# Hydroxylation state of fatty acid and long-chain base moieties of sphingolipid determine the sensitivity to growth inhibition due to AUR1 repression in Saccharomyces cerevisiae

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**Hydroxylation state of fatty acid and long-chain base moieties of sphingolipid determine the sensitivity to growth inhibition due to *AUR1* repression in *Saccharomyces cerevisiae***

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Abbreviations: Cer, ceramide; Dihydrosphingosine, DHS; Dihydrosphingosine 1-phosphate, DHS1P; Dox, doxycycline; IPC, inositol phosphorylceramide; LCB, long-chain base; MIPC, mannosylinositol phosphorylceramide; $M(IP)_2C$, mannosyldiinositol phosphorylceramide; MMA, monomethylamine; MSG, L-glutamic acid sodium salt hydrate; phytosphingosine, PHS

**ABSTRACT**

The structures of ceramide found in the yeast *Saccharomyces cerevisiae* are classified into five groups according to the hydroxylation states of the long-chain base and fatty acid moieties. This diversity is created through the action of enzymes encoded by *SUR2*, *SCS7*, and as yet unidentified hydroxylation enzyme(s). Aur1p is an enzyme catalyzing the formation of inositol phosphorylceramide in the yeast, and the defect leads to strong growth inhibition due to accumulation of ceramide and reductions in complex sphingolipid levels. In this study, we found that the deletion of *SCS7* results in the enhancement of growth inhibition due to repression of *AUR1* expression under the control of a tetracycline-regulatable promoter, whereas the deletion of *SUR2* attenuates the growth inhibition. Under *AUR1*-repressive conditions, *SCS7* and *SUR2* mutants showed reductions in the complex sphingolipid levels and the accumulation of ceramide, like wild-type cells. On the other hand, the deletion of *SCS7* had no effect on the growth inhibition through reductions in the complex sphingolipid levels caused by repression of *LIP1* encoding a ceramide synthase subunit. Furthermore, the deletion of *SUR2* did not suppress the growth inhibition under *LIP1*-repressive conditions. Therefore, it is suggested that the deletion of sphingolipid hydroxylases changes the toxicity of ceramide under *AUR1*-repressive conditions.

Key words: Sphingolipid, ceramide, *Saccharomyces cerevisiae,* hydroxylation, *SCS7*, *SUR2*

## INTRODUCTION

Sphingolipids, the major lipid components of the eukaryotic plasma membrane, play critical roles in many physiologically important events, such as signal transduction, membrane trafficking, and cell-to-cell interaction [1; 2]. Ceramide (Cer), the backbone of sphingolipids, is now recognized as an intracellular lipid second messenger that regulates various signal transduction systems [3]. Cer comprises a long-chain base (LCB) attached to a fatty acid via an amide bond. In mammalian tissues, Cer exhibits great structural diversity and complexity due to the chain lengths, degrees of saturations, and hydroxylation states of fatty acids and LCBs [4]. In contrast to the complexity of the Cer structure in mammals, the Cer species variation in yeast *Saccharomyces cerevisiae* is relatively simple. In yeast, the chain length of fatty acids in Cers is primarily C26. According to the hydroxylation state, the Cer in yeast can be classified into five types (types A, B, B', C, and D) (Fig. 1) [5]. Cer-A contains dihydrosphingosine (DHS) and a C26 fatty acid. Cer-A can be hydroxylated at the C-4 position of the DHS moiety by a sphingolipid hydroxylase, Sur2p, being converted into Cer-B [6]. Alternatively, Sur2p can hydroxylate DHS into phytosphingosine (PHS), which is then converted to Cer-B. Scs7p catalyzes the hydroxylation of the C-2 position fatty acid in Cer-A and Cer-B, Cer-B' and Cer-C being yielded, respectively [6]. Cer-D is generated through further hydroxylation, at an unknown position, of the fatty acid of Cer-C. The synthesis of Cer-D requires *CCC2* encoding a putative copper transporter [7], but the hydroxylase involved in this synthesis remains to be identified. Due to the limited molecular subtypes, yeast is a useful model for investigating the relationship between the structural diversity of Cer and its physiological function.

Yeast has three classes of complex sphingolipids, IPC (inositol phosphorylceramide), MIPC (mannosylinositol phosphorylceramide), and $M(IP)_2C$ (mannosyldiinositol phosphorylceramide), all of which include phosphoinositol. IPC, the simplest complex

sphingolipid in yeast, is formed by IPC synthase (Aur1p), an enzyme catalyzing the transfer of the head group of phosphatidylinositol to Cers (Fig. 1) [5; 8]. Repression of Aur1p causes strong growth inhibition due to accumulation of Cers and reductions of all complex sphingolipid levels [8; 9; 10]. In a previous study[11], we screened yeast mutant strains showing resistance to aureobasidin A, a specific inhibitor of Aur1p [8; 12]. It was found that deletion of *ELO3*, which is involved in the elongation of fatty acids for the synthesis of C26 fatty acids, confers resistance to growth inhibition caused by Cer accumulation under *AUR1*-repressive conditions, implying that the chain lengths of fatty acids in Cer are a critical factor for the Cer-induced growth defect in yeast [11]. In the present study, we found that deletion of a Cer hydroxylase, *SCS7* or *SUR2*, greatly affects the growth inhibition induced by *AUR1* repression. This indicated a relationship between the hydroxylation states of fatty acids and long-chain base moieties of Cer and the sensitivity of cells to *AUR1* repression.

## MATERIALS AND METHODS

***Yeast Strains and Media*** – The *S. cerevisiae* strains used are listed in Table 1. Disruption of *SCS7, SUR2,* and *CCC2* was performed by replacing their open reading frames with the *URA3* marker from pRS406 [13]. Disruption of *SUR2* was also performed by replacing its open reading frame with the *hphNT1* marker from pFA6a-hphNT1 [14]. To provide uniform auxotrophic conditions, *ura3* cells were transfected with the *URA3* fragment, thereby generating *URA3* cells. Disruption of *LCB4* was performed by replacing its open reading frame with the *natMX4* marker from p4339 (pCRII-TOPO::natMX4) [15]. The cells were grown in either YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or synthetic complete (SC/MSG) medium (0.17% yeast nitrogen base w/o amino acids and ammonium sulfate (BD Difco, Heidelberg, Germany), 0.1% L-glutamic acid sodium salt hydrate (MSG; Sigma), and 2% glucose) containing nutritional supplements [15].

***[$^3$H]myo-inositol Labeling*** – Yeast cells grown in YPD medium were collected by centrifugation and then washed with SC/MSG medium containing minimum inositol (2 μg/ml). Then the cells were resuspended in 500 μl of SC/MSG medium containing minimum inositol to 2 $A_{600}$ units/ml and then labeled with [$^3$H]*myo*-inositol (1 μCi/1 $A_{600}$ unit of cells; PerkinElmer Life Sciences, Norwalk, CT) for 1 h at 30°C. The cells were chilled on ice, collected by centrifugation, washed with distilled water, and then suspended in 150 μl of ethanol/water/diethylether/pyridine/15 N ammonia (15:15:5:1:0.018, v/v). Radioactivity was measured using a liquid scintillation system, and samples exhibiting equal radioactivity were used for further experiments. After 15-min incubation at 60°C, the samples were centrifuged at 10,000 x g for 1 min and the supernatants were withdrawn. Lipids were extracted from the residues once more in the same manner. The resulting supernatants were dried. For mild alkaline treatment, the lipid extracts were dissolved in 150 μl monomethylamine (MMA; 40% methanol

solution)/water (10:3, v/v) incubated for 1 h at 53°C, and then dried. The lipids were suspended in 20 μl of chloroform/methanol/water (5:4:1, v/v) and then separated on Silica Gel 60 TLC plates (Merck, Whitehouse Station, NJ) with chloroform/methanol/4.2 N ammonia (9:7:2, v/v) as the solvent system.

***[$^3$H]DHS Labeling -*** Yeast cells grown in YPD medium were collected by centrifugation and then resuspended in 600 μl of fresh YPD medium to 1.7 $A_{600}$ units/ml. 0.5 μCi [4,5-$^3$H]DHS (American Radiolabeled Chemical Inc., St. Louis, MO) in 167 μl of YPD medium containing 4 mg/ml BSA was added to the cultures, followed by incubation for 1 h at 30°C. The cells were chilled on ice, collected by centrifugation, and then washed with YPD medium containing 1 mg/ml BSA. The lipids were extracted, subjected to mild alkaline treatment, and then separated by TLC as described under "*[$^3$H]myo-inositol Labeling*".

***[$^{14}$C]serine Labeling –*** Yeast cells were cultured overnight in YPD medium, diluted (0.2 $A_{600}$ units/ml) in fresh YPD medium with or without 10 μg/ml doxycycline (Dox), and then incubated for 4 h. Cells were collected by centrifugation, washed with SC/MSG medium lacking serine, resuspended in SC/MSG medium lacking serine with or without 10 μg/ml Dox, and then incubated at 30°C for 1 h. Cells were collected by centrifugation and then resuspended in 700 μl of fresh SC/MSG medium lacking serine with or without 10 μg/ml Dox to 1 $A_{600}$ units/ml. Then the cells were labeled with L-[U-$^{14}$C]serine (0.7 μCi/1 $A_{600}$ unit of cells; PerkinElmer Life Sciences, Norwalk, CT) for 5 h at 30°C. The lipids were extracted and subjected to mild alkaline treatment as described under "*[$^3$H]myo-inositol Labeling*". The extracted lipids were separated on Silica Gel 60 TLC plates with chloroform/methanol/acetic acid (100:5.2:0.58, v/v) as the solvent system, and then analyzed with a Bio Imaging analyzer FLA-2000 (Fuji Photo Film, Kanagawa, Japan).

## RESULTS

### *Effect of deletion of sphingolipid hydroxylases on growth inhibition by AUR1-repression*

To determine whether or not hydroxylation of Cers affects the growth inhibition caused by *AUR1* repression, we used a mutant strain that carries the *AUR1* gene under the control of a tetracycline-regulatable (Tet) promoter (*tet-AUR1*) [11; 16]. *SCS7* and *SUR2,* sphingolipid hydroxylase genes, and *CCC2*, a putative copper transporter gene involved in Cer-D synthesis, were deleted in *tet-AUR1* cells, *tet-AUR1 scs7*Δ, *tet-AUR1 sur2*Δ, *tet-AUR1 sur2*Δ *scs7*Δ, and *tet-AUR1 ccc2*Δ cells being generated. As shown in Fig. 2A, the growth of *tet-AUR1* was inhibited in a concentration-dependent manner by 0.2 to 10 μg/ml doxycycline (Dox). Dox-treated *tet-AUR1 scs7*Δ cells exhibited more severe growth inhibition than Dox-treated *tet-AUR1* ones; that is, even in the presence of a low concentration of Dox (0.2 μg/ml), significant growth inhibition was observed in *tet-AUR1 scs7*Δ cells but not for *tet-AUR1* cells. In contrast, *tet-AUR1 sur2*Δ cells showed a significant resistance to Dox; that is, the strain could still grow in the presence of 10 μg/ml Dox. *tet-AUR1 scs7*Δ *sur2*Δ cells showed a similar growth inhibition pattern to *tet-AUR1* cells in the presence of Dox, indicating that the deletion of *SCS7* and *SUR2* counteracted the growth inhibition. Deletion of *CCC2* had no effect on the growth inhibition. Fig. 2B shows the time courses of growth of *tet-AUR1*, *tet-AUR1 scs7*Δ, *tet-AUR1 sur2*Δ, *tet-AUR1 sur2*Δ *scs7*Δ, and *tet-AUR1 ccc2*Δ cells in the presence or absence of Dox. The growth rate of *tet-AUR1* cells began to slow down at 6-9 h after the addition of 10 μg/ml Dox, and was affected by the deletion of *SCS7* or *SUR2* in the presence of Dox. These alterations of growth rate well coincide with the results of a spot test (Fig. 2A).

### *Sphingolipid metabolism of a sphingolipid hydroxylase-deleted mutant under AUR1-repressive conditions*

To determine the complex sphingolipid levels in Dox-treated *tet-AUR1*, *tet-AUR1 scs7*Δ, *tet-AUR1 sur2*Δ, and *tet-AUR1 sur2*Δ *scs7*Δ cells, cells were radiolabeled with [$^3$H]*myo*-inositol (Fig. 3A) or [$^3$H]DHS (Fig. 3B). In both radiolabeling experiments, reductions in complex sphingolipid levels, including IPCs, MIPCs and M(IP)$_2$Cs, were observed in all mutant strains tested after 9 h Dox treatment (Fig. 3 A-B). To examine the accumulation of Cer caused by *AUR1* repression, cells were radiolabeled with [$^{14}$C]serine (Fig. 3C and D). In Dox-treated *tet-AUR1* cells, the accumulation of Cer-C, which contains PHS and α-OH-C26 fatty acid, was observed. The deletion of *SCS7* and *SUR2* resulted in predominant accumulation of Cer containing PHS and nonhydroxylated C26 fatty acid (Cer-B), and DHS and α-OH-C26 fatty acid (Cer-B'), respectively, under *AUR1*-repressive conditions. The double deletion of *SCS7* and *SUR2* caused the accumulation of Cer-A, which contains DHS and nonhydroxylated C26 fatty acid (Fig. 3C and D). These results indicated that under *AUR1*-repressive conditions, reductions in complex sphingolipid levels and the accumulation of Cers occur in *scs7*Δ, *sur2*Δ, and *sur2*Δ *scs7*Δ cells.

***Growth of scs7Δ and sur2Δ cells under Cer synthesis-defective conditions***

*AUR1*-repressed cells show reductions in the complex sphingolipid levels and the accumulation of Cers, both of which are responsible for the growth defect. To investigate whether or not the deletion of *SCS7* or *SUR2* affects the growth of cells exhibiting with the reductions in the complex sphingolipid levels, *scs7*Δ and *sur2*Δ cells were grown under Cer synthesis-repressive conditions. *LIP1* encodes a subunit of Cer synthase, and the repression causes reductions in both the Cer and complex sphingolipid levels (Fig. 1) [17]. A mutant strain carrying the Tet-promoter-regulated *LIP1* gene (*tet-LIP1*) was used for the repression of Cer synthesis [11]. *tet-LIP1 scs7*Δ and *tet-LIP1 sur2*Δ cells were created and radiolabeled with [$^3$H]*myo*-inositol (Fig. 4A) or [$^3$H]DHS (Fig. 4B) in the presence or absence of Dox. When cells were radiolabeled

after overnight treatment with 10 μg/ml Dox, significant decreases in the complex sphingolipid levels were observed in all mutant strains (Fig. 4A and B). Fig. 4C shows the time courses of growth of *tet-LIP1*, *tet-LIP1 scs7*Δ, and *tet-LIP1 sur2*Δ cells after overnight treatment with 0 or 10 μg/ml Dox. In both *tet-LIP1* and *tet-LIP1 scs7*Δ cells, a slight but significant delay of cell growth was observed on treatment with Dox. In *tet-LIP1 sur2*Δ cells, the delay of cell growth caused by Dox was more severe than for *tet-LIP1* and *tet-LIP1 scs7*Δ cells. In the [$^3$H]DHS labeling experiments (Fig. 4B), a high accumulation of DHS 1-phosphate (DHS1P) was observed in Dox-treated *tet-LIP1 sur2*Δ cells, which was not seen in Dox-treated *tet-AUR1 sur2*Δ cells (Fig. 3B). Since LCB 1-phosphates, including DHS1P, exhibit various bioactivities, such as regulation of the cell cycle and stress responses [5], *LCB4*, encoding major LCB kinase [18], was deleted in *tet-LIP1*, *tet-LIP1 scs7*Δ and *tet-LIP1 sur2*Δ cells. In all cells, the deletion of *LCB4* resulted in dramatic reductions in the DHS1P levels (Fig. 4B) and slight enhancement of the growth defect when cells were treated with Dox (Fig. 4C and D). However, the tendency of growth defect by Dox treatment was similar between *LCB4* and *lcb4*Δ cells, that is, Dox-treated *tet-LIP1 sur2*Δ *lcb4*Δ cells showed more severe growth defect than Dox-treated *tet-LIP1 lcb4*Δ, and *tet-LIP1scs7*Δ *lcb4*Δ cells (Fig. 4D). Thus, it is suggested that the high accumulation of DHS1P in Dox-treated *tet-LIP1 sur2*Δ cells did not affect the growth defect under *LIP1*-repressive conditions. The effect of the deletion of *SCS7* or *SUR2* in *tet-LIP1* and *tet-LIP1 lcb4*Δ cells is quite different from that in *tet-AUR1* cells; that is, as compared with *tet-AUR1* cells, *tet-AUR1 scs7Δ* cells are more sensitive to Dox treatment, whereas *tet-AUR1 sur2*Δ cells are relatively resistant (Fig. 2A and B). Furthermore, the deletion of *SUR2* or *SCS7* did not have significant effect on growth inhibition by myriocin[5], which inhibits the first step of sphingolipid synthesis and causes reductions in all sphingolipid levels including those of complex sphingolipids (Fig. 4E). Taken together, these results suggested that

under *AUR1*-repressive conditions, the effect of *SCS7* or *SUR2* deletion on the growth inhibition is not irrelevant to the reductions in the complex sphingolipid levels.

## DISCUSSION

In the present study, it was found that the deletion of *SCS7* results in the enhancement of the growth inhibition induced by repression of *AUR1* expression under the control of a tetracycline-regulatable promoter, whereas the deletion of *SUR2* attenuates the growth inhibition. Dox-treated *tet-AUR1 scs7*Δ and *tet-AUR1 sur2*Δ cells showed reductions in the complex sphingolipid levels and the accumulation of Cers, like *tet-AUR1* cells. In contrast, with repression of *LIP1*, which causes a reduction in Cer synthesis activity, the deletion of *SCS7* did not enhance the growth inhibition induced by the impaired sphingolipid biosynthesis. The deletion of *SUR2* caused more severe growth inhibition under *LIP1*-repressive conditions. Furthermore, the deletion of *SUR2* or *SCS7* did not have significant effect on growth defect induced by inhibition of the first step of sphingolipid synthesis by myriocin. The both *LIP1* repression and treatment with myriocin commonly caused reductions in the complex sphingolipid levels, thus it was suggested that the deletion of *SCS7* or *SUR2* alters the toxicity of Cer under *AUR1*-repressive conditions. *SCS7* and *SUR2* encode hydroxylases involved in the hydroxylation at the C-2 position of fatty acids and the C-4 position of LCBs in Cer, respectively. Therefore, Cers containing α-hydroxylated fatty acids seemed to be less toxic than ones containing non-hydroxylated fatty acids, and PHS-based Cers are more toxic than DHS-based ones.

It should be noted that the accumulation of Cers in Dox-treated *tet-AUR1 sur2*Δ cells was significantly lower than in the other cells tested (Fig. 3D). Thus, one possible explanation for the resistance to growth inhibition of Dox-treated *tet-AUR1 sur2*Δ cells is the lower level of Cer accumulation. On the other hand, Dox-treated *tet-AUR1 scs7*Δ *sur2*Δ cells did not show enhancement of the growth inhibition despite a comparable level of Cer accumulation to that in Dox-treated *tet-AUR1 scs7*Δ cells (Figs. 2 and 3D). This means that the deletion of *SUR2* attenuates the growth inhibition caused by the

deletion of *SCS7* under *AUR1*-repressive conditions, supporting the notion that PHS-based Cers are more toxic than DHS-based ones.

Cers have been suggested to be intracellular lipid second messengers that regulate various signal transduction systems involved in cell differentiation, proliferation, and apoptosis in eukaryotes from yeast to mammals [3]. In yeast, DHS-based Cer is less effective than PHS-based one on the growth inhibition when cells were treated with exogenous cell-permeable C2-Cers [19]. However, the structure of C2-Cers is greatly different from that of natural Cers, which primarily contain fatty acids with carbon chain lengths of 26 in yeast [5]. In the present study, it was suggested that the accumulation of dihydroCers under *AUR1*-repressive conditions is less toxic than that of phytoCers. Thus, the weaker bioactivity of dihydroCers on the inhibition of yeast cell growth can also be applied to endogenous natural Cers.

So far, several information of the relationship between the toxicity of Cers and their hydroxylation at fatty acid moieties was provided by experiments involving the addition of exogenous Cers to cells. Szulc et al reported that cytotoxic activity of cell-permeable α-hydroxy-C6-Cer is more potent than that of C6-Cer in Human breast cancer MCF7 cells, cervical cancer Hela cells, and lung adenocarcinoma A549 cells [20]. In human neuroblastoma SH-SY5Y cells, and leukemia K562 and HL60 cells, exogenously added Cers containing long-chain α-hydroxylated fatty acids have more potent cytotoxic activity than Cers containing non-hydroxylated ones [21]. These effects are likely cell type-specific, as α-hydroxy Cers exhibit significantly lower activity than non-hydroxy Cers as to the induction of DNA fragmentation in human promonocytic U937 cells [22]. In many mammalian tissues, the levels of α-hydroxy Cers range from 1-3% of total Cers; however, in specific tissues, such as the nervous system, skin, and intestinal epithelial cells, the level is over 50% [23]. In contrast, in yeast cells, phytoCer containing α-OH-C26 fatty acid (Cer-C), but not that containing non-hydroxylated C26

fatty acid (Cer-B), is major constituent of cellular sphingolipids. The relationship between the tissue or organism-specific distribution of α-hydroxy Cers and their cell-type specific toxicity will become an important area for further study.

In summary, the results of this study revealed that the hydroxylation states of Cer moieties greatly affect the growth inhibition caused by *AUR1* repression in yeast. Further study will provide a new insight into the relationship between the structure and physiological function of Cers.

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## FIGURE LEGENDS

Fig. 1. **Ceramide synthesis pathway in yeast *Saccharomyces cerevisiae*.**

The pathway and responsible enzyme genes for the synthesis of yeast ceramides (Cers) are shown. Cers are classified into five types (A, B, B', C, and D) according to the hydroxylation state. *SUR2* is required for the production of Cer B. *SCS7* is required for the production of Cer-B' and -C. Although a hydroxylase involved in the production of Cer-D remains unknown, *CCC2*, the gene encoding an intracellular $Cu^{2+}$ transporter, is involved in the conversion of Cer-C into Cer-D. All these five types of Cer are used as precursors for complex sphingolipid (IPC, MIPC, and $M(IP)_2C$) formation.

Fig. 2. **Effect of deletion of *SCS7*, *SUR2*, and *CCC2* on growth inhibition caused by *AUR1* repression.**

A, *tet-AUR1*, *tet-AUR1 scs7*Δ, *tet-AUR1 sur2*Δ, *tet-AUR1 scs7*Δ *sur2*Δ, and *tet-AUR1 ccc2*Δ cells were cultured overnight in YPD medium, and then spotted onto YPD plates with or without the indicated concentrations of Dox, in 10-fold serial dilutions starting with a density of 0.7 $A_{600}$ units/ml. All plates were incubated at 30°C and photographed after 2 days. B, time course of cell growth. Cells were cultured overnight in YPD medium and then diluted (0.02 $A_{600}$ units/ml) in fresh YPD medium with or without 10 μg/ml Dox, and aliquots of cell suspensions were subjected to cell density measurements ($A_{600}$) at the indicated times. The dotted lines in panels b, c, d, and e indicate the time courses of cell growth of *tet-AUR1* cells with or without Dox. The results presented are for one experiment (triplicate) representative of at least two independent ones. In some instances, error bars are too small to be visible.

Fig. 3. **Sphingolipid metabolism under *AUR1*-repressive conditions.**

A, B, [$^3$H]*myo*-inositol or [$^3$H]dihydrosphingosine (DHS) labeling of *tet-AUR1*,

*tet-AUR1 scs7*Δ, *tet-AUR1 sur2*Δ, *tet-AUR1 scs7*Δ *sur2*Δ cells. Cells were cultured overnight in YPD medium, diluted (0.1 $A_{600}$ units/ml) in fresh YPD medium with or without 10 μg/ml Dox, incubated for 8 h, and then labeled with [$^3$H]*myo*-inositol (A) or [$^3$H]DHS (B) for 1 h. Radiolabeled lipids were extracted, treated with MMA, and then separated by TLC. C, cells were cultured with or without 10 μg/ml Dox, and then labeled with [$^{14}$C]serine. Radiolabeled lipids were extracted, treated with MMA, and then separated by TLC. The *asterisk* indicates an unidentified band. D, the contents of Cers in (C) were determined with an imaging analyzer. The band of Cer-C, Cer-B, Cer-B', and Cer-A, respectively, were quantified in *tet-AUR1*, *tet-AUR1 scs7*Δ, *tet-AUR1 sur2*Δ, or *tet-AUR1 scs7*Δ *sur2*Δ cells. The data are the averages for three independent experiments with standard deviation. The details are given under "Materials and Methods".

Fig. 4. **Growth and sphingolipid metabolism of *scs7*Δ and *sur2*Δ cells under *LIP1*-repressive conditions.**

A, B, [$^3$H]*myo*-inositol or [$^3$H]DHS labeling of *tet-LIP1* cells. Cells were cultured overnight in YPD medium with or without 10 μg/ml Dox, diluted (0.1 $A_{600}$ units/ml) in fresh YPD medium with or without 10 μg/ml Dox, incubated for 8 h, and then labeled with [$^3$H]*myo*-inositol (A) or [$^3$H]DHS (B) for 1 h. Radiolabeled lipids were extracted, treated with MMA, and then separated by TLC. The *asterisk* indicates an unidentified band. The details are given under "Materials and Methods". C, D, time course of cell growth. Cells were cultured overnight in YPD medium with or without 10 μg/ml Dox and then diluted (0.02 $A_{600}$ units/ml) in fresh YPD medium with or without 10 μg/ml Dox, and aliquots of cell suspensions were subjected to cell density measurements ($A_{600}$) at the indicated times. The results presented are for one experiment (triplicate) representative of at least two independent ones. E, inhibition of cell growth by

myriocin. Cells were cultured overnight in YPD medium, and then spotted onto YPD plates with or without 0.2, 0.25, 0.3 μg/ml myriocin in 10-fold serial dilutions starting with a density of 0.7 $A_{600}$ units/ml. All plates were incubated at 30°C and photographed after 1 day.

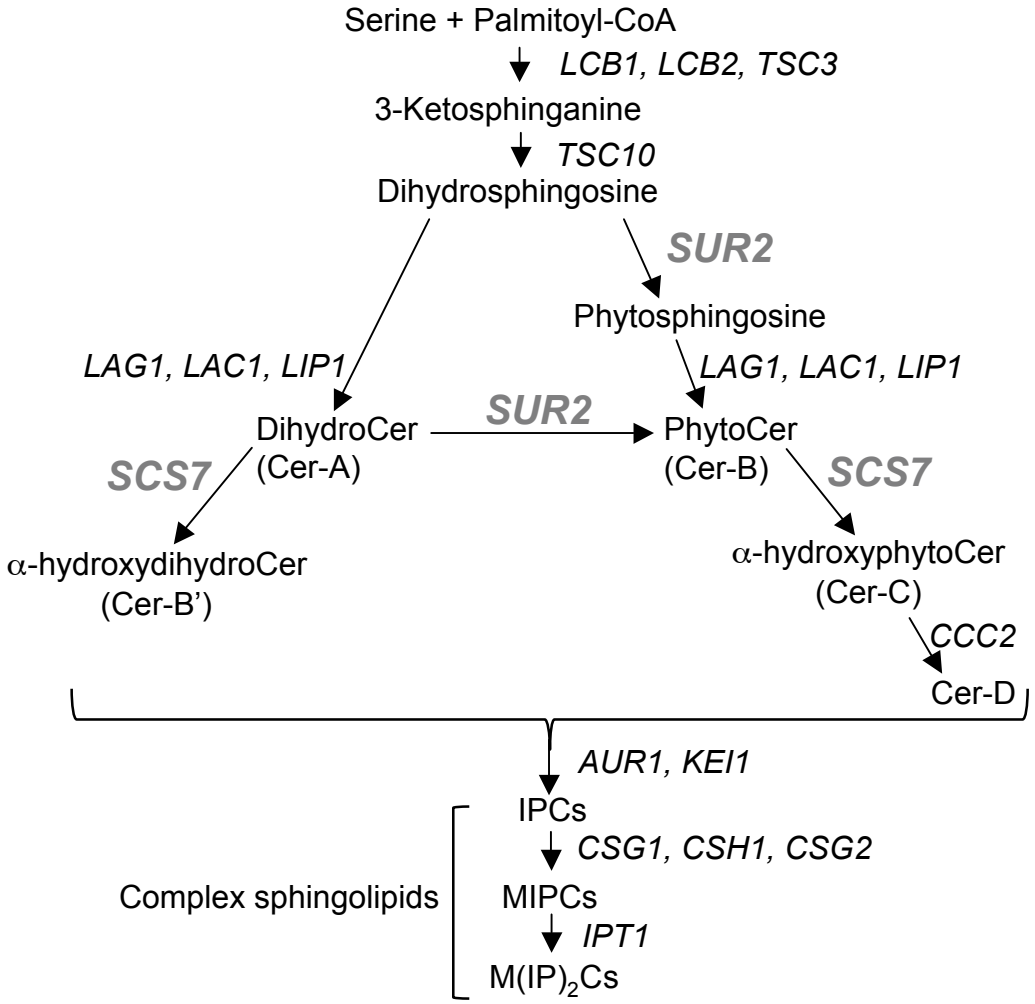

Serine + Palmitoyl-CoA
LCB1, LCB2, TSC3
3-Ketosphinganine
TSC10
Dihydrosphingosine
SUR2
Phytosphingosine
LAG1, LAC1, LIP1
LAG1, LAC1, LIP1
SUR2
DihydroCer
(Cer-A)
PhytoCer
(Cer-B)
SCS7
SCS7
α-hydroxydihydroCer
(Cer-B')
α-hydroxyphytoCer
(Cer-C)
CCC2
Cer-D
AUR1, KEI1
IPCs
CSG1, CSH1, CSG2
Complex sphingolipids
MIPCs
IPT1
M(IP)2Cs

**Fig. 1**

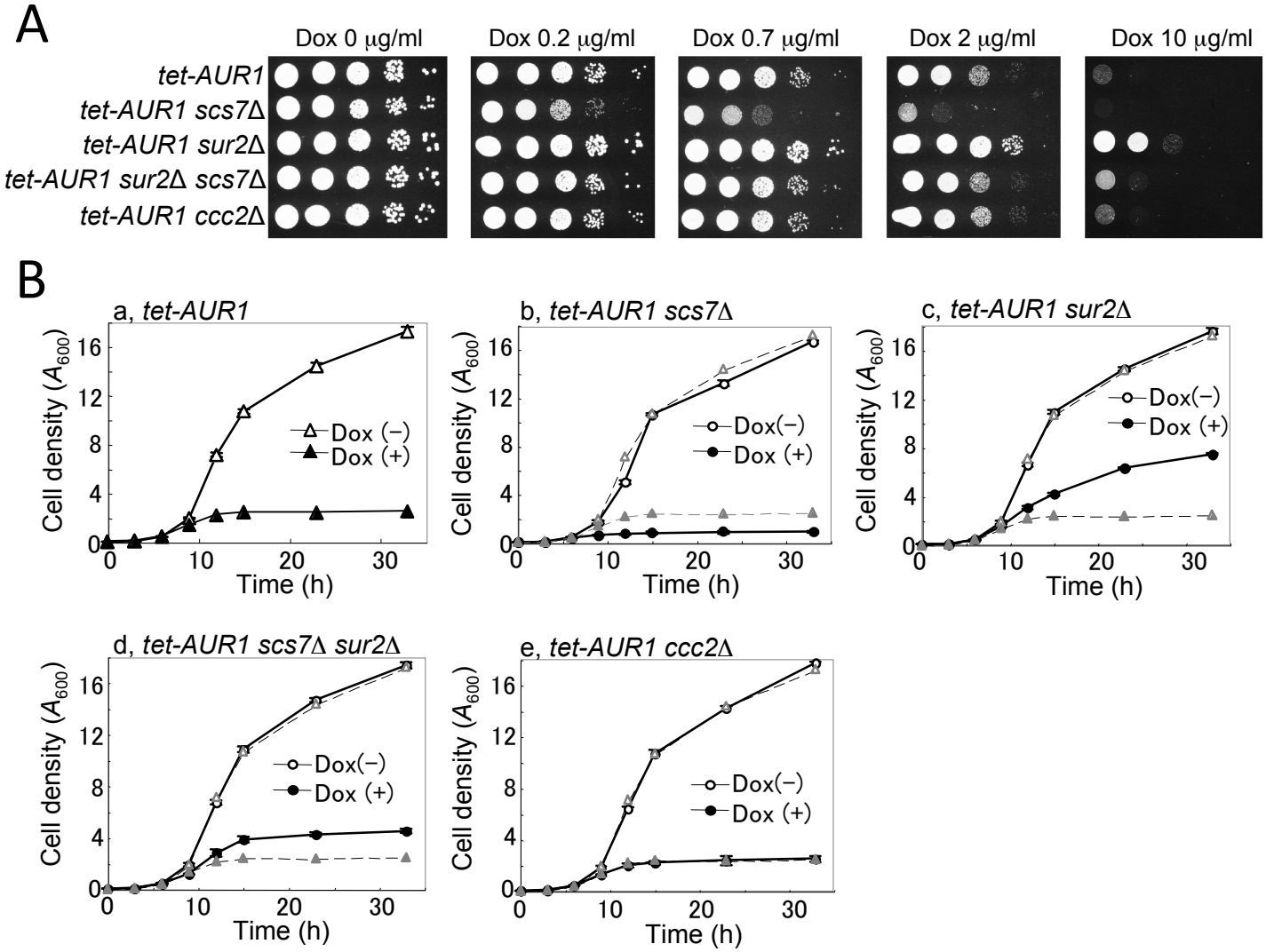

A
Dox 0 μg/ml
Dox 0.2 μg/ml
Dox 0.7 μg/ml
Dox 2 μg/ml
Dox 10 μg/ml
tet-AUR1
tet-AUR1 scs7Δ
tet-AUR1 sur2Δ
tet-AUR1 sur2Δ scs7Δ
tet-AUR1 ccc2Δ
B
a, tet-AUR1
b, tet-AUR1 scs7Δ
c, tet-AUR1 sur2Δ
d, tet-AUR1 scs7Δ sur2Δ
e, tet-AUR1 ccc2Δ
Cell density (A600)
Time (h)
Dox (−)
Dox (+)
Dox(−)
Dox (+)

Fig. 2

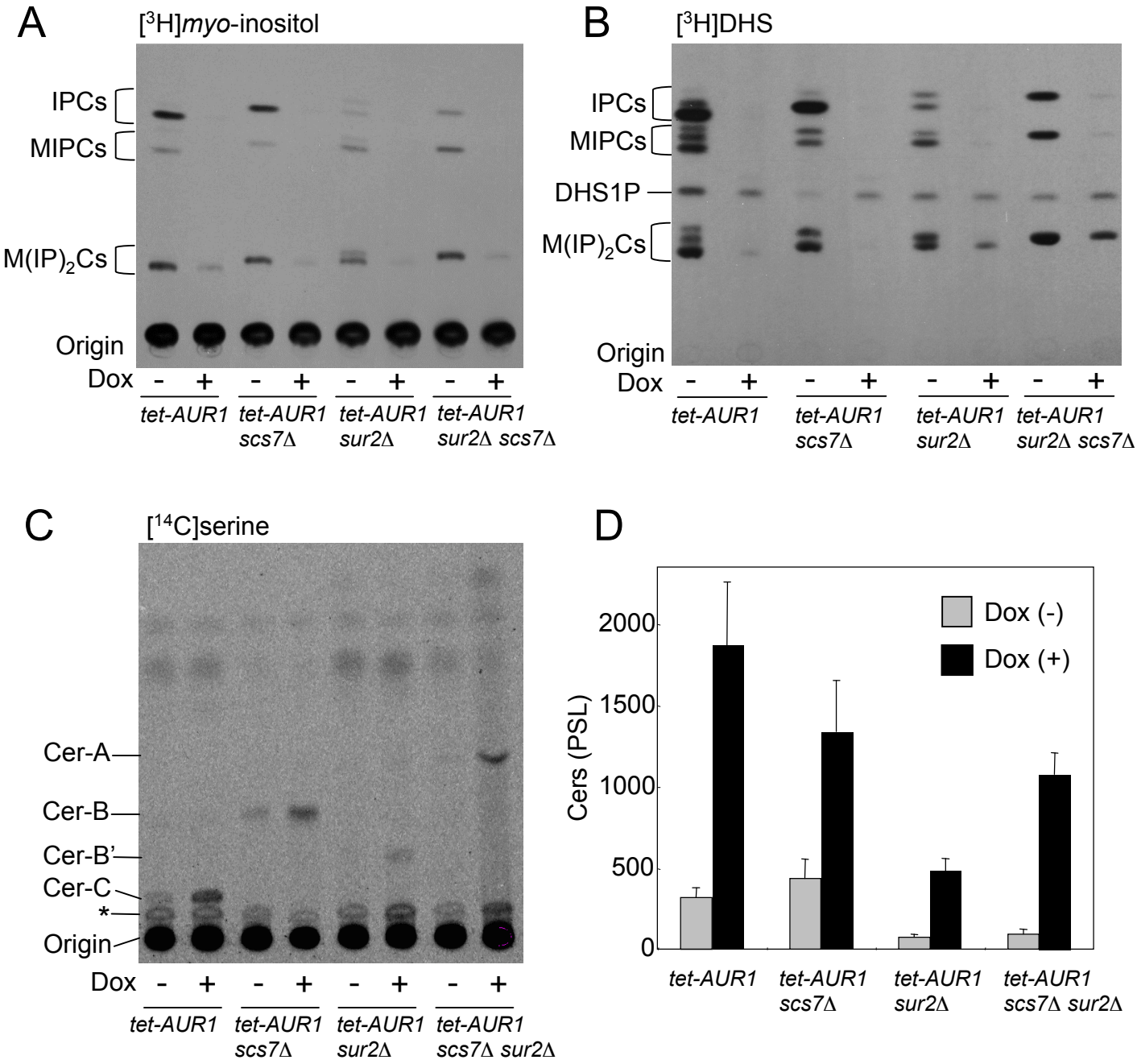
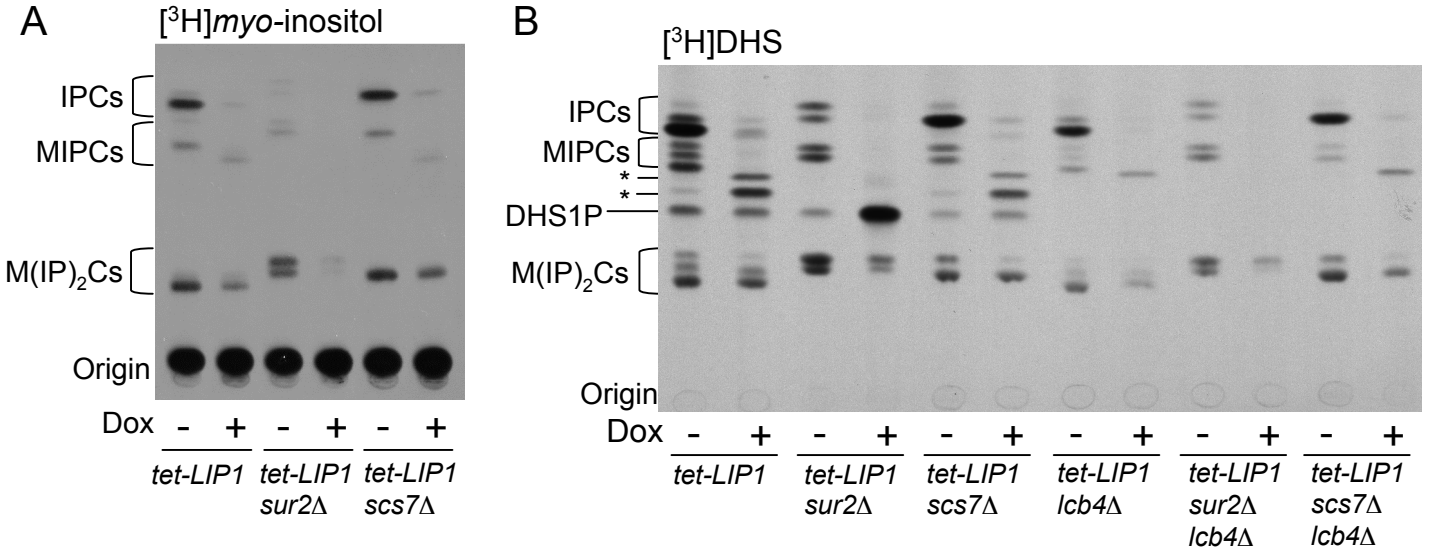
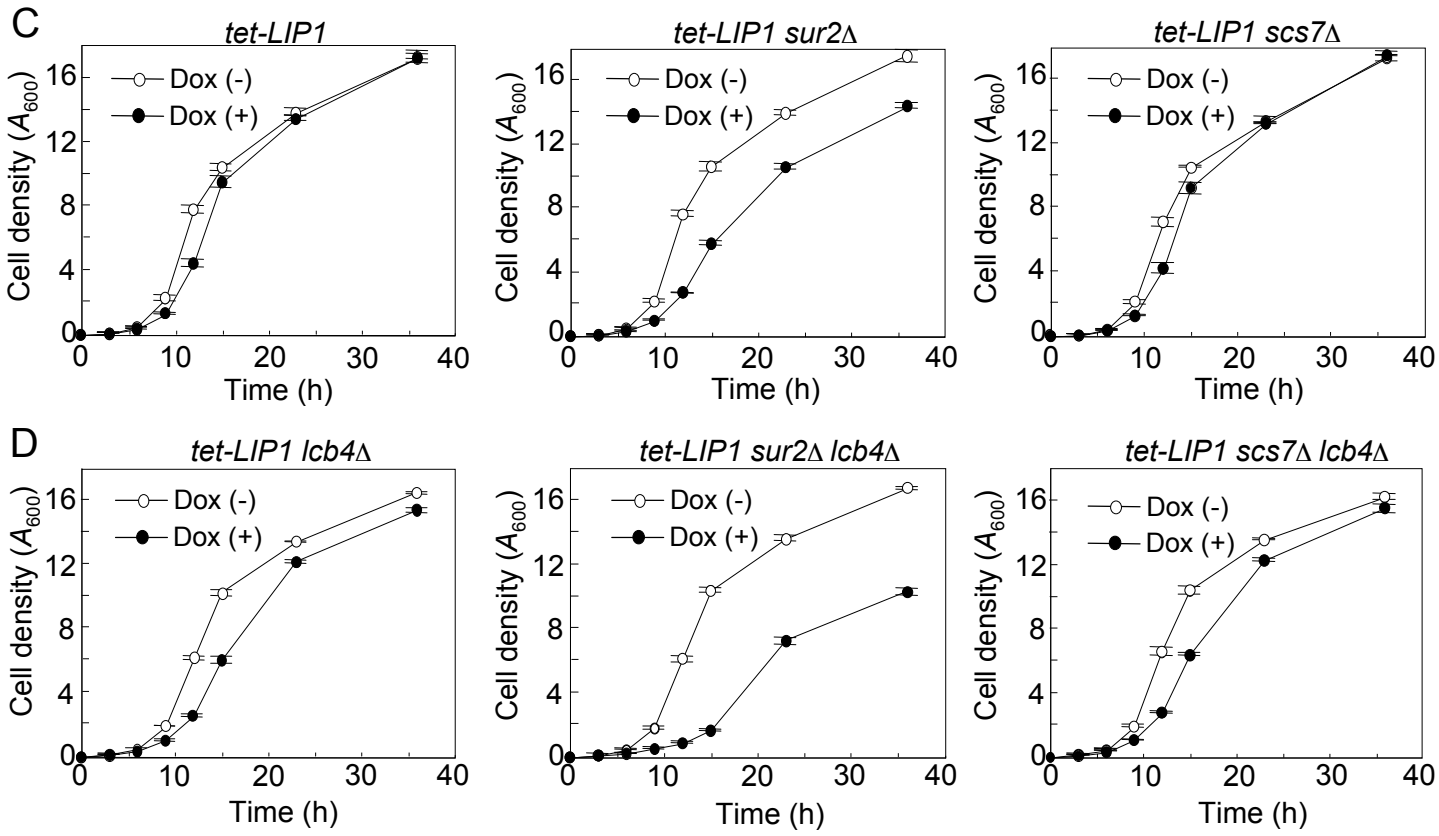

A
[3H]myo-inositol
IPCs
MIPCs
M(IP)2Cs
Origin
Dox - + - + - + - +
tet-AUR1
tet-AUR1 scs7Δ
tet-AUR1 sur2Δ
tet-AUR1 sur2Δ scs7Δ
B
[3H]DHS
IPCs
MIPCs
DHS1P
M(IP)2Cs
Origin
Dox - + - + - + - +
tet-AUR1
tet-AUR1 scs7Δ
tet-AUR1 sur2Δ
tet-AUR1 sur2Δ scs7Δ
C
[14C]serine
Cer-A
Cer-B
Cer-B'
Cer-C
*
Origin
Dox - + - + - + - +
tet-AUR1
tet-AUR1 scs7Δ
tet-AUR1 sur2Δ
tet-AUR1 scs7Δ sur2Δ
D
Cers (PSL)
2000
1500
1000
500
0
Dox (-)
Dox (+)
tet-AUR1
tet-AUR1 scs7Δ
tet-AUR1 sur2Δ
tet-AUR1 scs7Δ sur2Δ

**Fig. 3**


A
[3H]myo-inositol
IPCs
MIPCs
M(IP)2Cs
Origin
Dox - + - + - +
tet-LIP1
tet-LIP1 sur2Δ
tet-LIP1 scs7Δ
B
[3H]DHS
IPCs
MIPCs
*
*
DHS1P
M(IP)2Cs
Origin
Dox - + - + - + - + - + - +
tet-LIP1
tet-LIP1 sur2Δ
tet-LIP1 scs7Δ
tet-LIP1 lcb4Δ
tet-LIP1 sur2Δ lcb4Δ
tet-LIP1 scs7Δ lcb4Δ


C
tet-LIP1
tet-LIP1 sur2Δ
tet-LIP1 scs7Δ
Dox (-)
Dox (+)
Cell density (A600)
Time (h)
D
tet-LIP1 lcb4Δ
tet-LIP1 sur2Δ lcb4Δ
tet-LIP1 scs7Δ lcb4Δ

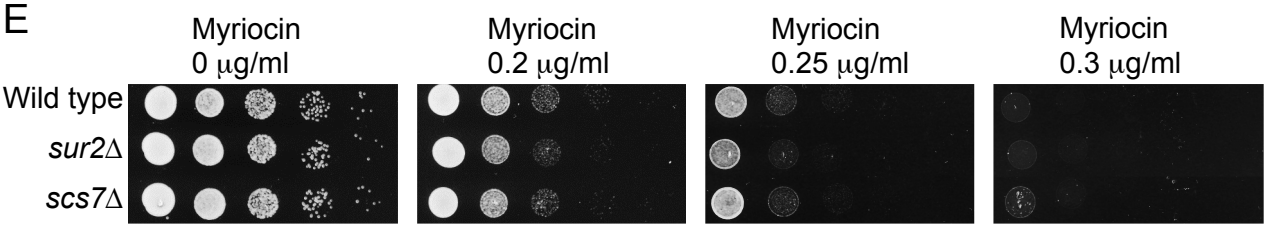

E
Myriocin 0 μg/ml
Myriocin 0.2 μg/ml
Myriocin 0.25 μg/ml
Myriocin 0.3 μg/ml
Wild type
sur2Δ
scs7Δ

**Fig. 4**

TABLE I. Strains used in this study.

| Strain | Genotype | Source |
|---|---|---|
| BY4741 | *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* | [24] |
| MTY175 | BY4741, *tetO$_7$-AUR1*::*kanMX4 URA3* | [11] |
| MTY210 | BY4741, *tetO$_7$-AUR1*::*kanMX4 scs7*Δ::*URA3* | This study |
| MTY220 | BY4741, *tetO$_7$-AUR1*::*kanMX4 sur2*Δ::*URA3* | This study |
| MTY633 | BY4741, *tetO$_7$-AUR1*::*kanMX4 scs7*Δ::*URA3 sur2*Δ*::hphNT1* | This study |
| MTY616 | BY4741, *tetO$_7$-AUR1*::*kanMX4 ccc2*Δ::*URA3* | This study |
| MTY176 | BY4741, *tetO$_7$-LIP1*::*kanMX4 URA3* | [11] |
| MTY579 | BY4741, *tetO$_7$-LIP1*::*kanMX4 scs7*Δ::*URA3* | This study |
| MTY618 | BY4741, *tetO$_7$-LIP1*::*kanMX4 sur2*Δ::*URA3* | This study |
| MTY941 | BY4741, *tetO$_7$-LIP1*::*kanMX4 URA3 lcb4* Δ::*natMX4* | This study |
| MTY942 | BY4741, *tetO$_7$-LIP1*::*kanMX4 sur2*Δ::*URA3 lcb4* Δ::*natMX4* | This study |
| MTY943 | BY4741, *tetO$_7$-LIP1*::*kanMX4 scs7*Δ::*URA3 lcb4* Δ::*natMX4* | This study |
| MTY128 | BY4741, *sur2*Δ::*URA3* | This study |
| MTY211 | BY4741, *scs7*Δ::*URA3* | This study |