Aureobasidin A arrests growth of yeast cells through both ceramide intoxication and deprivation of essential inositolphosphorylceramides

Vanessa Cerantola,[1] Isabelle Guillas,[1†]
Carole Roubaty,[1] Christine Vionnet,[1] Danièle Uldry,[1]
Jens Knudsen[2] and Andreas Conzelmann[1*]

[1]Department of Medicine, University of Fribourg,
Fribourg, Switzerland.
[2]University of Southern Denmark, Campusvej 55,
DK-5230 Odense M, Denmark.

Summary

All mature *Saccharomyces cerevisiae* sphingolipids comprise inositolphosphorylceramides containing C26:0 or C24:0 fatty acids and either phytosphingosine or dihydrosphingosine. Here we analysed the lipid profile of *lag1Δ lac1Δ* mutants lacking acyl-CoA-dependent ceramide synthesis, which require the reverse ceramidase activity of overexpressed Ydc1p for sphingolipid biosynthesis and viability. These cells, termed 2Δ.YDC1, make sphingolipids containing exclusively dihydrosphingosine and an abnormally wide spectrum of fatty acids with between 18 and 26 carbon atoms. Like wild-type cells, 2Δ.YDC1 cells stop growing when exposed to Aureobasidin A (AbA), an inhibitor of the inositolphosphorylceramide synthase *AUR1*, yet their ceramide levels remain very low. This finding argues against a current hypothesis saying that yeast cells do not require inositolphosphorylceramides and die in the presence of AbA only because ceramides build up to toxic concentrations. Moreover, W303*lag1Δ lac1Δ ypc1Δ ydc1Δ* cells, reported to be AbA resistant, stop growing on AbA after a certain number of cell divisions, most likely because AbA blocks the biosynthesis of anomalous inositolphosphorylsphingosides. Thus, data argue that inositolphosphorylceramides of yeast, the equivalent of mammalian sphingomyelins, are essential for growth. Data also clearly confirm that wild-type strains, when exposed to AbA, immediately stop growing because of ceramide intoxication, long before inositolphosphorylceramide levels become subcritical.

*For correspondence. E-mail andreas.
conzelmann@unifr.ch; Tel. (+41) 26 300 8630; Fax (+41) 26 300
9735. †Present address: UPMC Univ Paris 06, UMR 7180, PCMP,
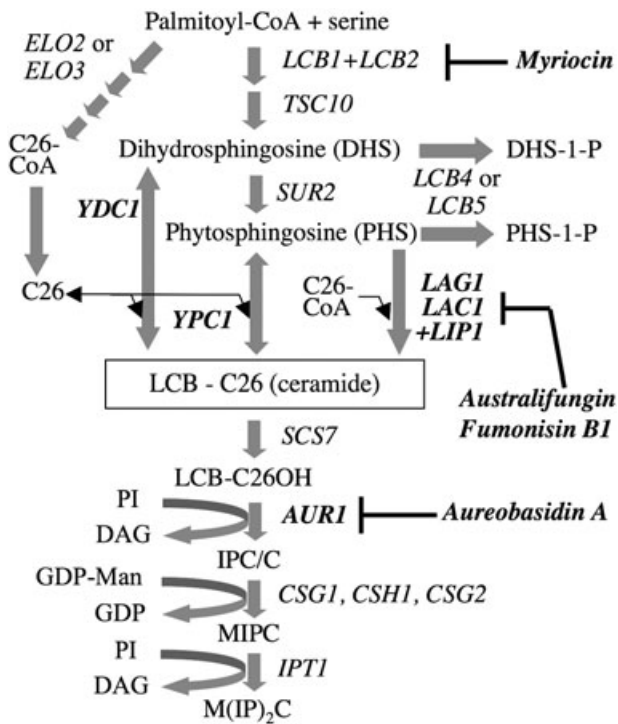F-75005, Paris, France and CNRS, UMR 7180, PCMP, F-75005,
Paris, France.

Introduction

In *Saccharomyces cerevisiae*, the inositolphosphorylceramides (IPCs), mannosyl-IPCs (MIPCs) and inositolphosphoryl-MIPCs [M(IP)$_2$Cs] are major components of the plasma membrane (Fig. 1) (Dickson and Lester, 1999). Moreover, as in mammalian cells, dihydrosphingosine (DHS), phytosphingosine (PHS), their 1-phosphorylated derivatives as well as ceramides have been proposed to act as signal transduction molecules governing heat stress responses, endocytosis, GPI protein transport, ubiquitin-dependent degradation of membrane channels and progression through G1 (Dickson and Lester, 2002; Sims *et al.*, 2004; Dickson *et al.*, 2006; Dickson, 2008).

The biosynthesis of ceramide is dependent on the longevity assurance gene *LAG1* and its close homologue *LAC1* (D'mello *et al.*, 1994) (Fig. 1), which are members of a large gene family with homologues present throughout the eukaryotic kingdom (Winter and Ponting, 2002; Obeid and Hannun, 2003). Yeast Lag1p and Lac1p are functionally redundant and deletions of *LAG1* or *LAC1* do not cause an abnormal growth phenotype, whereas the concomitant deletion of *LAG1* and *LAC1* causes a significant growth defect in the W303 genetic background and the same double deletion is lethal in the YPK9 background (Jiang *et al.*, 1998; Barz and Walter, 1999; Guillas *et al.*, 2001). Lag1p or Lac1p are functional only in complex with Lip1p (Fig. 1), a small, single-span endoplasmic reticulum (ER) membrane protein, deletion of which severely reduces the growth rate of yeast cells (Vallée and Riezman, 2005).

Using multicopy suppressor screens, it has been found that overexpression of *YPC1* or *YDC1* (Fig. 1) improves the slow growth phenotype of W303 *lag1Δ lac1Δ* (2Δ. W303) cells (Schorling *et al.*, 2001) and suppresses the lethality of the *lag1Δ lac1Δ* double mutation in the YPK9 background (Jiang *et al.*, 2004). Ypc1p and Ydc1p are two highly homologous membrane proteins of the ER. They show 54% identity over their entire length and catalyse

Fig. 1. Major pathways of sphingolipid biosynthesis in yeast. Gene names are in italic and enzyme inhibitors in bold italics. Note that Ypc1p utilizes all long-chain bases (LCBs) whereas Ydc1p only utilizes DHS (Mao *et al.*, 2000b). PI, phosphatidylinositol; DAG, diacylglycerol; GDP-Man, GDP-mannose.

the hydrolysis of ceramides *in vitro*, i.e. they act as ceramidases (Fig. 1). *In vitro*, as observed with many enzymes, Ypc1p can also catalyse the reverse reaction, i.e. the condensation of [³H]palmitate with PHS or DHS (Mao *et al.*, 2000a). Ydc1p, contrary to Ypc1p, has been shown to hydrolyse only DHS-containing ceramides and exhibits only very weak reverse activity *in vitro* (Mao *et al.*, 2000b). The reverse activities of Ypc1p and Ydc1p thus provide an alternative way of making ceramide (Fig. 1), which can explain why the overexpression of *YPC1* or *YDC1* can improve or rescue the growth of *lag1Δ lac1Δ* (2Δ) cells or render yeast cells resistant to the toxic effect of fumonisin B1, a specific inhibitor of the acyl-CoA-dependent ceramide synthase (Mao *et al.*, 2000a,b) (Fig. 1).

Metabolic labelling experiments suggest the existence of yet another, third way of making sphingolipid-like compounds. Indeed, W303 *lag1Δ lac1Δ ypc1Δ ydc1Δ* (4Δ.W303) cells are viable and able to incorporate [³H]DHS, [³H]inositol and ³²PO4 into lipids X1 and X2, two mild base-resistant, but not yet characterized lipids (Schorling *et al.*, 2001).

Initially, yeast cells were assumed to require IPCs because the IPC synthase *AUR1* is essential (Fig. 1) and because Aureobasidin A (AbA), a highly specific inhibitor of Aur1p, rapidly kills growing yeast cells (99% dead cells

within 1 h) (Endo *et al.*, 1997; Nagiec *et al.*, 1997). The requirement for IPCs has however been questioned, since W303 *lag1Δ lac1Δ*, W303 *lag1Δ lac1Δ ypc1Δ ydc1Δ* and *lip1Δ* cells grew well in the presence of high concentrations of AbA (Schorling *et al.*, 2001; Vallée and Riezman, 2005), and because sphingolipids are altogether dispensable for certain yeast cells harbouring a gain of function suppressor allele in the lyso-phosphatidate acyltransferase *SLC1* (Nagiec *et al.*, 1993). The AbA resistance of W303 *lag1Δ lac1Δ*, W303 *lag1Δ lac1Δ ypc1Δ ydc1Δ* and *lip1Δ* cells led to the hypothesis that IPCs are dispensable for cell growth and that AbA kills wild-type (wt) cells by causing the accumulation of toxic levels of ceramides rather than by depriving them of IPCs.

Here we set out to further investigate the physiological potential of alkaline ceramidases and in particular of *YDC1* by examining sphingolipid profiles and stress resistance of yeast cells, which neither possess acyl-CoA-dependent ceramide synthases nor a third way of making inositolphosphorylsphingoids and thus depend for ceramide biosynthesis on *YDC1*. In the course of this study we realized that such cells could be used to answer the question as to whether or not IPCs are essential for cell survival of yeast cells. We find that IPCs are essential for growth but this becomes only apparent in *lag1Δ lac1Δ* strains, which do not accumulate toxic ceramides when IPC synthase activity is compromised.

Results

YPK9 cells depend on LAG1 *and* LAC1 *to make the bulk of sphingolipids*

When YPK9 *lag1Δ lac1Δ* (2Δ.LAG1) cells containing a plasmid-born copy of *LAG1* behind the *GAL1* promoter were very strongly depleted of Lag1p on glucose and were then labelled with [³H]inositol, they made no normal sphingolipids anymore (Fig. 2, lane 8), but they incorporated as much [³H]inositol into lipids as when growing on galactose (Fig. 2, lanes 2 and 4). After depletion, the only mild base-resistant lipid still made was the uncharacterized 'lipid a' (Fig. 2, lane 8), but cells had stalled all synthesis of normal IPCs, MIPCs and lipid b that one finds in W303 *lag1Δ lac1Δ* cells (Guillas *et al.*, 2001). Thus, unlike W303 *lag1Δ lac1Δ*, the YPK9 *lag1Δ lac1Δ* background did not seem to contain any alternative pathways or enough endogenous Ypc1p and Ydc1p activity for making ceramides and mild base-resistant inositolphosphorylsphingoids, as is further detailed in *Supporting information*.

Overexpression of YDC1 *in YPK9* lag1Δ lac1Δ *allows for the synthesis of small amounts of IPCs*

YPK9 *lag1Δ lac1Δ* cells are not viable but the strain can be rescued by overexpression of *YDC1* and the thus
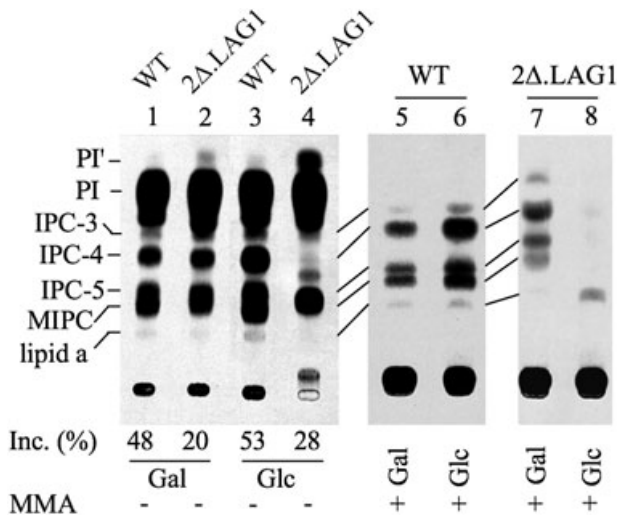
Fig. 2. Sphingolipid profile of 2Δ.LAG1 after strong depletion of Lag1p. 2Δ.LAG1, a strain harbouring *LAG1* behind the *GAL1* promoter, was depleted of Lag1p by pre-culture on glucose (Glc, SDaaA) for 30 h or left on galactose (Gal, SGaaA). YPK9 (WT) cells were also cultured in either SGaaA or SDaaA for 15 h before labelling, all pre-cultures being performed in media without inositol. Aliquots of 2.5 A_{600} units of cells were labelled with [^{3}H]inositol at 30°C for 2 h in the same type of media as used in the pre-culture. The lipids were extracted, desalted and treated with monomethylamine (+MMA, lanes 5–8) to remove glycerophospholipids or were mock incubated (−MMA, lanes 1–4). Then they were analysed by TLC using solvent system 3. The total incorporation of [^{3}H]inositol into lipids is given as a fraction (%) of the total radioactivity added to cells below lanes 1–4. IPC-3, -4, -5 containing ceramide moieties with three, four or five hydroxyl groups (sum of hydroxyls on LCB and the fatty acid) correspond to IPC/B, IPC/C and IPC/D respectively (Haak *et al.*, 1997). M(IP)$_2$C and glycerolphosphorylinositol stay at the origin in this TLC system.

Fig. 3. Overexpression of *YDC1* in YPK9 *lag1Δ lac1Δ* strains fails to restore a normal ceramide and sphingolipid profile.
A and B. Five A_{600} units of 2Δ.YDC1, 2Δ.LAG1 and YPK9 (WT) cells were grown on SDaaA without inositol and labelled with [^{3}H]inositol (A) or [^{3}H]DHS (B) in the same way as described in Fig. 2. [^{3}H]inositol labelling of 2Δ.YDC1 and YPK9 was stopped after 0.3 generation times, which corresponded to 2.3 and 1 h of labelling, respectively, in order to compensate for the slower growth of 2Δ.YDC1. Lipids were extracted, desalted, deacylated or not with monomethylamine (MMA) and run on TLC using solvent system 3 (A) or 1 (B). The bracket next to lane 3 of (B) indicates abnormal base-resistant lipids. The figures show one of two experiments, which gave identical results.
C. The indicated strains were pre-cultured in SDaaUA and inositol was removed during last hour of pre-culture. Cells were then labelled with [^{3}H]inositol and lipids were extracted. Aliquots of extract corresponding to 10^6 c.p.m. were deacylated with NaOH or control incubated; desalted lipids were separated by TLC in solvent system 2. Figures below the lanes indicate the amount of mild base-resistant lipids as a percentage of the total of labelled lipids in each lipid extract, determined by radioscanning.

rescued strain (2Δ.YDC1) grows, albeit at a reduced rate, as described (Jiang *et al.*, 2004). The profile of [^{3}H]inositol-labelled lipids of 2Δ.YDC1 is shown in Fig. 3A. It appears that the cells not only make 'lipid a', but also some mild base-resistant species co-migrating with IPC-3, IPC-4 and IPC-5, suggesting that Ydc1p can act as a ceramide synthase *in vivo*. Yet, the quantification of this radiogram showed that 2Δ.YDC1 made about 11 times less sphingolipids than wt (Table S1). When 2Δ.YDC1 cells were labelled with [^{3}H]DHS, they made several mild base, i.e. monomethylamine (MMA)-resistant lipids (Fig. 3B, lanes 3 and 4, bracketed), which were less hydrophobic than the normal PHS-C26:0-OH of wt cells (Fig. 3B, lane 2). These potential ceramides have not been further characterized but the ESI-MS analysis of IPCs suggests that they are ceramides containing fatty acids with less than 26 C atoms (see below).

In vitro ceramide synthase activity (reverse ceramidase activity) of Ydc1p was only detectable with DHS, but not with PHS as a substrate (Mao *et al.*, 2000b). Because DHS is continuously transformed into PHS by
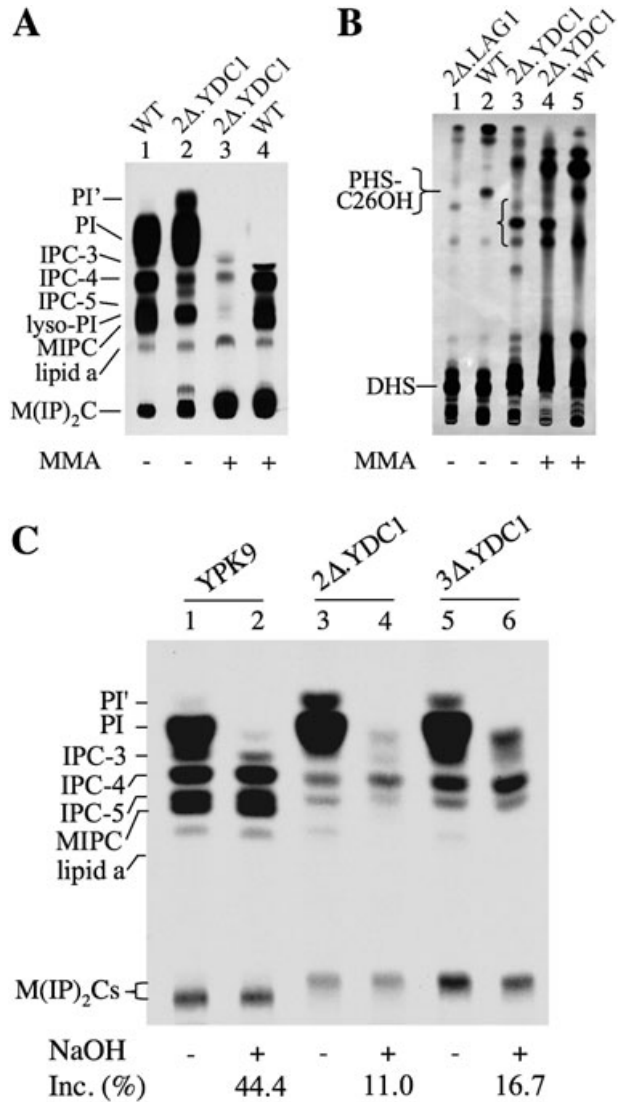
Sur2p (Fig. 1), we figured that the DHS concentration in 2Δ.YDC1 may be too low to allow the ΔG′ for ceramide synthesis by Ydc1p to become negative. We therefore deleted *SUR2* in 2Δ.YDC1, thus generating the YPK9 *lag1Δ lac1Δ sur2Δ* strain named 3Δ.YDC1. As shown in Fig. 3C and in a second identical experiment (Fig. S1), labelling with [³H]inositol and mild base deacylation revealed that deletion of *SUR2* in 2Δ.YDC1 increased the amount of sphingolipids 1.5-fold, but did not change the thin-layer chromatography (TLC) migration of the main species (Fig. 3C, lanes 3–6). Indeed, 2Δ.YDC1 and 3Δ.YDC1 showed a rather similar sphingolipid profile on TLC (Fig. 3C, lanes 4 and 6), which was different from the one of YPK9 (Fig. 3C, lane 2; Fig. S1). (It should be noted that the TLC system used is more sensitive towards the number of hydroxyls than to the length of the fatty acids.)

Mass spectrometric analysis of IPCs generated by Ydc1p in vivo

To further characterize the sphingolipid profile of 2Δ.YDC1 and 3Δ.YDC1, their lipid extracts were analysed by mass spectrometry (HPLC-ESI-MS/MS) in the negative ion mode. As shown in Fig. 4A, YPK9 cells only make IPCs with 42, 44 or 46 C atoms and mostly three, four or five hydroxyl groups in their ceramide moiety. (DHS and PHS are counted as contributing two and three hydroxyls, respectively, the remaining hydroxyl groups being attached to the fatty acid moiety). Since most yeast sphingolipids contain long-chain bases (LCBs) with 18, less frequently 20 C atoms, it is likely that the main IPCs of YPK9 wt cells (IPC44-3, 44-4 and 44-5) contain C26 and C24 fatty acids and their mono- and dihydroxylated derivatives. Indeed, already the first studies by Robert
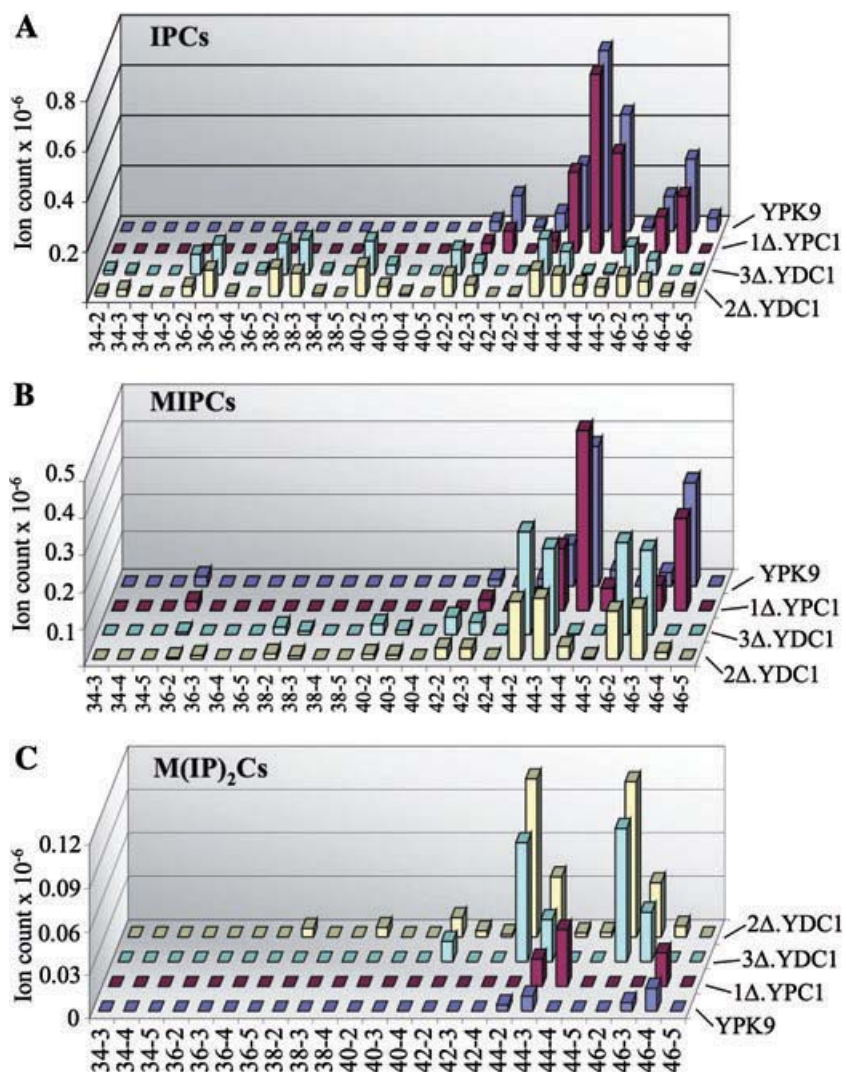

Fig. 4. HPLC-ESI-MS/MS analysis of sphingolipids of 2Δ.YDC1 and 3Δ.YDC1. Lipids were extracted quantitatively (Hanson and Lester, 1980) from cells growing exponentially in YPD at 30°C; lipids were deacylated with mild base, and analysed by HPLC-ESI-MS/MS. Parent ion and fragment ion masses (m/z) were recorded throughout the HPLC run and were screened for all theoretically possible sphingolipids containing different saturated, monounsaturated, and non-, mono- or dihydroxylated fatty acids combined with DHS or PHS as well as for conventional phospholipids. Sphingolipids are specified by two consecutive numbers standing for the number of C atoms and hydroxyls in their ceramide moiety respectively. The masses corresponding to these abbreviations are indicated in Table S2. The vertical axis indicates the ion counts. All species were identified as phosphoinositides by characteristic fragment ions at m/z 259 and 241, corresponding to inositolphosphate and [inositolphosphate-H₂O] except when ion counts were <2000. The x-axis only lists those species that indeed were found to be present in at least one of the analysed strains. Before injection, the lipid extracts were mixed with a fixed amount of lipid extract from wt cells grown in [¹³C]glucose as the only carbon source and the data were normalized using this internal standard. Note that intensities of DHS- and PHS-containing sphingolipids are not directly comparable since they ionize with different efficiency.

Lester showed that the most frequent IPC of yeast contains a PHS with 18 C atoms and a monohydroxylated C26 fatty acid, which translates into a 44-4 species (Smith and Lester, 1974). The species with 46 C atoms may contain a LCB with 20 C atoms or a fatty acid with 28 C atoms. [In other strains it was observed that 2% of LCBs contain 20 C atoms (Smith and Lester, 1974) and 2% of very long-chain fatty acids are C28 (Oh et al., 1997).] Overall, the profile of sphingolipids of our YPK9 cells is very similar to the one recently published for another yeast wt strain, RH690-15D, except that this latter contains more IPC46-4 than IPC44-5 (Ejsing et al., 2006). The profile of YPK9 is also very comparable to the one of BY4741 (Guan and Wenk, 2006).

Quite differently from YPK9, 2Δ.YDC1 cells contain similar amounts of IPCs with 36, 38, 40, 42, 44 and 46 C atoms in their ceramide moiety, suggesting that the fatty acids C18, C20, C22, C24 and C26 all are used with even frequency (Fig. 4A). Data further show that most IPCs of 2Δ.YDC1 have only two or three hydroxyls in their ceramide moiety and that the profile of 3Δ.YDC1 is almost the same as the one of 2Δ.YDC1, except for the lack of some minor IPC44-4 and IPC44-5 species. It should be noted that 3Δ.YDC1 cells cannot make PHS, but only DHS, so that all species containing three hydroxyls by necessity contain a monohydroxylated fatty acid linked to DHS. Since 2Δ.YDC1 have almost the same IPC profile as 3Δ.YDC1, we have to conclude that in vivo Ydc1p exclusively makes DHS-containing ceramides even in the 2Δ.YDC1 strain, in which PHS is quite abundant (Table 1).

As a control we used a YPK9 lag1Δ strain overexpressing YPC1 (1Δ.YPC1) from a LEU2 multicopy vector of the same type as present in 2Δ.YDC1 and 3Δ.YDC1. Figure 4 shows that its sphingolipid profile is very similar to the one of YPK9, indicating that neither the presence of a LEU2 vector nor the overexpression of a ceramidase/reverse ceramidase by itself has an impact on the sphingolipid profile in a strain still possessing an acyl-CoA-dependent ceramide synthase.

Table 1. Accumulation of free long-chain bases in lag1Δ lac1Δ cells.

	PHS	DHS	Fold increase
YPK9	7.5 ± 3.6	10.2 ± 7.2	1
2Δ.LAG1[a]	364.1 ± 175.5	98.7 ± 53.7	26
1Δ.YPC1	57.3 ± 37.6	22.8 ± 22.3	4.5
2Δ.YDC1	387.5 ± 150.2	254.4 ± 149.5	36

a. The data on 2Δ.LAG1 are from cells grown on glucose.
Numbers indicate pmoles per A_{600} unit of cells. Results are the mean of three experiments and five different determinations. The last column compares the sum of PHS and DHS with the normal levels set to 1.

Low amounts of IPCs with a double bond in their ceramide moiety were found in all cell lines (not shown). It has been known for a long time that a small fraction of yeast sphingolipids contains an LCB with a double bond (Smith and Lester, 1974), whereas unsaturated C24 and C26 are undetectable (Ejsing et al., 2006; Guan and Wenk, 2006) or very scarce (Oh et al., 1997).

MIPCs of all cells contained the same number of hydroxyls as present in ceramides of their IPCs (except that there is no 46-5 MIPC) (Fig. 4B). In contrast, in all strains, the ceramides of MIPCs mostly had C24 or C26 fatty acids and IPCs with shorter fatty acids, very prevalent in 2Δ.YDC1 and 3Δ.YDC1, were not efficiently utilized for MIPC synthesis (Fig. 4B). Data also show that the ceramides of M(IP)$_2$Cs are very similar to the ones of MIPCs in almost all cells, except that cells rarely make M(IP)$_2$Cs from MIPC 44-5 (Fig. 4B versus Fig. 4C). Curiously, M(IP)$_2$C levels were much higher in 2Δ.YDC1 and 3Δ.YDC1 than in wt cells (Fig. 4C).

Mass spectrometry also showed that 2Δ.YDC1 had two times higher amounts of phosphatidylinositol (PI), in accordance with the slower IPC biosynthesis observed in metabolic labelling experiments (Fig. 3C, Fig. S1).

Mass spectrometric analysis indicates the presence of C26:0 in phosphatidylinositol of 2Δ.YDC1

2Δ.YDC1 cells harbour abnormally high amounts of PI′ (Fig. 3A, lane 2 versus 1, and Table S1; Fig. 3C, lane 3 versus 1). PI′ is characteristic for 2Δ cells and was proposed to represent a hydrophobic PI species containing C26:0 fatty acids instead of the normal C16 and C18 species (Guillas et al., 2001; Cerantola et al., 2007). This is confirmed in our MS analysis of 2Δ.YDC1 by the presence of strong m/z signals at 947.7 and 975.8, signals that were not seen in wt. These signals correspond to PI species with a total of 42 or 44 C atoms and one double bond in their two fatty acids (PI 42:1 and PI 44:1) respectively. Fragmentation of the 975 parent ion yielded fragment m/z signals at 281, 395, 579, 693 and 711, corresponding to [M-H] masses of C18:1, C26:0, [lyso-PI(C18:1)-H$_2$O], [lyso-PI(C26:0)-H$_2$O] and lyso-PI(C26:0), respectively, confirming the identity of 975.7 as PI with a C26:0 and C18:1 fatty acid, the latter presumably residing in sn-2. This type of lipid is also observed in low amounts in normal cells (Schneiter et al., 2004). The high levels of PI′ in 2Δ.YDC1 suggest that C26:0-CoA or C26:0 levels are abnormally high in this strain. Higher than normal levels of C26:0 are probably necessary in order to generate a negative ΔG′ for ceramide synthesis by Ydc1p. As reported before for other strains (Schneiter et al., 2004; Cerantola et al., 2007), C26:0-containing species were found only in PI but no other glycerophospholipid species.

2Δ.YDC1 and 3Δ.YDC1 cells are relatively resistant to stresses known to block growth of sphingolipid-deficient cells

The robustness of 2Δ.YDC1 and 3Δ.YDC1 strains with regard to stress conditions known to block the growth of cells having an abnormal sphingolipid biosynthesis was tested as detailed in Figs S2 and S3. We found that neither the 2Δ.YDC1 nor the 3Δ.YDC1 strain was sensitive to temperature (Fig. S2) or low pH (not shown). Moreover, 3Δ.YDC1 showed similar resistance to most

stress conditions as parental YPK9 cells. 2Δ.YDC1 cells grew slightly less well than 3Δ.YDC1 and YPK9 already in the absence of any stress, and their growth was also weaker in most stress conditions. However, 2Δ.YDC1 cells gained almost normal growth at all temperatures between 17°C and 37°C on a specially designed medium containing high concentrations of amino acids and inositol, here termed Lester medium (LM) (Pinto *et al.*, 1992) (Fig. 5A and data not shown). LM also restored better growth on glucose to 2Δ.LAG1 (Fig. 5A). Metabolic labelling experiments demonstrated that LM
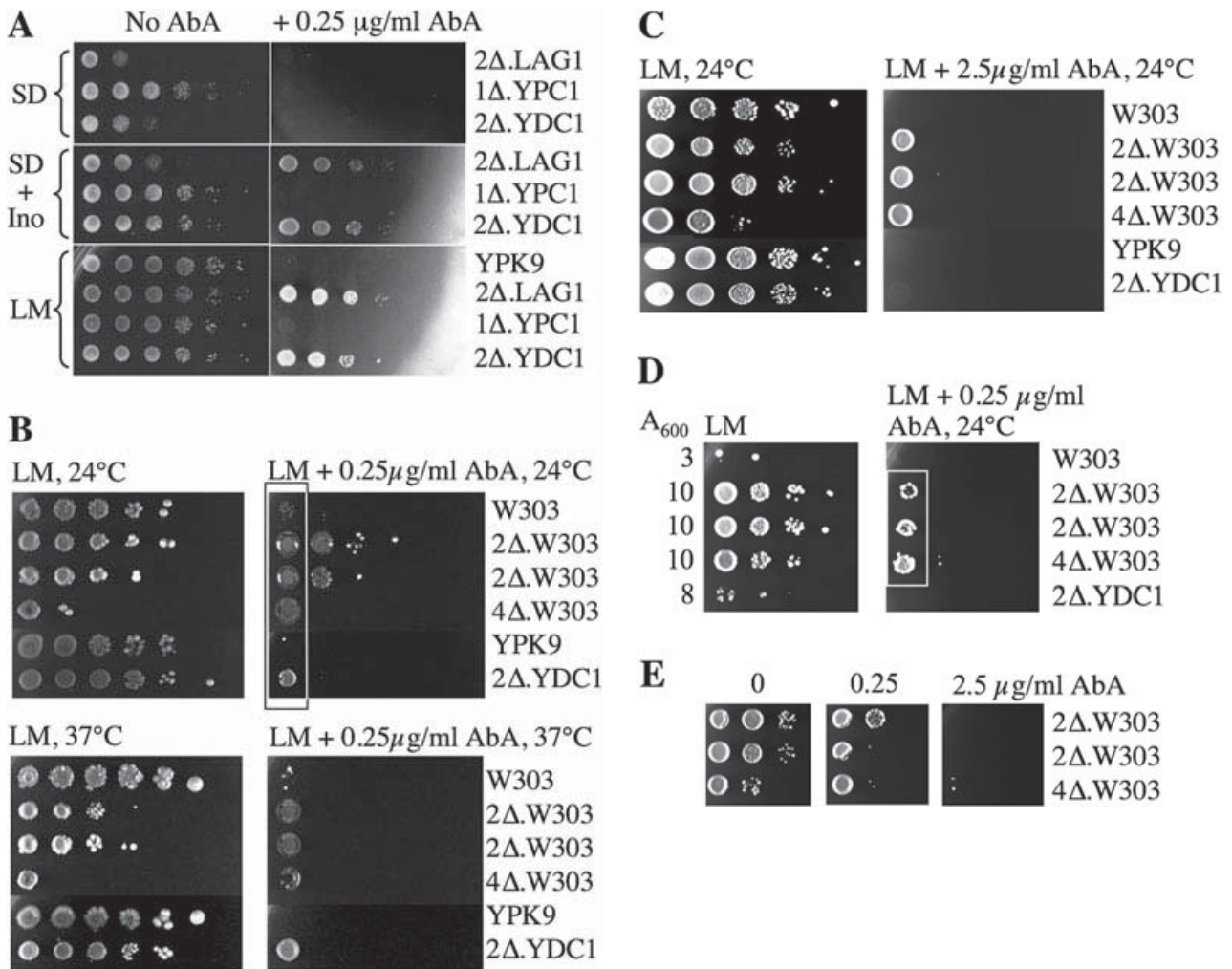

Fig. 5. Aureobasidin A (AbA) resistance of various *lag1Δ lac1Δ* strains. Aliquots of cell suspensions having an A_{600} of 10 and 10-fold serial dilutions thereof were plated on various media in (A), and on Lester medium (LM) in all other panels.
A. Cells were plated on SDaaUA medium without inositol (SD) or with inositol (2 μg ml⁻¹; SD+Ino), or on LM in the presence or absence of AbA. Plates were incubated at 24°C for 4 days.
B. Strains were plated with or without AbA (0.25 μg ml⁻¹) and incubated for 5 days at 24°C or 37°C. (Two different subclones of W303 *lag1Δ lac1Δ* were tested.)
C. Cells were plated in the presence or absence of 2.5 μg ml⁻¹ AbA and incubated for 5 days.
D. Cells having been growing on 0.25 μg ml⁻¹ AbA were scraped from the plate shown in (B) (boxed), were counted and replated on the same medium or on LM and incubated at 24°C for 4 days. For some cells the A_{600} value of the starting cell suspension was less than 10 as indicated.
E. Cells having been replated on AbA (boxed in D) were counted and replated on LM plates with various concentrations of AbA (0, 0.25 and 2.5 μg ml⁻¹) and incubated for 5 days at 24°C.
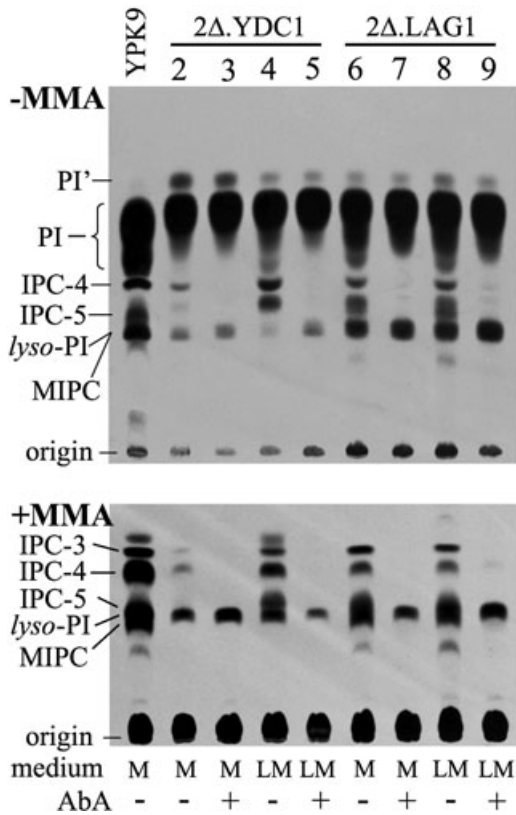
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Fig. 6. Aureobasidin A (AbA) efficiently abolishes IPC synthesis in *lag1Δ lac1Δ* cells. 2Δ.YDC1 and 2Δ.LAG1 cells were grown for 16 h in minimal medium (M, SDaaA without inositol) or LM, leading to partial depletion of Lag1p in 2Δ.LAG1. Four hours before adding radioactivity all cells were transferred to SDaaA lacking inositol to induce the inositol transporter Itr1p. Cells were labelled with [³H]inositol in SDaaA, in the presence or absence of AbA (2 µg ml⁻¹); lipids were extracted, treated (bottom) or not (top) with monomethylamine (MMA) and separated by TLC in solvent 3. Deacylation was incomplete in this experiment, as some lyso-PI was left after deacylation, but this material was no more present after deacylation using methanolic 0.1 M NaOH at 37°C for 1 h (not shown). The experiment also shows that pre-culture in LM stimulated the incorporation of [³H]inositol into 2Δ.YDC1, a finding that may explain the positive effect of LM on cell growth.

increased the capacity of 2Δ.YDC1 to make IPCs (Fig. 6, lane 4 versus 2). Yet, 2Δ.LAG1 cells could not loose pBM150-*LAG1* on SDaaUA in the presence of 5′-fluoroorotic acid and high concentrations of inositol, even in the presence of 10% glycerol, which can act as a chemical chaperon (not shown). On the contrary, omission of inositol from synthetic medium (SD) diminished the growth rate of 2Δ.LAG1 and 2Δ.YDC1, while growth of 1Δ.YPC1 was independent of exogenous inositol (Fig. 5A).

Additional experiments showed that the notable cell wall thickening of *lac1Δ lag1Δ* cells described by Barz and Walter (1999) was not corrected in 2Δ.YDC1 (Fig. S4E and F versus Fig. S4C and D).

Lac1Δ lag1Δ cells cannot grow on AbA for prolonged periods

W303 *lag1Δ lac1Δ* and W303 *lag1Δ lac1Δ ypc1Δ ydc1Δ* grew reasonably well in the presence of high concentrations of AbA, an inhibitor of the IPC synthase *AUR1* (Nagiec *et al.*, 1997; Schorling *et al.*, 2001; Vallée and Riezman, 2005). As shown in Fig. 5A, right panels, when placed on LM or inositol-containing SDaaUA medium, those YPK9 cells having difficulty in making ceramide (2Δ.LAG1) or those making ceramide through reverse ceramidase activity (2Δ.YDC1) were relatively resistant to AbA. In contrast, wt YPK9 and 1Δ.YPC1 cells, making sphingolipids using an acyl-CoA-dependent ceramide synthase, were sensitive. To exclude the possibility that 2Δ.YDC1 cells did not take up AbA efficiently, we evaluated the effect of AbA on the incorporation of [³H]inositol into sphingolipids. As can be seen in Fig. 6, biosynthesis of IPCs in 2Δ.YDC1 and 2Δ.LAG1 cells was completely inhibited by AbA (Fig. 6, lanes 2, 4, 6, 8 versus 3, 5, 7, 9). This argues that the uptake of AbA in 2Δ.YDC1 and 2Δ.LAG1 strains is normal. Further studies however revealed that the AbA resistance of 2Δ.YDC1 and 2Δ.LAG1 is only relative. (i) Temperature appeared to be critical as 2Δ.LAG1 cells grew only poorly in the presence of AbA (0.25 µg ml⁻¹) at 30°C or 37°C (not shown). (ii) Other tests also showed that 2Δ.YDC1 were not resistant to very high concentrations of AbA (2.5 µg ml⁻¹, Fig. 5C) and that the cells growing on plates containing 0.25 µg ml⁻¹ AbA were not able to further grow when inoculated a second time on 0.25 µg ml⁻¹ AbA (Fig. 5D and not shown). Thus, AbA resistance of 2Δ.YDC1 cells was only relative, not absolute.

We wondered if the same is true for W303 *lag1Δ lac1Δ* (2Δ.W303) and W303 *lag1Δ lac1Δ ypc1Δ ydc1Δ* (4Δ.W303) cells. As can be seen on Fig. 5B, at 24°C the 2Δ.W303 cells were much more resistant to 0.25 µg ml⁻¹ AbA than the parental W303 wt strain, and 4Δ.W303, growing less well than 2Δ.W303, nevertheless grew as well on AbA as in its absence, albeit only at high cell density. At 37°C, without AbA, 2Δ.W303 and 4Δ.W303 grew almost as well as at 24°C, but their growth was more reduced in the presence of AbA than at 24°C. When higher concentrations of AbA were used (2.5 µg ml⁻¹), 2Δ.W303 and 4Δ.W303 still grew but 2Δ.W303 grew less than on 0.25 µg ml⁻¹ AbA (Fig. 5C). When we tried to propagate 2Δ.W303 and 4Δ.W303 cells having grown on 0.25 µg ml⁻¹ AbA at 24°C by replating them onto the same medium and incubating them at 24°C, they grew less well than when they had been put onto 0.25 µg ml⁻¹ AbA for the first time, although they grew very well in the absence of AbA (Fig. 5D). Replating 2Δ.W303 and 4Δ.W303 cells a third time on AbA demonstrated that cells continued to survive on 0.25 µg ml⁻¹ but did not grow at all on

A

Ceramides

B

Ceramides

C

C26:0-OH
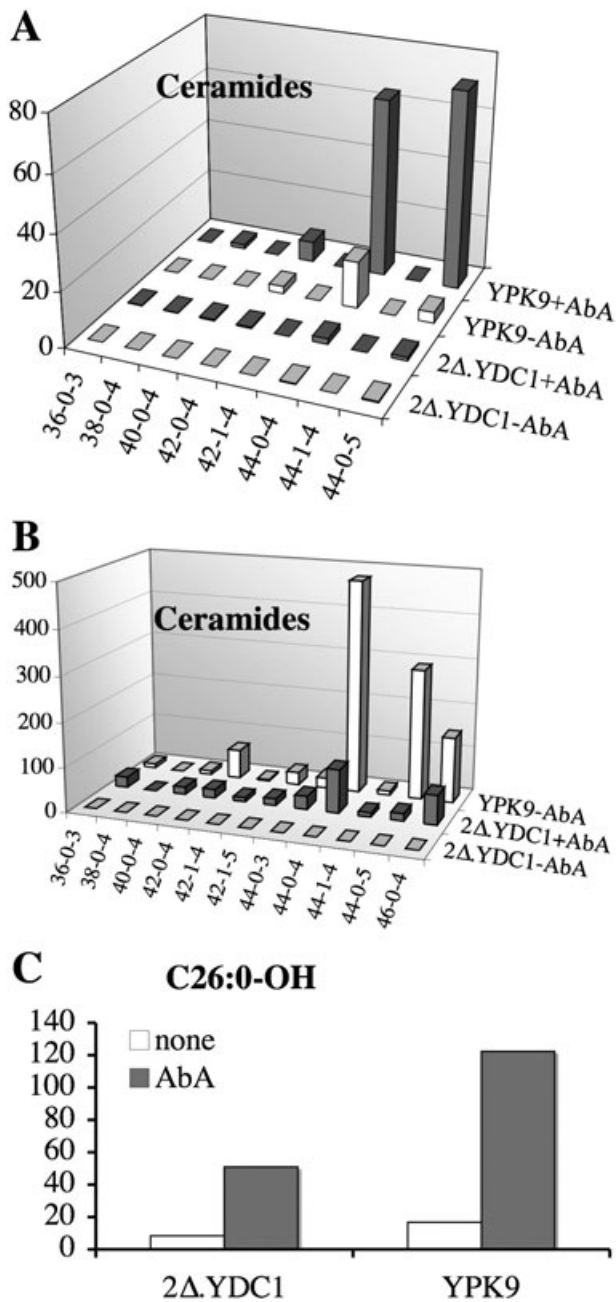
□ none
■ AbA

2Δ.YDC1   YPK9

Fig. 7. Ceramide levels in 2Δ.YDC1. Lipids were extracted from cells having been growing exponentially in YPD at 30°C, and grown further either in the presence or in the absence of 0.25 µg ml^{-1} AbA during 4 h at 30°C. Extracts were mixed with internal ^{13}C standard, deacylated and analysed by HPLC-ESI-MS/MS as in Fig. 4. Negative ions were scanned and screened for all theoretically possible ceramide species containing different saturated, monounsaturated, and non-, mono- or dihydroxylated fatty acids combined with DHS or PHS.
A and B. Ceramides shown in (A) and (B) are qualified by three numbers indicating the number of C atoms, double bonds and hydroxyls. (B) Some extracts were re-run using a HPLC program favouring detection of ceramides.
C. C26:0-OH levels (m/z = 411) of same cell extracts are depicted.
In (A) and (B) the x-axis only lists those ceramide species, which indeed were found to be present in at least one of the analysed strains. The vertical axis in all panels indicates ion counts × 10^{-3} per A$_{600}$ unit of cells.

2.5 µg ml^{-1} AbA (Fig. 5E). Thus, 2Δ.W303 and 4Δ.W303 are more resistant against AbA but they cannot be grown for prolonged periods on very high concentrations of AbA.

AbA does not cause the accumulation of ceramides in 2Δ.YDC1 cells

To understand if 2Δ.YDC1 stopped growing in the presence of AbA because of the accumulation of toxic levels of ceramides or for lack of IPCs, we measured ceramide levels in cells incubated with AbA at 30°C. In the experiment shown in Fig. 7A and B, the ceramides increased significantly during incubation with AbA in all strains, while during the same time IPC levels decreased (3.4- and 3-fold in YPK9 and 2Δ.YDC1, respectively, not shown). Importantly, ceramides were below the detection limit in 2Δ.YDC1 in the absence of AbA, i.e. the strain had at least 530 times less ceramide 44-4-0 than wt cells. Even when treated with AbA, the most prominent species appearing, 44-4-0, remained five times lower than the one of untreated wt cells (Fig. 7A and B). This probably reflects the fact that ceramide synthesis in 2Δ.YDC1 ceases as soon as ceramide concentrations reach a level, at which the free energy change (ΔG′) for ceramide synthesis by a reverse ceramidase activity becomes zero [ΔG′ = ΔG°′ + RT·ln([ceramide]/[fatty acids]·[DHS])]. In contrast, the ΔG°′ for the hydrolysis of acyl-CoAs is in the order of −35 kJ mol^{-1}, and in wt cells this energy is utilized by the acyl-CoA-dependent ceramide synthases Lag1p and Lac1p so that the ΔG′ of ceramide synthesis remains negative even when ceramide reaches very high concentrations. The very low ceramide levels in AbA-treated 2Δ.YDC1 cells strongly argue that these cells do not die from accumulating ceramides but rather from the absence of IPCs.

The lipid profiles also revealed that cells contained significant amounts of C26:0-OH (Fig. 7C). As Scs7p (Fig. 1), the α-hydroxylase for C26:0 fatty acids, is believed to utilize as substrates only ceramides or IPCs (Haak et al., 1997), the free C26:0-OH fatty acids must be breakdown products and this suggests that part of sphingolipids is continually hydrolysed even in the absence of AbA.

Curiously, titration of AbA showed that the 2Δ.YDC1 and 3Δ.YDC1 cells were more sensitive than wt at low concentrations of AbA (0.03 µg ml^{-1}) although they resisted AbA better than wt at high concentrations (0.3 µg ml^{-1}) (Fig. 8). We interpret these data in the sense that IPCs are essential for yeast cells and that, because of a less efficient ceramide and IPC biosynthesis, IPC levels become
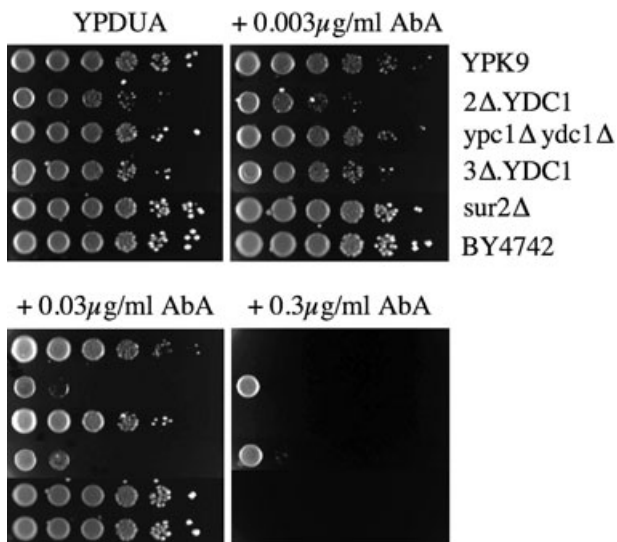
Fig. 8. 2Δ.YDC1 and 3Δ.YDC1 cells are hypersensitive to Aureobasidin A (AbA). Tenfold serial dilutions of indicated strains were plated on YPDUA plates containing various concentrations of AbA and incubated for 3 days at 24°C.

subcritical in 2Δ.YDC1 and 3Δ.YDC1 already at 0.03 μg ml^{-1} AbA, concentration at which wt cells continue to grow. At higher concentrations (0.3 μg ml^{-1}) of AbA however, wt cells acutely stop cell growth because of ceramide accumulation and this latter phenomenon does not occur in 2Δ.YDC1 and 3Δ.YDC1 cells (Figs 5A, 7 and 8). After prolonged growth at this concentration however, also 2Δ.YDC1 cells cease to grow because synthesis of IPCs is blocked or becomes limiting (Fig. 5D).

Synthesis of abnormal inositolphosphorylsphingoids in 4Δ.W303 is blocked by AbA

Metabolic labelling of 4Δ.W303 in the absence or presence of AbA with [³H]inositol demonstrated that these cells still make mild base-resistant inositolphosphorylsphingoids, and this in spite of the absence of all known enzymes catalysing ceramide synthesis (Fig. 9A, lane 3 versus 1). Some of this material migrates in the region of IPC-4 and IPC-5, whereas several more abundant and more polar lipids migrate between MIPC and M(IP)₂C (Fig. 9A, lanes 3 and 7 versus 1). In two different solvent systems it appears that there are at least five different mild base-resistant lipids made in 4Δ.W303 (Fig. 9A, lanes 3 and 7; Fig. 9B, lanes 2 and 6). Importantly, the synthesis of these abnormal inositolphosphorylsphingoids is inhibited by AbA (Fig. 9A, lanes 5 and 9 versus 3 and 7; Fig. 9B, lanes 4 and 8 versus 2 and 6). This suggests that the growth of 4Δ.W303 is blocked by AbA (Fig. 5E) since the drug blocks the biosynthesis of essential inositolphosphorylsphingoids which can functionally replace IPCs. The biosynthetic pathway for generation of these various

abnormal sphingolipids apparently only exists in the genetic background of W303, not in the one of YPK9 (Fig. 9, lanes 3 and 7 versus Fig. 2, lane 8).

Accumulation of LCBs is not toxic but rather essential for growth of 2Δ.YDC1 cells

The accumulation of endogenous LCBs or the addition of exogenous LCBs to the growth medium has been reported to be toxic for certain strains, whereby PHS is
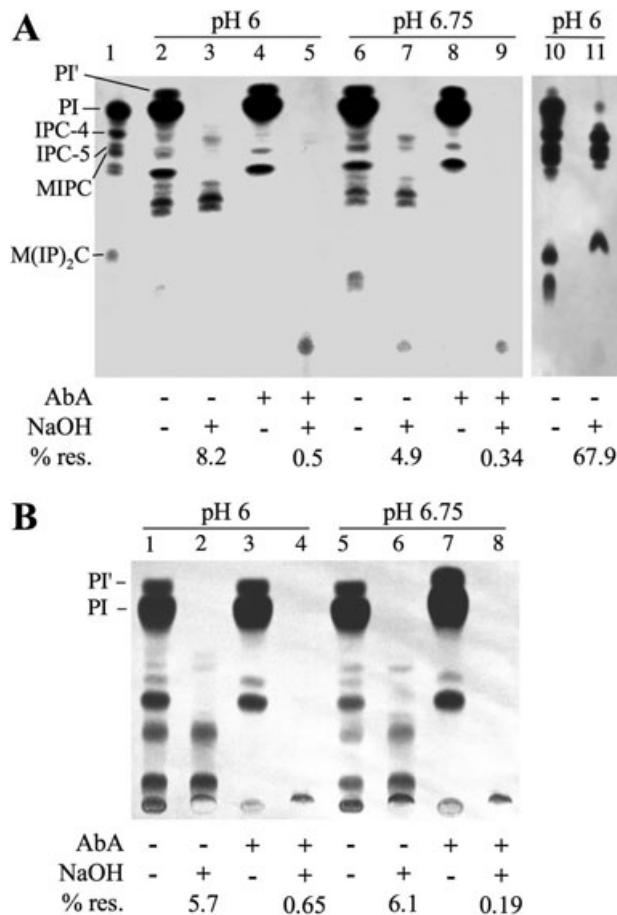

Fig. 9. Synthesis of abnormal inositolphosphorylsphingoids in 4Δ.W303 is blocked by Aureobasidin A (AbA).
A. Wild-type (lane 1) or 4Δ.W303 cells (lanes 2–9) were grown in Lester medium (lanes 2–5) or LM adjusted to pH 6.75 with 3-(*N*-Morpholino)-propanesulphonic acid (MOPS) (lanes 6–9), and labelled with [³H]inositol in inositol-free LM for 4 h at 30°C, either in the presence or in the absence of AbA (2.5 μg ml^{-1}). [Medium with pH 6.75 was utilized because of the difficulty of sphingolipid-deficient strains to maintain the cytosolic pH in acidic media (Patton *et al.*, 1992).] Lipids were extracted, deacylated with NaOH or mock incubated, desalted and analysed by TLC in solvent system 2, radioscanning and fluorography. Figures indicate the amount of base-resistant lipids present after NaOH treatment as percentage of the total c.p.m. present in the not deacylated lipid extract. For comparison, when parental W303 cells were labelled under identical conditions, 68% of radioactivity was in mild base-resistant lipids (not shown).
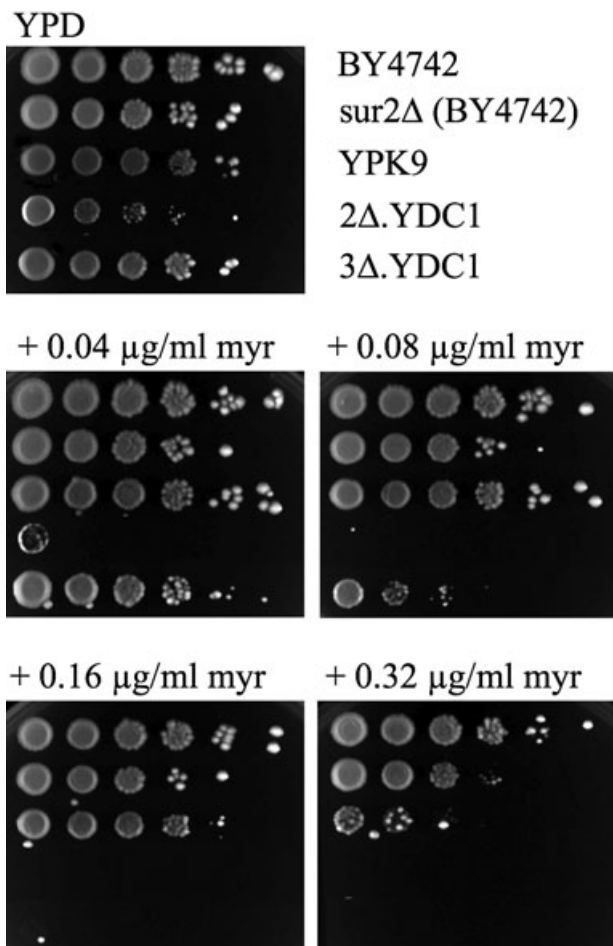B. As (A), but TLC separation was performed in solvent system 3.

Fig. 10. 2Δ.YDC1 and 3Δ.YDC1 cells are hypersensitive to myriocin. Tenfold dilutions of the various strains were deposited onto plates containing between 0 and 0.32 μg ml⁻¹ myriocin (myr) plus 0.5% CH₃OH. Plates were incubated at 30°C for 3 days.

more toxic than DHS (Mao *et al.*, 2000b; Chung *et al.*, 2001). Possible reasons may be that PHS causes the inhibition or degradation of essential nutrient transporters (Skrzypek *et al.*, 1998; Chung *et al.*, 2000; 2001), or that PHS compromises glucan biosynthesis (Abe *et al.*, 2001). As shown in Table 1, 2Δ.YDC1 cells have very high levels of LCBs, levels that are in the same range as those reported for W303 *lag1Δ lac1Δ* cells (Guillas *et al.*, 2001). To address the question as to whether the high concentrations of PHS in 2Δ.YDC1 (Table 1) may contribute to the slower growth of these cells, we tested if the growth of 2Δ.YDC1 could be improved by partial inhibition of LCB biosynthesis in the presence of myriocin (Fig. 1). As seen in Fig. 10, myriocin did not improve the growth of 2Δ.YDC1 cells, quite to the contrary: 2Δ.YDC1 cells were extremely hypersensitive to myriocin and even 3Δ.YDC1 were hypersensitive. A likely explanation is that the very high levels of DHS in those cells are required in order to grant a negative ΔG′ for reverse ceramidase, i.e. ceram-

ide synthesis by Ydc1p, and that any reduction of ceramide and therefore IPC biosynthesis causes IPC levels to become subcritical. Furthermore, YPK9, 2Δ.YDC1 and 3Δ.YDC1 cells were completely resistant to 25 μM DHS (Fig. S5, top) or 25 μM PHS (not shown), which ought to further elevate LCB levels in the cells.

A recent study demonstrates that exogenous and endogenous LCBs form functionally different intracellular pools (Brace *et al.*, 2007). To see whether exogenous LCBs can access Ydc1p for formation of sphingolipids, we tested whether 2Δ.YDC1 can survive on exogenous LCBs when endogenous LCB biosynthesis is blocked by myriocin. Addition of exogenous DHS could only partially compensate the myriocin hypersensitivity of 2Δ.YDC1 and 3Δ.YDC1 cells (Fig. S5), i.e. only at very low concentrations of myriocin (0.1 μg ml⁻¹). One explanation is to assume that the uptake mechanism cannot concentrate DHS sufficiently to force reverse ceramidase activity, at least not in the pool of LCBs, which is accessible for Ydc1p. Yet, at higher concentrations of myriocin, also wt cells died, possibly because of the same insufficiency of LCB uptake or because of non-specific toxicity of the drug.

Discussion

Before its target was known, it was established that AbA at high concentrations kills 99% of wt yeast cells within 1 h of exposure (Endo *et al.*, 1997), but subsequent studies showed that *lag1Δ lac1Δ* cells can grow in the presence of AbA and proposed that IPCs may not be required for the growth of yeast cells (Schorling *et al.*, 2001; Vallée and Riezman, 2005). Our data confirm, using newly made strains, this previously reported AbA resistance of W303 *lag1Δ lac1Δ* and W303 *lag1Δ lac1Δ ypc1Δ ydc1Δ* cells at 24°C and show that high concentrations of AbA (0.3 μg ml⁻¹) also are not immediately arresting the growth at 24°C of *lag1Δ lac1Δ* double mutants in the more sensitive YPK9 background. These previous and our present findings are most reasonably explained by assuming that high ceramide levels cause a rapid growth arrest in wt cells containing acyl-CoA-dependent ceramide synthases. Yet, our results now indicate that prolonged exposure to AbA or exposure to higher concentrations of AbA stops the growth of cells of all genetic backgrounds, even those that lack acyl-CoA-dependent ceramide synthases and therefore do not accumulate ceramides in the presence of AbA (Figs 5B–E and 6A). Moreover, in the presence of AbA, 2Δ.YDC1 cells abruptly stop the biosynthesis of IPCs (Fig. 6), while their ceramide levels remain very low (Fig. 7A). These data argue therefore that AbA deprives 2Δ.YDC1 cells of IPCs and clearly points to the importance of IPCs for the survival of YPK9 cells. Data further suggest that this essential role of IPCs can be

taken over by anomalous inositolphosphorylsphingoids, which also seem to be made by an *AUR1*-dependent pathway (Fig. 9). On the other hand, the ceramide levels of 3Δ.YDC1 cells are extremely low, and yet, the cells show normal growth and stress resistance; this argues that ceramides are probably not required as signalling molecules during the stresses tested. Interestingly, a series of seminal studies performed 15 years ago led to a similar conclusion, namely that yet another kind of anomalous lipids can take over the role of sphingolipids. These reports showed that sphingolipids are altogether dispensable for certain yeast cells harbouring a gain of function suppressor allele in the lyso-phosphatidate acyl-transferase *SLC1* (Nagiec *et al.*, 1993). The *SLC1-1* allele allows *lcb1Δ* cells to synthesize PI with a C26 fatty acid at the *sn*-2 position of the glycerol moiety, a form of PI that subsequently can be mannosylated in the same way as IPCs, whereas PI of wt cells does not get mannosylated (Fig. 1) (Lester *et al.*, 1993). It is very likely that this C26-containing form of PI can functionally replace IPCs in their essential roles.

It may be hypothesized that the high concentrations of AbA required to stop the growth of 2Δ.W303 and 4Δ.W303 cells (2.5 μg ml⁻¹) are exerting non-specific toxic effects. While theoretically possible, this seems to be rather unlikely because various single-point mutations in *AUR1* were reported to make wt cells resistant to very high concentrations (> 20 or 25 μg ml⁻¹) of AbA, i.e. 100- to 500-fold more resistant than wt cells, and this without affecting their sensitivity to other drugs (Heidler and Radding, 1995; Hashida-Okado *et al.*, 1996).

Acute AbA intoxication leads to a defect in bud growth and release of vacuolar and cytosolic amino acids into the medium indicating a loss of the vacuolar and plasma membrane barriers, a loss that cannot be relieved by osmotic support (Endo *et al.*, 1997). Thus, IPCs or IPC-like sphingolipids may just be required for adequate membrane structure, but they may also have more specific roles in GPI protein and membrane protein transport (Watanabe *et al.*, 2002; Gaigg *et al.*, 2005; 2006) and stabilization of membrane channels (Lee *et al.*, 2002; Chung *et al.*, 2003). Depletion of Aur1p also leads to the disappearance of microtubuli and of tubulin, but it is not clear if this is due to an increase of ceramide or a decrease of IPCs (Hashida-Okado *et al.*, 1996).

YPC1 and *YDC1* were originally identified in a screen for multicopy suppressors of the growth inhibition caused by the lack of acyl-CoA-dependent ceramide synthase activity, and were readily recognized as genes responsible for an alternative, acyl-CoA-independent ceramide biosynthesis pathway (Mao *et al.*, 2000a,b) (Fig. 1). The present study further characterizes the enzymatic potential of Ydc1p in the YPK9 genetic background where the acyl-CoA-dependent ceramide synthesis was genetically

inactivated and no alternative sphingolipid biosynthesis pathways are operating (Fig. 2). In this context our data first demonstrate that Ydc1p can efficiently synthesize ceramides *in vivo*. Indeed, it has previously been assumed that Ydc1p has little reverse ceramidase activity. This assumption was based on its low reverse ceramidase activity *in vitro* as measured with [³H]C16:0 as a substrate (Mao *et al.*, 2000b), on the fact that only *YPC1* but not *YDC1* was isolated in two independent screens for high-copy suppressors of a *lag1Δ lac1Δ* growth defect (Schorling *et al.*, 2001; Jiang *et al.*, 2004) and from the comparison of the growth behaviour of *YPC1*- versus *YDC1*-complemented *lag1Δ lac1Δ* strains. Data shown here demonstrate that Ydc1p can produce significant amounts of sphingolipids *in vivo* and can, when overexpressed, restore a normal growth and stress resistance to 3Δ.YDC1 cells. Moreover, data in Figs 3 and 4 document that *in vivo* Ydc1p utilizes only DHS, not PHS, as was found *in vitro* (Mao *et al.*, 2000b). Furthermore, Ydc1p can use fatty acids of any length between C18 and C26 with similar efficiency. It is noteworthy that the various ceramides of 2Δ.YDC1 cells comprise the major ceramide species encountered in mammalian cells so that 2Δ.YDC1 cells, by offering appropriate ceramide substrates, may be a suitable host for the functional expression of mammalian sphingolipid-synthesizing enzymes.

Data shown in Fig. 5A indicate that the reported relative inositol auxotrophy of *lcb1Δ SLC1-1* lacking all LCBs is also present in strains which have a deficiency in ceramide biosynthesis (2Δ.YDC1 and glucose-grown 2Δ.LAG1) and which therefore contain very high concentrations of LCBs. Thus it would appear that inositol is not required to compensate for the lack of PHS-mediated signalling through the Pkh1p/Pkh2p signalling pathway (Dickson *et al.*, 2006) or for the lack of signalling through PHS-1-phosphate, but for some other function. Removal of inositol from the growth medium is known to upregulate the unfolded protein response (UPR) (Cox *et al.*, 1997) and to inactivate Opi1p, a transcriptional repressor for numerous genes involved in lipid biosynthesis (Loewen *et al.*, 2004). A UPR response, caused by the lack of inositol in the medium, may be harmful in a situation where sphingolipid biosynthesis is restricted. In contrast, addition of inositol to the growth medium downregulates the UPR and strongly increases PI biosynthesis (Gaspar *et al.*, 2006), not only by providing more substrate for the PI synthase Pis1p, but possibly also by downregulating *PSD1* and *OPI3*, which draw, as Pis1p, on the primal substrate CDP-diacylglycerol (Santiago and Mamoun, 2003). Addition of inositol to the growth medium has also been reported to increase the IPC synthase activity twofold (Ko *et al.*, 1994). The increased biosynthesis of PI, the substrate of Aur1p (Fig. 1) and the increased Aur1p activity may thus stimulate IPC

Table 2. Strains and plasmids.

Name	Genotype/type and markers	Source/previous name
Strains		
YPK9	*MAT***a** *ade2-101*[ochre] *his3-Δ200 leu2-Δ1 lys2-801*[amber] *trp1-Δ63 ura3-52*	Jiang *et al.* (1998)
YPK11	Same as YPK9, but *MAT*α	M. Jazwinski
2Δ.LAG1	Same as YPK9 but *lac1Δ::LEU2 lag1Δ::TRP1* containing plasmid pBM150-*LAG1*	Guillas *et al.* (2001)
1Δ.YPC1	YPK9 *lag1Δ::TRP1* kan[r] containing plasmid pPK183 for overexpression of Ypc1p	M. Jazwinski
1Δ.YPC1 sur2Δ (FBY1209)	Same as 1Δ.YPC1 but containing also *sur2::KanMX4*	This study
2Δ.YDC1	YPK9 *lag1Δ::TRP1 lac1Δ::URA3* containing plasmid pJJ26 for overexpression of Ydc1p	M. Jazwinski
3Δ.YDC1 (FBY1220)	Same as 2Δ.YDC1, but containing also *sur2::kanMX4*	This study
ypc1Δ ydc1Δ (FBY1224)	Same as YPK9 but *ydc1::natMX ypc1::kanMX4*	This study
BY4742	*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCARF collection
sur2Δ	Same as BY4742, but containing *sur2::kanMX4*	EUROSCARF
vps4Δ	Same as BY4742, but containing *vps4::kanMX4*	EUROSCARF
W303-1A	*MAT***a** *can1-100 ade2 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Guillas *et al.* (2001)
2Δ.W303-clone 17	*can1-100 ade2 his3-11,15 leu2-3,112 trp1-1 ura3-1 lac1::LEU2 lag1::TRP1*	This study
2Δ.W303-clone 19	*can1-100 ade2 his3-11,15 leu2-3,112 trp1-1 ura3-1 lac1::LEU2 lag1::TRP1*	This study
4Δ.W303 (FBY958)	*can1-100 ade2 his3-11,15 leu2-3,112 trp1-1 ura3-1 lac1::LEU2 lag1::TRP1 ydc1::natMX ypc1::kanMX4*	This study
Plasmids		
pPK183	2 μ, *YPC1* behind its own promoter, *LEU2*	Jiang *et al.* (2004)
pJJ26	2 μ, *YDC1* behind its own promoter, *LEU2*	Jiang *et al.* (2004)
pBM150-*LAG1* named pPB5 in Jiang *et al.* (2004)	*ARS1 CEN4 URA3 GAL1*[UAS]-*LAG1*	Jiang *et al.* (1998)

biosynthesis and thereby increase the robustness of 2Δ.YDC1 cells.

Our studies also shed some light on the potential physiological roles of *YPC1* and *YDC1*. Although their physiological expression levels are too low as to allow cell survival of YPK9 *lag1Δ lac1Δ* cells, they markedly enhance growth of 2Δ.W303 cells, in that these cells grow much better than 4Δ.W303 (Fig. 5B). Thus, they may contribute to ceramide biosynthesis if Lac1p and Lac1p are not operational. Under natural conditions *YPC1* and *YDC1* may help cells to grow when they encounter inhibitors such as Australifungin or Fumonisin B1 (Wang *et al.*, 1991; Mandala *et al.*, 1995) by providing reverse ceramidase activity. As to their role as ceramidases, *ypc1Δ* cells and *ydc1Δ* cells were reported to contain significantly more labelled IPCs after [³H]palmitate labelling, suggesting that under physiological conditions, Ypc1p and Ydc1p may continuously degrade part of ceramides, which otherwise would be used for IPC biosynthesis (Mao *et al.*, 2000b). The presence of free C26:0-OH in wt cells reported here suggests that normal cells degrade some of their ceramides and that this degradation can be increased in the presence of AbA (Fig. 7C). *YPC1* and *YDC1* may have also some non-overlapping functions, as their deletion does not generate the same phenotype, as they show different genetic interactions and as they are oppositely regulated by calcineurin (Mao *et al.*, 2000b; Yoshimoto *et al.*, 2002; Schuldiner *et al.*, 2005).

Our data establish, clarify or confirm numerous aspects concerning the specificity of the enzymes involved in sphingolipid biosynthesis in yeast, which are mainly interesting to the specialist. These aspects are detailed in *Supporting information*.

Further studies will be required to fully appreciate the roles of *YPC1* and *YDC1* in sphingolipid homeostasis of eukaryotes.

Experimental procedures

Yeast strains, media, reagents

Strains used in this study are listed in Table 2. Cells were grown on rich medium (YPD, YPG) or defined minimal media (SD, SG) containing 2% glucose (D) or galactose (G) as a carbon source and uracil (U), adenine (A) and amino acids (aa) at 30°C (Sherman, 2002). Media contained inositol unless indicated otherwise. Alternatively, LM, a medium specially designed for cells unable to make sphingolipids, was used (Pinto *et al.*, 1992). Myriocin was from Sigma, St Louis, MO, AbA from Takara Shuzo (Shiga, Japan).

Construction of strains

W303 *lag1Δ lac1Δ* cells proved to be highly unstable as many clones, when labelled with [³H]inositol, displayed lipid profiles quite different from the one previously found in the original W303 *lag1Δ lac1Δ* strain and contained some new microsomal activity allowing to acylate [³H]DHS through an uncharacterized pathway (Guillas *et al.*, 2001). Some clones started to produce a major mild base-resistant lipid co-migrating with M(IP)$_2$C and/or ceased to make the residual amounts of IPC/C, IPC/D, MIPC or the abnormal lipids a and b that had

been found in the original W303 *lag1Δ lac1Δ* cells (not shown). We concluded that these cells rapidly got outraced by spontaneous suppressors, which survived freezing and thawing better than unsuppressed cells, and that W303 *lag1Δ lac1Δ* cells were genetically unstable and unsuitable to study the effects of overexpression of *YDC1*. We therefore decided to work in the YPK9 background, in which the *lag1Δ lac1Δ* double deletion is lethal (Jiang *et al.*, 1998), but which can be maintained by pBM150-*LAG1*, a centromeric plasmid bearing *LAG1* under the control of the *GAL1* promoter (Guillas *et al.*, 2001). The YPK9 *lag1Δ lac1Δ* pBM150-*LAG1* (2Δ.LAG1) strain grows at a normal rate on galactose and therefore is probably not accumulating suppressors; when put on glucose-containing media, cells undergo numerous cell divisions but then switch to very slow growth. The YPK9 *lag1Δ lac1Δ* rescued by overexpression of *YDC1* (2Δ.YDC1) strain was received from M. Jazwinski and constructed by deleting *LAC1* in YPK9 *lag1Δ* overexpressing *YDC1* from plasmid pJJ26 (Jiang *et al.*, 2004). To obtain the YPK9 *lag1Δ lac1Δ sur2Δ* strains overexpressing *YDC1*, we transfected a *sur2*::kanMX4 cassette amplified from BY4742 *sur2*::kanMX4 (EUROSCARF) into 2Δ.YDC1. PCR was used to prepare *ydc1*::natMX and *ypc1*::kanMX4 deletion cassettes. To obtain the W303 *lag1Δ lac1Δ ypc1Δ ydc1Δ* strain, we deleted *YPC1* and *YDC1* in a W303 *lag1Δ lac1Δ* containing a galactose-inducible *LAG1* gene on the plasmid pBM150-*LAG1*. The resulting W303 *lag1Δ lac1Δ ypc1Δ ydc1Δ* pBM150-*LAG1* strain was grown in liquid LM with uracil for several weeks. (5-Fluoroorotic acid counter-selection does not work on LM.) After plating we isolated W303 *lag1Δ lac1Δ ypc1Δ ydc1Δ* strains, which were uracil auxotroph and in which PCR analysis showed that the pBM150-*LAG1* had been lost. All deletions were verified by PCR.

Cell labelling, lipid extraction, MMA treatment, NaOH treatment and TLC

Unless stated otherwise, metabolic radiolabelling, cell disruption, lipid extraction and lipid analysis were performed as described (Reggiori *et al.*, 1997; Guillas *et al.*, 2001). Ascending TLC on silica 60 gel glass plates was performed with the following solvents: solvent 1 = chloroform : methanol : NH$_4$OH 40:10:1; solvents 2 and 3 = chloroform : methanol : 0.25% KCl at ratios of 55:45:10 and 55:45:5 respectively. Radioactivity was detected by fluorography and quantified by one- or two-dimensional radioscanning in a Berthold radioscanner.

Quantification of LCBs

Quantification of LCBs was performed as described (Merrill *et al.*, 1988).

Mass spectrometry analysis of lipid extracts

Exponentially growing cells (A$_{600}$ of 1–2) were extracted as described (Hanson and Lester, 1980), using procedure IIIB. At this stage an equivalent amount of a similar lipid extract from wt cells grown in [^{13}C]glucose, supplemented with [^{12}C]inositol, was added as an internal standard. Aliquots in CHCl$_3$-CH$_3$OH were heated and stirred at 65°C for 15 min to solubilize M(IP)$_2$C and immediately injected into a normal phase PVA SIL HPLC column (YMC Europe GmbH, D-46514 Scherbeck, Germany, 1 × 150 mm) eluted at 45 µl min^{-1}. Solvents A, hexane-isopropanol (98:2); B, CHCl$_3$-isopropanol (65:35); C, CH$_3$OH were changed linearly over time to give ratios A : B : C as follows: 0 min 100:0:0; 22 min 12:88:0; 30 min 10:74:16; 45 min 8:61:31; 50 through 65 min 0:0:100; 70 through 72.5 min 0:100:0; 75 through 82.5 min 100:0:0. To improve sensitivity of ceramide detection the gradient was modified to 0 min 12:88:0; 3 min 10:74:16; 4 min 8:61:31; 5 through 11 min 0:0:100; 13 min 0:100:0; 17 through 24 min 12:88:0. Ions in the effluent were ionized by electrospray ionization with an electrode potential of 3500 volt and the masses of negative ions were detected by a Bruker Esquire-LC quadrupole ion trap mass spectrometer. The signals from the well-known and well-resolved lipids of the ^{13}C internal standard were used to normalize the intensities of ^{12}C signals derived from the same or closely related lipids of the test extract being analysed. Total ion counts represent the integration of the particular ion throughout the elution region. For detection of IPCs and ceramides, lipid extracts were deacylated using methanolic NaOH and desalted. For glycerophospholipid and sphingolipid detection, aliquots corresponding to 0.01 and 0.15–1.0 A$_{600}$ units of cells were injected respectively.

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