997a), a chitin synthase inhibitor, Calcofluor white (Roncero and Duran, 1985), and rapamycin, which affects the cell wall integrity response pathway (Krause and Gray, 2002), GhLag1 showed a strong growth defect. All of these inhibitors are expected to have stronger effects in mutants with defects in cell wall biosynthesis or monitoring of cell wall integrity, suggesting that the replacement of C26 sphingolipids with C18 sphingolipids affects proper formation of the cell wall. We cannot rule out that the cause of the problem might be other abnormalities in the strain, such as the elevated amounts of IPCs or ceramides, reduced amounts of free long-chain bases (LCBs) due to increased transformation into ceramides and sphingolipids, or reduced amounts of LCB-1-phosphates or a combination of these.

Since there was an apparent defect in cell wall integrity, we next tested if the change in sphingolipid chain length affected basic membrane trafficking pathways like
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A. RH 2888 (WT), RH7166 (\( \text{lag1}\Delta \text{lac1}\Delta \)) and RH6979 (\( \text{lag1}\Delta \text{lac1}\Delta \) TDH3::GhLag1::TRP1), abbreviated as GhLag1 were diluted to a final concentration of \( 1 \times 10^7 \) cells ml\(^{-1} \), and 10 \( \mu l \) of 10-fold serial dilutions were spotted on YPD plates at indicated temperatures. The plates were incubated for 3 days.

B–D. Identification and quantification by ESI-MS of the ceramides, IPC and M(IP)\(_2\)C produced in WT and GhLag1p strains. Results are presented as relative amounts (RA) normalized by the control condition that was set to 100.

E. WT and GhLag1 cells were labelled with \(^{3}H\)DHS, ceramides were analysed on TLC and detected using a phosphorimager.

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endocytosis or delivery of transmembrane proteins to the plasma membrane. We examined the ability of the GhLag1 strain to internalize Lucifer Yellow (LY), a fluid-phase endocytic marker (Riezman, 1985) and FM4-64, a membrane-bound endocytic marker (Vida and Emr, 1995) (Fig. S1B and C). In the GhLag1 strain, LY was internalized and accumulated in the vacuole as in the WT strain. FM4-64 was also internalized and after a chase localized to the vacuole membrane in both strains. Next we speculated that the cell wall defect could be due to the mistargeting of membrane proteins. We used a series of constructs in which yeast membrane proteins were cloned and tagged with fluorescent markers (Fig. S1D–G). Can1p, an arginine transporter, Hxt1p, a hexose transporter, and Mid2p, a cell wall integrity sensor, were tagged with the fluorescent protein Venus and grown to logarithmic phase. They all localized to the plasma membrane in both WT and GhLag1 strains, with some proteins also being delivered to the vacuole, presumably after endocytosis. Gap1p, a general amino acid transporter, was tagged with GFP and localized directly to the vacuole under these growth conditions. We also localized Tat2p, Sfk1p, Pma1p, Gas1p and Cwp2p

Fig. 2. The GhLag1 strain is resistant to aureobasidin A (AbA).
A. WT, lag1Δ lac1Δ and GhLag1 strains were grown on plates containing 500 ng ml⁻¹ AbA.
B. A small amount of GhLag1 was taken from plates containing AbA and replated on the plate shown below. This was done three consecutive times. WT controls were taken from a fresh culture.
C. Growth curves of WT and GhLag1 in the presence or absence of 250 ng ml⁻¹ AbA. Cultures were started at a concentration of 0.2 OD₆₀₀ and aliquots were measured at indicated times.
D and E. (D) WT was grown for 2 h and (E) GhLag1 was grown overnight on medium with or without 0.25 μg ml⁻¹ AbA. Cells were collected, treated with TCA and lipids were extracted, identified and quantified by ESI-MS. All cultures were grown at 30°C.
of AbA treated and untreated cells using ESI-MS/MS sphingolipid levels, we measured the ceramides and IPCs the resistance to AbA and its relation to ceramide and
To rule out these possibilities and to further characterize the resistance to AbA and its relation to ceramide and sphingolipid levels, we measured the ceramides and IPCs of AbA treated and untreated cells using ESI-MS/MS (Fig. 2D–E). We first looked at the WT strain after a two-hour treatment with AbA. As expected, we saw an accumulation of C26 ceramide (4.2-fold) and a strong decrease in the amount of IPC. Levels of M(IP)2C, however, remained constant. This discrepancy between the effects of AbA on IPC levels, which are strongly reduced, and on M(IP)2Cs, which are not reduced, can be attributed to the fact that IPCs not only cease to be made, but also are converted into MIPCs and M(IP)2Cs during the incubation on AbA. Next, we looked at the sphingolipid levels of the GhLag1 strain grown overnight. As expected, after overnight treatment the GhLag1 strain accumulated ceramides to levels at least threefold higher than untreated cells and showed greatly decreased amounts of IPC (less than 0.1%). As in the WT, M(IP)2C levels did not show a significant decrease. These results reinforced our suggestion that sphingolipids, at least IPCs, may not be essential for yeast growth and that the accumulation of C18 ceramide at the levels tested is not toxic for yeast. This leads us to conclude that yeast cells die when treated with AbA due to the accumulation of very-long-chain ceramides rather than the reduction of sphingolipids.

Disruption of AUR1 gene in a GhLag1 background leads to a viable strain with decreased levels of complex sphingolipids

AUR1 is one of the yeast genes responsible for catalysing the reaction that converts ceramides into IPCs. The gene is essential in yeast (Hashida-Okado et al., 1996), presumably because of both the accumulation of ceramide and the lack of sphingolipids. Our experiments showing that the GhLag1 strain is resistant to AbA, led us to re-evaluate the essential nature of the AUR1 gene in the context of the GhLag1 strain background. To address this issue, we created a diploid strain that was heterozygous for aur1Δ::URA3, lac1Δ::ADE2 and lag1Δ::HIS3 deletions and expressed the cotton CerS (GhLAG1::TRP1). After sporulation the tetrads were dissected. If the AUR1 gene is essential one expects to obtain two viable and two inviable spores from each tetrad. However, we found 3 out of 48 tetrads that had 3 viable spores. An example from a dissection plate is shown in Fig. 3A. This result supports the idea that in a normal yeast background the AUR1 gene is essential. However, in a strain that contains only short-chain ceramides, requiring co-segregation of the four different mutations, the disruption of AUR1 is not lethal. The disruption of AUR1 was confirmed by PCR. Growth of the quadruple mutant strain (termed cSLΔ strain for its lack of complex sphingolipids) is slower than the WT cells at 24°C and 30°C and no growth was detected at 37°C (Fig. 3B). ESI-MS/MS of sphingolipids of the cSLΔ strain showed an increased level of C18 cera-
mides and a decreased level of IPCs (no detection in cells grown on plates and less than 1% of the levels found in the GhLag1 mutant when cells were grown in liquid medium). No M(IP)2Cs of any chain length were detected in the cSLD strain (Fig. 3C). This result allows us to conclude that indeed sphingolipids are not absolutely essential for yeast cell viability under these particular conditions. The cSLD strain, which contains virtually no sphingolipids, allows us to address a long-standing question concerning the cellular functions of sphingolipids.

Lack of sphingolipids leads to a defect in cytokinesis

Above we noticed that, in liquid medium, the GhLag1 strain grew only at lower AbA concentrations than on solid medium. We explored whether a similar phenotype could be found for the cSLD strain. As for the GhLag1 strain treated with AbA, the cSLD strain showed strongly impaired growth in liquid media when agitated. We observed that the problem was due to the agitation and not to the medium being liquid, because cSLD cells grew in liquid medium without agitation (Fig. S2A). When grown in liquid at 30°C without agitation, the cSLD strain was able to grow from 0.2 to approximately 5 OD600 units ml−1. If the same amount was grown with agitation the maximum OD600 reached was 1 OD600 units ml−1 in the best experiments. Agitating the flasks after an adaptation period of 6 hours only helped to a certain extent (Fig. S2A). Analysis of DIC images of cells grown under these conditions showed that mother and daughter cells seemed to remain connected and that agitation might confer a mechanical stress that the resultant connections could not withstand (Fig. S2B).

To understand this phenomenon we followed growth and cell division. Cells were grown on a layer of solid rich medium and observed under a bright field microscope. A montage for each strain is shown in Fig. 4A–C. In WT and GhLag1 strains, cells divide and move aside to give rise to new buds only when the previous division is finished (Movies S1 and S2). After approximately 4 h WT and GhLag1 cells have divided 4 times and have new buds (Fig. 4A and B). When we observed cSLD cells, the final stages of cytokinesis seemed to be defective. The central cell takes 9 h to produce 4 buds (while the WT and GhLag1 took only 4 h), and the daughter cells are never fully separated from the mother cell (Fig. 4C). Several of the daughter cells seem to die (Movie S3). The surviving daughters start producing buds before they are completely separated from the mother cell (Fig. 4C and Movie S3). This leads to the conclusion that the lack of sphingolipids causes a defect in cell separation. It seems that a new cell cycle might begin before the previous one has been entirely completed.

Analysis of the causes of defective cytokinesis in sphingolipid deficient cells

Next we speculated that a possible cause for the death of daughter cells during cSLD cell division might be a problem in nuclear division or improper recruitment of septins. DAPI stainings of fixed cSLD cells showed that even in cells that have not finished the last phases of cytokinesis and which form a complicated structure of interconnected cells, each cell has a nucleus (Fig. 5A). Although we cannot conclude that all of the genetic material is properly segregated, it does not seem that there is...
Fig. 4. Depletion of sphingolipids leads to a defect in cytokinesis. Division of each strain was followed by microscopy. For WT (A) and GhLag1 (B) strains each frame shown was taken with an interval of 30 min. For cSLΔ strain each frame shown was taken with an interval of 90 min. Movies with complete cell division can be found in supplementary data. Bars correspond to 10 μm.
a defect in nuclear segregation into the bud. In order to visualize whether septa were laid down and resolved at the appropriate times, we introduced a plasmid carrying a GFP labelled septin marker protein, Cdc10p-GFP plasmid. In the cSLΔ strain, the septin protein was properly localized to the bud neck (Fig. S3) although the fraction of cells with Cdc10p-GFP at the bud neck was significantly higher than that observed in WT (Fig. 5B). This can be explained by the longer time necessary for this strain to finalize cell division as shown in Fig. 4C and

**Fig. 5.** Cell division in mutant and WT strains.
A. DAPI staining of WT, GhLag1 and cSLΔ strains. The DIC is shown on the left and a Z-projection of the DAPI staining in shown on the right. Bars correspond to 10 μm.
B. Logarithmic growing cells were analysed for presence of Cdc10p-GFP in the bud neck. The graph shows the fraction of cells where the Cdc10p-GFP can be seen at the bud neck. The asterisk (*) indicates differences among groups that were considered significant for \( P < 0.05 \) according to Student’s \( t \)-test.
C. Strains harbouing plasmid pRS315 RPL5-GFP, a cytoplasmic GFP tagged protein. Dividing cells, either WT or cSLΔ, were photobleached. Recovery of fluorescence was checked after 5 min. Cells with interconnected cytoplasms recovered fluorescence after this time lapse, while unconnected cells did not. Arrows identify bleached cells.
Movie S3. To investigate if the connected cells with large buds still had physical continuity between their cytoplasmic compartments we introduced a plasmid with a cytoplasmic GFP tagged protein (Rpl5p-GFP) and photobleached the apparently connected daughter cells. In the WT strain, buds that did not yet have a primary septum were able to recover fluorescence after 5 min (Fig. 5C, upper panel), regardless of which cell (mother or daughter) was bleached. In the cSLA strain we chose cells with both small and big buds that were apparently interconnected to a third cell. It is important to note that this situation (a single cell with two buds) was never detected in the WT strain. The small buds of two budded cells were able to recover fluorescence but the bigger buds were not (Fig. 5C, lower panel). This happened to the budded cells, whenever the bleached bud was the bigger one (n = 7). This demonstrates that although cells remain physically connected, the cytoplasmic compartments of mother-daughter cells are completely separated at the end of cytokinesis. From the above data we conclude that even though cytokinesis seems to take longer, as judged from the increased fraction of cells containing septin proteins, it still occurs, but the cells do not physically separate, indicating that final steps of resolving the cell walls cannot be completed. We cannot exclude other defects, particularly leading to the death of the daughter cells, such as defective partitioning of other organelles including the ER and peroxisomes.

Accumulation of lipid bodies

When treated with AbA yeast cells accumulate ceramide which has been proposed to be toxic. It has been shown that ceramide has the potential to perturb membrane structure (Lopez-Montero et al., 2010), which might be the explanation for ceramide toxicity. However, from our study it is clear that ceramide toxicity depends upon the acyl chain length and cells with the cotton CerS, when treated with AbA, accumulate even higher amounts of ceramide than WT yeast. Since lipid bodies are organelles that stock neutral lipids, more specifically sterols and triacylglycerols, we investigated whether the accumulation of ceramide correlated with changes in lipid bodies. To our knowledge there are no published data available, suggesting that lipid bodies could also store ceramide. We analysed our mutant strains for changes in lipid bodies using the vital dye, Nile red. Nile red staining of lipid bodies increased in WT cells treated with AbA, which correlates with ceramide accumulation (Fig. 6A). Furthermore, Nile red staining of lipid bodies in the GhLag1 strain was as strong as in WT cells treated with AbA and staining was further greatly increased in the cSLA strain (Fig. 6B). When lipid bodies were purified by flotation and analysed by ESI-MS, only trace amounts of ceramides were found, and this only in the AbA treated WT and in the GhLag1 strains (Table 1). We also analysed the extracts by TLC, and lipid body preparations from the GhLag1 strain seemed to have more sterols and triacylglycerides than WT cells (data not shown). Most of the ceramides found in lipid bodies isolated from those cells are fully hydroxylated and have an acyl chain length of 24 carbons, even though the most abundant cellular ceramide has 26 carbons and one less hydroxylation in WT cells and 18 carbons in GhLag1 cells. These results suggest that small amounts of ceramides can be found in lipid bodies, but also suggests that the excess C26 ceramides and C18 ceramides synthesized in presence of AbA are not effectively sequestered there. An alternative explanation for the increase in lipid bodies is the possibility that cells with higher amount of sphingolipids might be under stress, which has been shown to increase lipid bodies (Gubern et al., 2009).

Discussion

Complex sphingolipids have been suggested to play roles in cell development, membrane trafficking, signal transduction (Kitatani et al., 2008; Bartke and Hannun, 2009). They have been proposed to be essential (Cerantola et al., 2009), even though mutants that prevent ceramide synthesis, a prerequisite precursor for their synthesis, are resistant to inhibitors of complex sphingolipid synthesis (Schorling et al., 2001). The interpretation of those data was that ceramide toxicity is only one of the reasons for cell death. In our studies to reveal novel cellular functions of plant ceramide synthases in vivo, we characterized the cotton CerS, GhLag1, by heterologous expression in an S. cerevisiae lag1Δ lac1Δ mutant lacking endogenous CerS activity. The cotton CerS led to the production of almost exclusively C18 ceramides and sphingolipids. The expression of GhLag1 in the yeast cells did not profoundly affect membrane protein localization or endocytosis, implying that the substitution of C18 sphingolipids for C26 sphingolipids is capable of sustaining many cellular functions. The growth rate on rich medium of the C18 and C26 producing yeast is almost identical. GhLag1 cells produced much higher levels of ceramides than the WT strain under normal growth conditions, suggesting that the C18 ceramide is not toxic to yeast cells. This allowed us to address the function of complex sphingolipids in cells where the problem of ceramide toxicity is eliminated. The major outcome of the study of complex sphingolipid function is that sphingolipids play an important role in cell division, most likely at a late stage of cytokinesis and certainly in cell separation. Inhibition of complex sphingolipid biosynthesis renders the cells highly sensitive to mechanical stress.

In order to study the plant CerS enzymes we rescued viability of lag1Δ lac1Δ by expression of GhLag1. The
results demonstrate that this protein is a *bona fide* ceramide synthase. LC-MS/MS analysis of the sphingolipids synthesized in the transgenic yeast strain indicates that the functional complementation of the double deletion strain is brought by production of sphingolipids containing mainly C18 fatty acid (Fig. 1B), instead of the normal C26 fatty acid. A CerS homologue *Asc-1* from tomato was able to rescue the yeast *lag1Δ lac1Δ* mutant (Spassieva et al., 2002), but the sphingolipid species produced by Asc-1 were not examined. Asc-1 was proposed to bring resistance to the ceramide synthase inhibitors, *Alternaria*

### Table 1. Trace amounts of ceramides detected in isolated lipid bodies (given as percentage of detected ceramides).

<table>
<thead>
<tr>
<th>Ceramide</th>
<th>WT Control</th>
<th>2 h AbA</th>
<th>GhLag1 Control</th>
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<tbody>
<tr>
<td>18:0/18:0 ceramide</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>18:0/24:0 ceramide</td>
<td>ND</td>
<td>85.6</td>
<td>99</td>
</tr>
<tr>
<td>20:0/24:0 ceramide</td>
<td>ND</td>
<td>6.8</td>
<td>0.01</td>
</tr>
<tr>
<td>18:0/26:0 ceramide</td>
<td>ND</td>
<td>7.3</td>
<td>0.09</td>
</tr>
</tbody>
</table>

ND, not detected.
Alternaria toxin and fumonisín B1 (Brandwagt et al., 2000), implying its role as a disease resistance gene involved in plant pathology. Previously, overexpression of individual mammalian CerS homologues in the yeast lag1Δ lac1Δ mutants resulted in production of sphingolipids with characteristic fatty acid chain lengths (Levy and Futerman, 2010). The difference in substrate specificity of the CerS(s) is speculated to be linked to their specialized physiological roles. Among them, mammalian CerS1 generates mainly C18 ceramides (Venkataraman et al., 2004). In contrast, accumulation of C18 ceramides in yeast did not promote cell death. However, in yeast C26 ceramides appear to be toxic. Therefore, toxicity of ceramide or ceramide appears to be chain length specific and the toxic chain length seems to be species specific. The precise reason for this specificity is unknown, but it is likely that the dependence on chain length suggests that the toxicity is not simply due to membrane perturbation.

To reveal cellular functions of C18 complex sphingolipids, application of AbA, a highly specific inhibitor of the IPC synthase, or deletion of AUR1 in a GhLag1 background was performed. Surprisingly, yeast cells producing a high level of C18 ceramides, shown by LC-MS/MS analysis, were resistant to AbA (Fig. 2). Initially, it has been speculated that yeast cells require IPCs because AUR1 is essential (Nagiec et al., 1997), and later that the high levels of ceramides caused by AbA treatment trigger wild-type cell death (Schorling et al., 2001). Here, we have been able to examine the important roles of complex sphingolipids in a strain where the absence of complex sphingolipids (<0.1% WT levels) was not accompanied by ceramide toxicity.

The cSLΔ cells grew only under certain conditions, without vigorous shaking and better on plates than in liquid medium. When grown on solid medium cSLΔ cells form a multicellular, interconnected structure, suggesting that they are defective in cell division. The defect in cell division is apparently quite late in the cycle because nuclear division seems to occur normally. Even though there did not seem to be a defect in septin recruitment, septin profiles at the bud neck were three times as abundant in mutant versus WT cells, implying that the septin-dependent membrane fusion steps may be specifically slowed down. We do not know if the entire process of cytokinesis takes longer than in WT cells. However, once the cytoplasms are separated, the next step, which involves resolution of the cell walls and cell separation, appears to be almost completely defective in cSLΔ cells. The two steps, cytokinesis and cell separation, have a common point that could be affected by the lack of complex sphingolipids. Both steps require

Experimental procedures

Yeast strains and plasmids, media and reagents

The strains used in this study are the following: RH2888 (MATa leu2 his3 lys2 trp1 ura3 can 1 bar1), RH6979 (MATa TDH3::GhLAG1::TRP1 lac1::ADE2 lag1::HIS3 leu2 ura3 lys2), cSLΔ RH7300 (MATa TDH3::GhLAG1::TRP1 lac1::ADE2 lag1::HIS3 leu2 ura1::URA3 lys2), and RH7166 (MATa lac1::ADE2 lag1::HIS3 leu2 ura3 lys2 trp1). All mutations were constructed using standard gene disruption procedures, with complete removal of open reading frames, in our laboratory strain background, mainly derived from S288C. Double and triple mutants, as well as introduction of plasmids into the cSLΔ strain, were obtained by genetic crosses. The Lag1p homologue GhLag1-1 (GenBank JQ080174) sequence was purchased from GeneArt (Germany), as yeast codon optimized ORFs (Fath et al., 2011), cloned for expression from the TDH3 promoter and inserted into the genome at the TRP1 locus to make stable cell lines. Rich medium (20 g l⁻¹ glucose, 20 g l⁻¹ peptone and 10 g l⁻¹ yeast extract with adenine, uracil and tryptophan at 40 mg l⁻¹) was used in all experiments. AbA was purchased from Takara Shuzo (Shiga, Japan). All synthetic lipid standards were obtained from Avanti Polar Lipids.
Visualized under a 100X 5 min. Cells were washed and placed on coverslips to be projected of the maximum intensity for each aggregate. Because aur1 forms 3D aggregates micrographs were acquired sequentially in a Z-plane and figures shown are a Z projection and images are a maximum intensity projection.

**Fluorescent protein visualization**

Visualization of fluorescent proteins was done as previously described (Castillon et al., 2009). Briefly, cells were grown overnight in SD-URA and diluted in YPD for 2 h before visualization using the AXIOZ1 microscope (ZEISS). For Fig. S2, cells were grown on rich liquid media under static condition and put into a shaker at 220 rpm for the indicated times. For Fig. S3 images were processed in the same way but were acquired using the Leica SP2 confocal microscope. Cells were counted using ImageJ cell counter software and cells were said to be Cdc10 positive when a visible band of GFP was visualized at the bud neck. This number was then divided by the total number of cells found in the field. Over 5 fields, each with over 20 cells was analysed.

**Cell division microscopy**

For cell division assay, cells were taken from logarithmic growing cultures and placed on small solid patches of rich medium to be visualized. We used a Leica AF6000 LX wide field microscope with a Coolsnap HQ camera (Photometrics, Roper Scientific, CA, USA), 63× objective with a 2.6× magnification, which acquired micrographs every 30 s to make movies of the dividing cells.

**DAPI staining**

For DAPI staining, logarithmic growing cells were collected, fixed with 100% ethanol for 1 h at room temperature, washed with PBS and stained with a DAPI solution of 10 μg ml⁻¹ for 5 min. Cells were washed and placed on coverslips to be visualized under a 100X 1.4NA oil objective with the AXIOZ1 microscope (ZEISS) and the Zeiss AxioCam MRm CCD camera controlled by the software AxioVision Rel. 4.6. Because aur1 forms 3D aggregates micrographs were acquired sequentially in a Z-plane and figures shown are a Z projection of the maximum intensity for each aggregate.

**Photobleaching assay**

To detect if mother–daughter cells were still connected, cells harbouring plasmid pRS315 PRL5-GFP were collected from logarithmic growing cultures. Using a Leica SP2 confocal microscope we selected cells to be bleached and gave a 10-second pulse to completely eliminate fluorescence from selected areas. Micrographs were taken immediately before and after bleaching and 5 min post bleach.

**Nile red staining**

For the Nile red staining assay, log growing cells were collected, washed with PBS and incubated for 10 min with final concentration of 0.1 mg ml⁻¹ Nile red. Cells were washed again and visualized using a Leica SP2 confocal microscope. Again micrographs were taken in a sequential manner in Z-plane and images are a maximum intensity projection.

**Plating assay and growth curves**

For the plating assays, yeast strains were grown to stationary phase and diluted to 10⁷ cells per ml in water, and 10-fold serial dilutions were pipetted onto agar plates containing rich medium with or without the indicated additives. Each experiment was repeated at least three times and the pictures shown are representative of the results found. For growth curves cells were grown to stationary phase and diluted to the indicated initial concentration in liquid rich medium with shaking at 220 rpm. OD600 was measured at the indicated time points. Each experiment was repeated at least three times and standard deviations are shown on the graphs.

**Statistical analysis and software**

All statistical analyses were performed using Student’s t-test, and differences among groups were considered significant for P < 0.05 (*). All data analysis and quantification were done using Image J.

**Mass spectrometry**

Yeast strains were grown in rich medium (YPD) to a final concentration of 1–2 OD600 units per ml and 50 OD600 units were harvested for lipid extraction as described (Guan et al., 2010). Briefly, samples were spiked with C17 ceramide and C₈ glucosylceramide and lipids were extracted using a pyridine solvent and base treated using monomethylamine. Lipids were desalted with butanol and introduced into a Varian 320MS triple quadrupole in LC mode by direct infusion in CH₃OH: CHCl₃:H₂O (7:2:1, containing 2 mM ammonium acetate). To quantify lipid species multiple ion monitoring was used for each species (Menuz et al., 2009).

Data were collected from at least three independent experiments each with triplicate measurements by mass spectrometry. The recoveries and amounts of different ceramides and complex sphingolipid species were calculated relative to the GlcCer (d₁₈:₁, 8:0) or to Cer (d₁₈:₁, 17:0) standard according to a standard curve. Results are presented as relative amounts (RA) normalized by the control condition that was set to 100, because detected absolute amounts could not be determined without an identical internal standard. A full description of the quantification method is presented elsewhere (Epstein et al., 2011).

In vivo lipid labelling

In vivo lipid labelling with ³H-sphinganine was performed as previously described (Zanolari et al., 2000). Sphingolipids were extracted by chloroform/methanol (1/1) in presence of glass beads, base treated as described above and then desalted by butanol/water partitioning. Lipids were analysed by TLC using chloroform : methanol : ammonia, 40:10:1 v/v/v
(Lahiri and Futerman, 2005) and visualized using tritium-sensitive screens and a Cyclone phosphorimager (Packard, Meriden, CT, USA). An unlabelled C18 dihydroceramide was used as standard to detect running of the ceramide species. The fold increase of ceramides over WT was the average of six determinations.

**Plasmids and reagents**

The plasmids expressing Hxt1-venus (RH3075), Can1-venus (RH3068) and Mid2-venus (RH3079) are from our lab collection (Castillon et al., 2009). Gap1p-GFP was a kind gift from Dr Bruno Andre. CDC10p-GFP was a gift from Maria Molina (Cid et al, 2001) and pRS315 RPL5-GFP from Vikram Panse (Kraft et al., 2008).

**Isolation of lipid bodies**

Isolation of lipid bodies was done as previously described (Zinser and Daum, 1995). The fraction recovered at the top of the sucrose gradient was extracted with 1 ml of chloroform and 1 ml of methanol. The organic phase was separated and dried under a stream of nitrogen. Lipids were resuspended in 1 ml of methanol and 1 ml of methanol. The organic phase was separated and dried under a stream of nitrogen. Lipids were resuspended and measured by ESI-MS using a TSQ Vantage spectrometer (Thermo Fisher Scientific) equipped with a Nanomate (Advion) as described elsewhere (Epstein et al., 2011). Amounts are given as percentage of ceramide species detected.

**Scanning electron microscopy**

Harvested yeast cells were fixed with 1% osmium in PBS and incubated at room temperature for 1 h. Cells were dehydrated with 30%, 50%, 75%, 85%, 95% and 100% (3¥) ethanol for 10 min each. Cells were coated with chromium and visualized using a JEOL JSM-6510LV low vacuum scanning electron microscope.

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**References**


Supporting information

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