Isc1 regulates sphingolipid metabolism in yeast mitochondria

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Abstract

The *Saccharomyces cerevisiae* inositolsphingolipid phospholipase C (Isc1p), a homolog of mammalian neutral sphingomyelinases, hydrolyzes complex sphingolipids to produce ceramide in vitro. Epitope-tagged Isc1p associates with the mitochondria in the post-diauxic phase of yeast growth. In this report, the mitochondrial localization of Isc1p and its role in regulating sphingolipid metabolism were investigated. First, endogenous Isc1p activity was enriched in highly purified mitochondria, and western blots using highly purified mitochondrial membrane fractions demonstrated that epitope-tagged Isc1p localized to the outer mitochondrial membrane as an integral membrane protein. Next, LC/MS was employed to determine the sphingolipid composition of highly purified mitochondria which were found to be significantly enriched in α-hydroxylated phytoceramides (21.7 fold) relative to the whole cell. Mitochondria, on the other hand, were significantly depleted in sphingoid bases. Compared to the parental strain, mitochondria from *isc1∆* in the post-diauxic phase showed drastic reduction in the levels of α-hydroxylated phytoceramide (93.1 % loss compared to WT mitochondria with only 2.58 fold enrichment in mitochondria compared to whole cell). Functionally, *isc1∆* showed a higher rate of respiratory-deficient cells after incubation at high temperature and was more sensitive to hydrogen peroxide and ethidium bromide, indicating that *isc1∆* exhibits defects related to mitochondrial function. These results suggest that Isc1p generates ceramide in mitochondria, and the generated ceramide contributes to the normal function of mitochondria. This study provides a first insight into the specific composition of ceramides in mitochondria.

Keywords

Mitochondria; Sphingolipid; Sphingomyelinase; Lipidomics; *ISC1*; Yeast

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1. Introduction

Sphingolipids play crucial roles as signaling molecules in eukaryotic cells, regulating such processes as stress responses, cell growth, differentiation, cell cycle arrest, endocytosis, and apoptosis [1-7]. Additionally, complex sphingolipids serve structural functions in membranes, and they may also undergo dynamic clustering with sterols to form lipid microdomains or 'rafts' within the membrane bilayer. This domain structure has been proposed to play a role as platforms for signal transduction and/or protein sorting [8].

Most, if not all, organelles contain sphingolipids as well as several sphingolipid-metabolizing enzymes [9-11]. Moreover, the hydrophobicity of bioactive sphingolipids restrains them to their specific compartments (unless transported by specific mechanisms). These considerations led to the proposal that sphingolipid function is compartment-specific [1]. In this context, many studies in eukaryotes have recently started suggesting important roles for mitochondrial sphingolipids [11-13].

Ceramide in mitochondria has been shown to regulate apoptosis in mammalian cells. For example, when bacterial sphingomyelinase was targeted to mitochondria, it induced mitochondrial ceramide generation and apoptosis [13], and mitochondrial ceramide has been implicated in the activation/translocation of the pro-apoptotic Bax [14]. Moreover, many studies have reported that ceramide has biophysical effects on mitochondrial membranes, by increasing the permeability of the outer and inner mitochondrial membranes [15], suppressing respiratory chain activity [16], or forming channels [17]. Therefore, the role of ceramide in mitochondria is attracting wide interests in terms of basic biology and medical application. Other studies have suggested mitochondrial localization of some enzymes of sphingolipid metabolism, including ceramide and dihydroceramide synthase [12,18], ceramidase [19], glycosyltransferase [20], human sialidase [21], and sphingosine kinase [22].

Saccharomyces cerevisiae has proven to be an invaluable model for studying sphingolipid biosynthesis, in part because of its simplicity in sphingolipid metabolism relative to mammalian systems [3,23]. Interestingly, the *S. cerevisiae* Isc1p, the homolog of mammalian neutral sphingomyelinases, was recently shown to associate with mitochondria in the post-diauxic phase of yeast growth [24]. Isc1p produces ceramide from the complex sphingolipids inositol phosphorylceramide (IPC), mannosylinositol phosphorylceramide (MIPC), and mannosyldiinositol phosphorylceramide (M(IP)$_2$C) [25-28]. Isc1p was also shown to have a role in utilization of non-fermentable carbon sources, as an *isc1⊿* strain grew very slowly in the post-diauxic phase or when grown in media containing non-fermentable carbon sources [29]. Utilization of non-fermentable carbon sources requires intact mitochondrial function, thus suggesting a role for Isc1p in mitochondrial function.

In this study we sought to elucidate the submitochondrial localization of Isc1p, its role in mitochondrial sphingolipid metabolism, and mitochondria-related phenotypes of *isc1⊿*. These studies have implications for compartment-specific sphingolipid metabolism and function in vivo.

2. Materials and Methods

2.1. Yeast strains and media

JK93α WT (MATα *leu2−3, 112 ura3−52 rme1 trp1 his4*) and JK93α *isc1⊿* (MATα *leu2−3, 112 ura3−52 rme1 trp1 his4 ISC1::G418*) [25] were used in this study. Standard yeast culture medium was prepared using Difco YPD broth. Plates were prepared using Difco YPD agar and stored at 4°C. YPGE plates contained 1% yeast extract, 2% bactopeptone, 2% ethanol, 2%

glycerol and 2% agar. Yeast cells were cultured at 30°C with shaking unless otherwise indicated.

2.2. Isolation of purified mitochondria

Isolation of purified mitochondria was basically performed essentially as described [30-32]. Briefly, yeast cells were incubated to saturation at 30°C. Six liter flask containing approximately 1.25 L YPD were inoculated with an overnight culture to $OD_{600}=0.1$ and incubated at 30°C for 24 h with vigorous aeration. Yeast cells were centrifuged at 2,000 g for 5 min, washed with water, resuspended in 100 mM Tris-SO$_4$, pH 9.4, 10 mM DTT (2 ml/g wet cell), incubated at 30°C for 15 min, centrifuged at 2,000 g for 5 min, washed with SP buffer (1.2 M sorbitol, 20 mM K-P pH 7.4), dissolved in SP buffer containing zymolyase 100T (7 ml/g cells, 1.5 mg/g cells), incubated at 30°C for 60 min, centrifuged at 2,500 g for 5 min, washed with SP buffer (7 ml/g wet cell) twice, resuspended in homogenization buffer (0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) (6.5 ml/g cells), homogenized in a 40 ml dounce homogenizer coated with Teflon using 25 strokes, diluted two fold with homogenizing buffer, centrifuged for 5 min at 1,500 g to remove cell nuclei and debris, and centrifuged again at 2,500 g for 5 min. The supernatant was centrifuged at 12,000 g for 15 min. The supernatant was saved as the post-mitochondrial fraction and TCA-precipitated for further analysis. The pellet was resuspended in 30 ml SM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) and centrifuged at 2,500 g for 5 min. The supernatant was then centrifuged at 12,000 g for 15 min, and the pellet was resuspended in 10 ml SM buffer as crude mitochondria. For purification of mitochondria, the amount of mitochondrial protein was measured by OD_{280}, adjusted to 5 mg/ml protein in SM buffer, homogenized with 10 strokes in a dounce homogenizer coated with Teflon, and subsequently loaded onto a four step sucrose gradient [1.5 ml of 60%, 4 ml of 32%, 1.5 ml of 23%, 1.5 ml of 15% of sucrose in 1 mM EDTA, 10 mM MOPS-KOH (pH 7.2)], and centrifuged at 134,000g for 1 h with no braking. The 32/60% interface was then recovered with a Pasteur pipette and washed with 2 vol of SM buffer at 12,000 g for 15 min. The mitochondrial fraction was again homogenized with 10 strokes in a dounce homogenizer coated with Teflon and then applied to a four step sucrose gradient as described above. The 32/60% interface was recovered, resuspended in 2 volumes of SM buffer, and centrifuged at 12,000 g for 15 min. The final pellet was resuspended in SM buffer, divided into small aliquots, and frozen at −80°C.

2.3. LC/MS measurements

Purified mitochondria or yeast cells in the post-diauxic phase were snap-frozen in a methanol-dry ice bath and stored at −80°C. ESI/MS/MS analysis of endogenous phyto- and dihydrosphingosine bases, their phosphates, α-hydroxylated phytoceramide, and non-hydroxylated phyto- and dihydroceramide species were performed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer, operating in a Multiple Reaction Monitoring positive ionization mode, using modified version [28,34]. Briefly, biological samples were fortified with internal standards (ISs: C_{17} base D-erythro-sphingosine, C_{17} sphingosine-1-phosphate, N-palmitoyl-D-erythro-C_{13} sphingosine, heptadecanoyl-D-erythro-sphingosine and C_6-Phyto-ceramide), then extracted with 70% isopropanol:ethylacetate:pyridine:25% ammonia (60:20:2:0.5 by volume) solvent system. After evaporation and reconstitution in 150 μl of methanol, samples were injected on the HP1100/TSQ 7000 LC/MS system and gradient eluted from the BDS Hypersil C8, 150×3.2 mm, 3 μm particle size column, with 1.0 mM methanolic ammonium formate / 2 mM aqueous ammonium formate mobile phase system. Peaks corresponding to the target analytes and ISs were collected and processed using the Xcalibur software system. Quantitative analysis was based on the calibration curves generated by spiking an artificial matrix with the known amounts of the target analyte synthetic standards and an equal amount of IS. The target analyte/IS peak areas ratios were plotted against analyzed concentration. The target analyte/IS peak area ratios from the samples were similarly

normalized to their respective ISs and compared to the calibration curves, using a linear regression model.

2.4. Submitochondrial localization of Flag-Isc1p

Submitochondrial localization of Flag-Isc1p was determined as previously described [35]. Briefly, 200 μg of highly purified mitochondria from *isc1⊿*+pYES/FLAG-*ISC1* were pelleted at 10,000 g for 15 min and suspended in 40 μl SM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2). Samples were split into two equal portions. One hundred and eighty microliters of SM buffer or EM buffer (1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) was added, pipetted up and down 10 times to facilitate the rupture of the outer membrane from the swollen mitochondria, and incubated for 15 min on ice. Samples were split into two equal portions and treated with or without 50 μg/ml proteinase K for 30 min on ice. Phenylmethylsulfonyl fluoride was added to the solution (final concentration 2 mM). The sample was then centrifuged at 12,000×g for 5 min, washed once and analyzed by western blotting using anti-Flag antibody.

2.5. Determination of association of proteins with membrane

Highly purified mitochondria, purified from *isc1⊿*+pYES/FLAG-*ISC1*, were treated without osmotic shock (250 mM sucrose, 10 mM HEPES, pH 7.2, 1 mM EDTA), with osmotic shock (10 mM HEPES, pH 7.2, 1 mM EDTA), with osmotic shock and high salt (0.1 M NaCl, 10 mM HEPES, pH 7.2, 1 mM EDTA), or with osmotic shock and alkali pH (0.1 M Na_2CO_3, pH 11.2, 10 mM HEPES, pH 7.2, 1 mM EDTA). They were then centrifuged at 100,000×g for 10 min, and the pellet and supernatant were analyzed by western blotting using antibody against Flag.

2.6. Statistical analysis

The amounts of sphingolipid species of mitochondria prepared from WT and *isc1⊿* are presented as means ± standard error of the mean (SEM) of three lipid measurements from two independent cultures and mitochondrial purifications. The amounts of sphingolipid species of the whole cell are presented as means ± SEM of lipid measurements from three independent cultures. Statistical differences were evaluated by Student's unpaired *t* test.

2.7. Miscellaneous

Antibodies against Cox3p, Porin, Dpm1p, V-ATPase (100kDa subunit), and Pgk1p were obtained from Molecular Probes, and that against FLAG was from Sigma-Aldrich. The antibody against Mge1p was a kind gift from Dr. C. M. Koehler.

3. Results

3.1. Isc1p as an integral membrane protein localized to the outer membrane of mitochondria

Since epitope-tagged Isc1p was previously shown to associate with mitochondria in the post-diauxic phase, it became important to determine the mitochondrial localization of endogenous Isc1p. WT and *isc1⊿* cells were incubated to the post-diauxic phase, collected by centrifugation, and mitochondria were isolated to a high degree of purity using multi-step centrifugation in conjunction with a two-step sucrose gradient, which has been verified by mitochondrial proteome studies [30-33]. The purity of the mitochondria was confirmed by western blotting with an antibody against a cytosolic marker protein, Pgk1p, a vacuolar marker protein, V-ATPase, an endoplasmic reticulum (ER) marker protein, Dpm1p, and a mitochondrial marker protein, Cox3p. The results showed that the purified fraction was devoid of Pgk1p, V-ATPase and Dpm1p and highly enriched in Cox3p (Fig. 1A), indicating that mitochondria were purified to a high degree, consistent with previous reports [30-32]. Subsequent studies were performed using these highly purified mitochondria prepared from

post-diauxic phase cells. In order to determine the localization of endogenous Isc1p, Isc1p enzymatic activity in the purified mitochondria was determined. Isc1p specific activity was highly concentrated in the mitochondrial fraction (2.8 pmol/μg/hr) when compared to the specific activity of the whole cell homogenate (1.0 pmol/μg/hr) (Fig. 1B). Moreover, cells and mitochondria from the *isc1Δ* strain contained only background activity, demonstrating the Isc1p is the primary enzyme responsible for the detected activity in mitochondria. Taken together, these results demonstrate that Isc1p activity is indeed localized to mitochondria (Fig. 1B).

Next it became important to determine if Isc1p is loosely associated with mitochondria or if it is an integral mitochondrial membrane protein. Mitochondria were treated with osmotic shock (to lyze the mitochondrial outer membrane) and then treated with either high salt or alkali pH extraction, centrifuged at 100,000 g for 10 min, and pellets were analyzed by western blotting. N-terminally tagged Flag-Isc1p [25] was not dissociated from the membrane with osmotic shock (Fig. 1C lanes 4 and 5), high salt (Fig. 1C lanes 6 and 7) or alkali pH (Fig. 1C lanes 8 and 9). Cox3p, a known integral membrane protein behaved similarly whereas Mge1p, a peripheral mitochondrial protein became significantly dissociated from membranes in response to treatment with alkaline pH. Taken together, these results indicate that Isc1p is a *bona fide* membrane protein.

Next, the submitochondrial localization of Isc1p was determined using protease protection assays. Proteinase K was added to purified mitochondria with or without osmotic shock, and the degradation of several marker proteins was examined by western blotting. Using this assay without osmotic shock, exogenously added proteinase K can only access and degrade proteins exposed from the outer mitochondrial membrane. On the other hand, with osmotic shock, proteinase K causes degradation of proteins contained in the outer membrane, in the intermembrane space, or exposed out of the inner membrane since osmotic shock causes rupture of the outer mitochondrial membrane while leaving the inner mitochondrial membrane intact. A previously characterized inner membrane protein, Cox3p, was not degraded even after osmotic shock (Fig. 1D, lane 4), demonstrating that under these conditions, no protease cleavage sites are exposed out of the inner membrane. On the other hand, porin, a marker protein for outer mitochondrial membrane proteins, was degraded only after osmotic shock (Fig. 1D, lane 4), suggesting that it is inserted in the outer membrane so that it is degraded only after rupture of the outer membrane. To the contrary, Flag-Isc1p was efficiently degraded even without osmotic shock (Fig. 1D, lane 2), indicating that Flag-Isc1p localized to the outer membrane of mitochondria with exposed protease cleavage sites, or, alternatively, only N-terminally tagged FLAG is exposed from the outer membrane and Isc1p is localized inside the outer membrane. From all the above data, it could be concluded that Flag-Isc1p localizes to the outer membrane of mitochondria and is integrated into the outer mitochondrial membrane.

3.2. Mitochondria are enriched in very long chain phytoceramides

In previous reports, we showed that Isc1p degrades complex sphingolipids such as IPC, MIPC and M(IP)$_2$C to form ceramides in vitro [25-27] and that Isc1p is activated by phosphatidylglycerol and cardiolipin [29], two phospholipids that reside in mitochondria [36,37]. These results, together with the mitochondrial localization of Isc1p, raised the possibility that Isc1p may be responsible for generation of ceramides in mitochondria in vivo. To address this issue, we first determined the sphingolipid composition of mitochondria and compared it to that of whole cells. WT cells were incubated to the post-diauxic phase, and the mitochondria were isolated to a high degree of purity using a two-step sucrose gradient as described above. The sphingolipid profile of the whole cell of WT and the mitochondria purified from WT was determined using LC-MS [34]. The masses of sphingolipids were calculated based on the phospholipid content.

In whole cells, α-hydroxylated C_{26}-phytoceramide, phytosphingosine, dihydrosphingosine, and α-hydroxylated C_{14}-phytoceramide constituted the major species (Fig. 2A), α-hydroxylated $C_{24:1}$-, C_{24}- and C_{18}-phytoceramides and non-hydroxylated C_{26}-phytoceramides were next in abundance (Fig. 2B), and α-hydroxylated C_{20}- and C_{22}-phytoceramides and most of the non-hydroxylated dihydroceramides and phytoceramides showed significantly lower levels (Fig. 2C).

In contrast, the results revealed, for the first time, that mitochondria contain predominantly α-hydroxylated C_{26}-phytoceramide (Fig. 3A) with significantly lower levels of other ceramides or sphingoid bases (Fig. 3B and 3C). These results were expressed relative to mitochondrial total phospholipids, and therefore, they represent the relative surface concentration of these sphingolipids in membranes. It should be noted that even for the highest ceramide, the ratio to phospholipids was less than 3%, thus demonstrating that phospholipids are indeed in excess and that mitochondrial ceramides are minor species.

In order to determine the relative enrichment of mitochondrial ceramides to that of whole cells, we calculated the degree of 'surface enrichment' of these sphingolipids in mitochondria to whole cells by dividing the ceramide/phospholipid ratio in mitochondria over that in whole cells. This calculated ratio of mitochondrial/whole cell for α-hydroxylated C_{26}-phytoceramides was 21.7 fold, and this enrichment in mitochondria was also observed for other ceramide species (Fig. 3D), indicating that mitochondria were highly enriched with ceramide species mainly composed of α-hydroxylated phytoceramides. This result also suggests that the major source of α-hydroxylated phytoceramides of the whole cell is derived from the mitochondria. Non-hydroxylated ceramides also showed increases in the mitochondria relative to the whole cells (Fig. 3D). Notably, the levels of the non-hydroxylated phytoceramides and dihydroceramides were significantly lower than those of α-hydroxylated phytoceramides both in whole cells and mitochondria (Fig. 2 and Fig. 3).

Reciprocally, the levels of phytosphingosine, the predominant 'simple' sphingolipid of yeast, were significantly decreased in mitochondria relative to whole cells (Fig. 3E, note inverted y-axis compared to 3D).

Taken together, the results demonstrate that in *S. cerevisiae*, mitochondria show selective enrichment in α-hydroxylated phytoceramides and selective depletion of sphingoid bases. These results were basically similar when sphingolipid levels were normalized by protein amount (data not shown), indicating that these results are not due to selective difference in total phospholipid levels between the whole cell and mitochondria.

3.3. Mitochondria of isc1⊿ show an altered sphingolipid profile

Next, the sphingolipid profile of mitochondria prepared from *isc1⊿* was determined and compared to that of WT mitochondria in order to determine the contribution of Isc1p to mitochondrial sphingolipids. The major sphingolipids detected in whole cells of the *isc1⊿* strain were α-hydroxylated C_{26}-phytoceramide, phytosphingosine, α-hydroxylated C_{14}-phytoceramide and dihydrosphingosine (Fig. 4A) followed by non-hydroxylated C_{26}-phytoceramide, α-hydroxylated C_{24}-phytoceramide and non-hydroxylated $C_{18:1}$-dihydroceramide (Fig. 4B), with other ceramides showing very low levels (Fig. 4C).

The predominant species of sphingolipids in mitochondria from *isc1 ⊿* were α-hydroxylated C_{26}-phytoceramide, non-hydroxylated C_{26}-phytoceramide, α-hydroxylated C_{14}-phytoceramide and α-hydroxylated $C_{24:1}$-phytoceramide (Fig. 4D), followed by α-hydroxylated C_{24}-phytoceramide, non-hydroxylated $C_{26:1}$-phytoceramide, phytosphingosine and dihydrosphingosine (Fig. 4E), with other sphingolipids being minor species (Fig. 4F).

Many of these species showed significant reduction in their enrichment in mitochondria. Most notably, the content of α-hydroxylated C_{26}-phytoceramide in *isc1Δ* mitochondria was only 6.94% of that of WT mitochondria (Fig. 5A), and this decrease was significantly rescued by expression of FLAG-*ISC1* (Fig. 5C). As a consequence, the enrichment of α-hydroxylated C_{26}-phytoceramide in mitochondria was decreased to 2.58 fold in *isc1Δ* (Fig. 4A and 4D) relative to the 21.7 fold in WT (Fig. 3D). This decrease in *isc1Δ* mitochondria was also observed for other ceramides (Fig. 5A).

These results suggest that Isc1p functions in cells to generate mitochondrial ceramides mainly composed of α-hydroxylated phyotceramides. Reciprocally, α-hydroxylated C_{14}-phytoceramide and non-hydroxylated C_{26}-phytoceramide were increased in *isc1Δ* mitochondria (Fig. 5B), suggesting a complementary pathway to overcome the decrease of other ceramide species. These lipid changes in *isc1Δ* mitochondria were partially suppressed by the expression of FLAG-*ISC1* (Fig. 5C), although some of the lipids were not recovered to 100%, probably because FLAG-*ISC1* was expressed under the *GAL1* promoter. Nontheless, this result indicates that the lipid changes observed in *isc1Δ* mitochondria relative to WT mitochondria are not derived from artifact of mitochondrial purification. Phospholipid content per protein in WT mitochondria was 34.9+/−3.14 (nmol/mg), while that in *isc1Δ* mitochondria is 36.7+/−0.96 (nmol/mg), indicating that the mass profile changes induced by the deletion of *ISC1* are not due to phospholipid changes in the mitochondria of *isc1Δ*. These results demonstrate that the *isc1Δ* has an altered mitochondrial sphingolipid profile, and suggest that Isc1p produces selectively α-hydroxylated phytoceramides in mitochondria in vivo.

3.4. Disruption of ISC1 induces mitochondrial-associated defects

The alteration of sphingolipid profile of mitochondria in vivo observed above suggested that Isc1p may influence mitochondrial related functions. In order to address this hypothesis, we examined the formation of petite colonies in *isc1Δ*. In *S. cerevisiae,* mitochondrial defects often manifest by growing as respiratory deficient, "petite" colonies on fermentable carbon sources. In addition, cells with compromised mitochondria form large numbers of petite colonies after prolonged culture at elevated temperature when grown on either fermentable or non-fermentable carbon sources [38,39]. To determine whether *isc1Δ* would form petites under these conditions, the *isc1Δ* strain was grown at normal (30°C) and high temperature (39°C), and the fraction of petite colonies was determined by counting the ratio of colonies which have smaller size than normal and are unable to grow on non-fermentable carbon source plates (YPGE). The results showed that the *isc1Δ* strain exhibited a significantly increased ratio of petite colonies at normal temperature ($p<0.01$, Fig. 6A) and this phenotype was significantly exacerbated by growth at high temperature ($p<0.12$, Fig. 6A).

Yeast cells with compromised mitochondria have also been shown to be sensitive to oxidative stress such as hydrogen peroxide [40]. To test whether the *isc1Δ* strain was sensitive to hydrogen peroxide, yeast in log phase growth were treated with 1 mM hydrogen peroxide for 45 min, and the number of colonies was measured. Figure 6B demonstrates that the number of colonies of WT cells treated with hydrogen peroxide was 58.0% of that of non-treated cells whereas the number of colonies of *isc1Δ* cells treated with hydrogen peroxide was 41.2% of that of non-treated cells, indicating that *isc1Δ* is significantly sensitive to hydrogen peroxide relative to WT ($p<0.01$).

Ethidium bromide has been reported to specifically damage mitochondrial DNA, and mutants that have dysfunctional mitochondria show altered sensitivity to this substance [41,42]. To elucidate if *isc1Δ* is sensitive to ethidium bromide, WT and *isc1Δ* cells were treated with 50 μM ethidium bromide and spotted onto a YPD plate. The results showed that *isc1Δ* was more sensitive to ethidium bromide than WT (Fig. 6C). Together, these results indicate that *isc1Δ*

has defective mitochondrial phenotypes and reveal a role of sphingolipid in mitochondrial function in vivo.

4. Discussion

Data in this report reveal that Isc1p of *Saccharomyces cerevisiae* localizes to the outer membrane of mitochondria as an integral membrane protein, and its activity is enriched in purified mitochondria. Moreover, this study is the first to measure α-hydroxylated ceramide species and to show that mitochondria are highly enriched in very long chain α-hydroxylated phytoceramides. Importantly *isc1Δ* showed an altered mitochondrial sphingolipid profile, indicating that Isc1p regulates sphingolipid metabolism of mitochondria, especially the formation of α-hydroxylated very long chain phytoceramides. Finally, *isc1Δ* displayed defects in mitochondrial functions, suggesting, for the first time, a role for mitochondrial sphingolipid metabolism in mitochondrial metabolic function in vivo.

Recent advances in methods for mitochondrial purification as well as newly developed lipidomic strategies have enabled a more comprehensive and precise assessment of simple sphingolipids in mitochondria in *Saccharomyces cerevisiae*. Previous data indicated that mitochondria contain 7.4% of the total complex sphingolipid content, with an additional 30% in plasma membrane [10]. Another report demonstrated that mitochondria contain 4.6 nmol of IPC/mg protein, 7.7 nmol of MIPC/mg protein and 5.6 nmol of $M(IP)_2C$/mg protein, while plasma membrane contains 34.9 nmol of IPC/mg protein, 69.9 nmol of MIPC/mg protein and 120.5 nmol of $M(IP)_2C$/mg protein [9]. It is important to note, however, that the mitochondrial fractions used for these studies were not derived from density gradient centrifugation, and thus, it is difficult to know the degree of contamination of these preparations with microsomal membranes. Moreover, these previous reports studied only complex sphingolipids and did not address ceramides and sphingoid bases, the subjects of this study.

With this current analysis, it was found that mitochondria from WT cells are highly enriched, and specifically, with very long chain α-hydroxylated phytoceramides. This unexpected result raises a number of questions as to the metabolism of very long chain sphingolipids in yeast and their function. Very little is known about the regulation of incorporation of very long chain fatty acids into sphingolipids or the subcellular sites of migration of these lipids. Both Lag1p and Lac1p ceramide synthases, thought to reside in the ER, are capable of introducing very long chain fatty acids into ceramides [43], which in turn are incorporated in IPC, MIPC, and $M(IP)_2C$ [44-47]. While the bulk of these complex sphingolipids eventually arrives at the plasma membrane, published results show that a significant component of these lipids may reside in the mitochondria [9]. Together with the current results that Isc1p is localized to the outer membrane of mitochondria (Fig. 1) and mitochondria purified from *isc1Δ* exhibit a lower content of α-hydroxylated phytoceramides than that from WT (Fig. 5A), it is suggested that these complex sphingolipids become substrates for Isc1p either at the outer mitochondrial membrane or in a compartment that comes in contact with the outer membrane (e.g. mitochondrial associated membranes or others). Further studies are required to determine where and how Isc1p interacts with yeast complex sphingolipids. Moreover, this study did not address the issue of topology of Isc1p, and therefore, it is not known whether its active site faces into the mitochondria or out of the mitochondria. This proposal that sphingolipids are generated at the outer membrane of mitochondria is also in agreement with the previous report [48] that detected threefold enrichment of ceramide in the outer membrane relative to the inner membrane of rat liver mitochondria. Considering that Isc1p is activated by cardiolipin and phosphatidylglycerol [26,29] and that these lipids are synthesized in the inner membrane of mitochondria [49-51], Isc1p may be activated at the membrane contact site of outer and inner membrane [52,53]. Alternatively, up to 36.6% of cardiolipin has been detected in the outer membrane of mitochondria [54,55] and this could then activate Isc1p. Further investigation is

required for elucidation of pathways of subcellular transport and metabolism of complex sphingolipids.

The results also showed that mitochondria are relatively depleted in sphingoid bases, and this is consistent with the known ER sites for de novo sphingolipid synthesis [56] or ceramide degradation in the ER and Golgi [57,58].

The current results as well as previous results [29] implicating Isc1p in regulation of metabolism of non-fermentable carbon sources suggest a role for either the precursor sphingolipids and/or the generated α-hydroxylated phytoceramides in regulating metabolic functions of mitochondria. These could include signaling functions of the ceramides and/or structural functions. The latter are consistent with the recent suggestion that many mitochondrial proteins reside in 'rafts' [59]. Moreover, genome-wide analysis revealed that most of ergosterol synthetic mutants showed defective mitochondrial morphology [60]. Since, ergosterol has been reported to form rafts together with sphingolipids [61], the results in this study raise the possibility of sphingolipid/sterol structures/raft in the function of mitochondria.

The specific ceramide generated by the action of Isc1p could also play regulatory functions. In mammalian cells, ceramide, exogenously added or generated endogenously by overexpression of mitochondrially-targeted sphingomyelinase, has been shown to induce/regulate Bax translocation and to be involved in mitochondrial dysfunction, such as increases in the permeability of the outer membrane and release of cytochrome c [13,15,17,62]. However, whether endogenous ceramides are generated in eukaryotic mitochondria and exert effects on mitochondria in vivo has not been well evaluated and has been under significant discussion [63]. The results in this study show, for the first time, that native alteration in sphingolipid metabolism in mitochondria causes dysfunctions of mitochondria in vivo, suggesting an intrinsic role of some specific ceramide in regulating mitochondrial functions. However, further studies are required for elucidation of sphingolipid-mediated functions in yeast.

In conclusion, we have determined the submitochondrial localization of Isc1p and its effect on mitochondrial sphingolipid metabolism, and elucidated that *isc1Δ* exhibits mitochondria-related phenotypes. The findings contribute key knowledge toward complete understanding of the metabolism of mitochondrial sphingolipids and their roles in mitochondrial function in eukaryotic cells.

Abbreviations

IPC, inositol phosphorylceramide; MIPC, mannosylinositol phosphorylceramide; M(IP)$_2$C, mannosyldiinositol phosphorylceramide; YPD, yeast/peptone/dextrose; YPGE, yeast/peptone/glycerol/ethanol; SEM, standard error of mean; ER, endoplasmic reticulum; IS, internal standard; a-HO-, α-hydroxylated; Cer, ceramide; DHSph, dihydrosphingosine; Phyto-Sph, phytosphingosine; dhCn-Cer, dihydroceramide with Cn fatty acid; phytoCn-Cer, phytoceramide with Cn fatty acid.
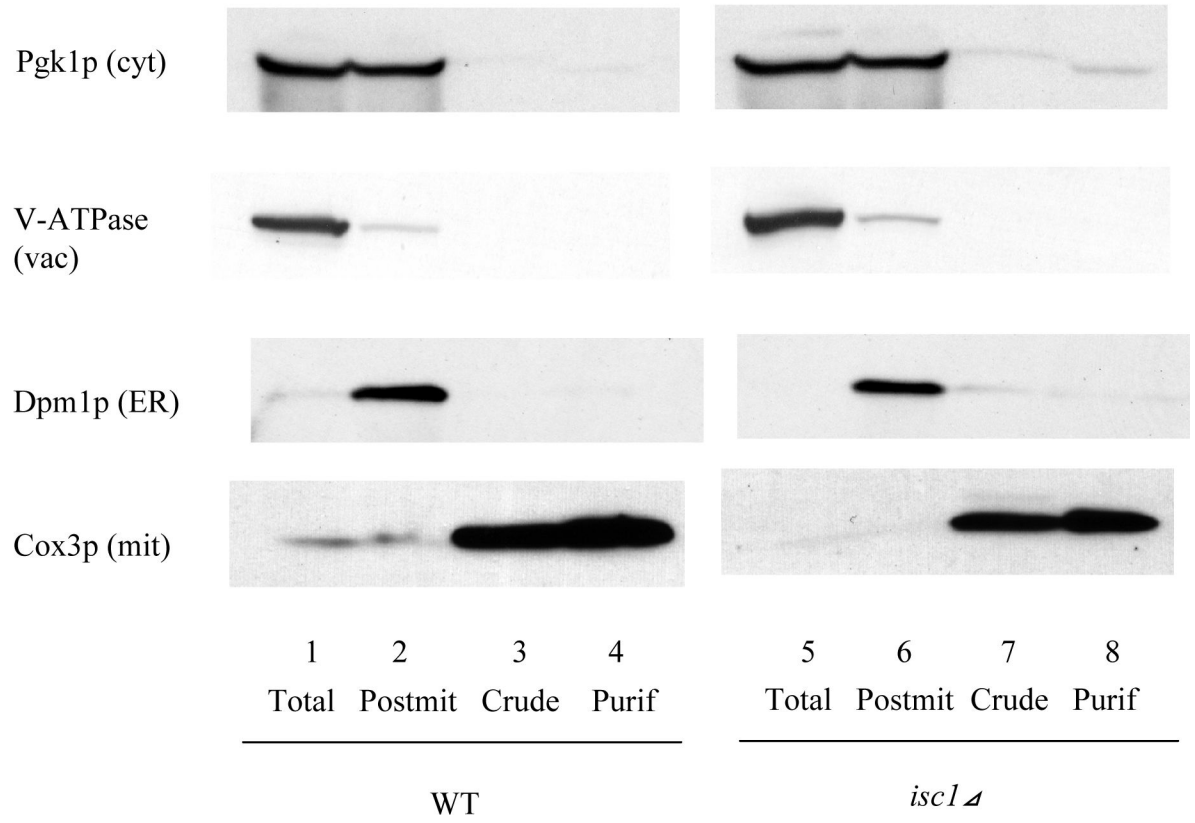
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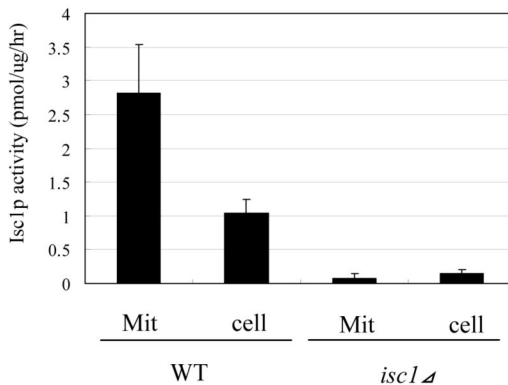
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A

	1	2	3	4		5	6	7	8
	Total	Postmit	Crude	Purif		Total	Postmit	Crude	Purif
			WT					*isc1Δ*	

B

C

	1	2	3	4	5	6	7	8	9
	Total	Memb	Super	Memb	Super	Memb	Super	Memb	Super
Osmotic shock	-			+		+		+	
NaCl	-			-		+		+	
Na$_2$CO$_3$	-			-		-		+	

D

	1	2	3	4
Proteinase K	-	+	-	+
Osmotic shock	-	-	+	+
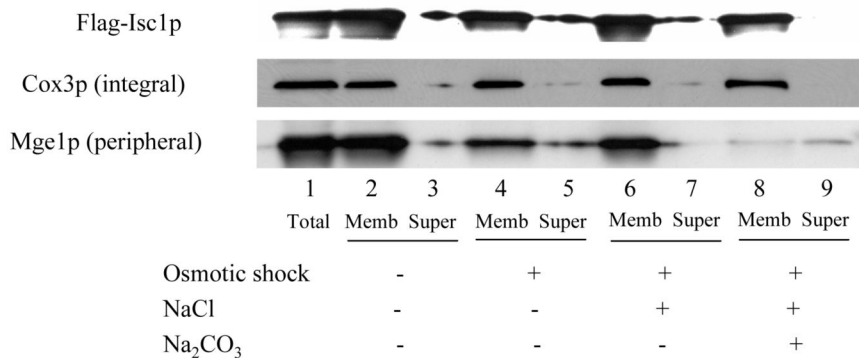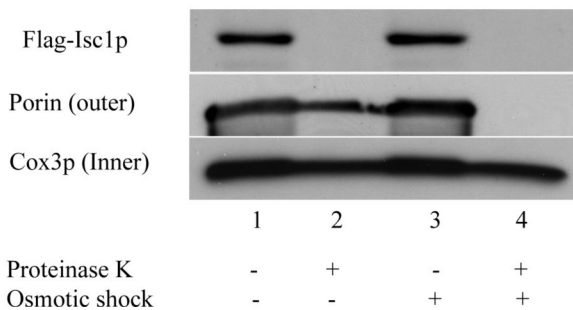
Fig. 1. Purification of mitochondria and submitochondrial fractionation of Flag-Isc1p
(A) Validation of the purity of mitochondria. Forty micrograms of each sample (lanes 1,5; total cell lysate, lanes 2,6; post-mitochondrial fraction, lanes 3,7; crude mitochondria and lanes 4,8; purified mitochondria) from WT (lanes 1−4) and *isc1Δ*(lanes 5−8) were subjected to western blotting against a cytosolic protein, Pgk1p, a vacuolar protein, V-ATPase, an ER membrane protein, Dpm1p, and a mitochondrial membrane protein, Cox3p. (B) Isc1p activity of purified mitochondria. Isc1p activity of purified mitochondria and the whole cell from WT and *isc1Δ* was measured basically as previously described (24). (C) Membrane association of Flag-Isc1p. Mitochondria purified from *isc1 Δ*+pYES/FLAG-*ISC1* were treated without osmotic shock

(lanes 2, 3; 250 mM sucrose, 10 mM HEPES, pH 7.2, 1 mM EDTA), with osmotic shock (lanes 4, 5; 10 mM HEPES, pH 7.2, 1 mM EDTA), with osmotic shock and high salt (lanes 6, 7; 0.1 M NaCl, 10 mM HEPES, pH 7.2, 1 mM EDTA), or with osmotic shock and alkali pH (lanes 8, 9; 0.1 M Na_2CO_3, pH 11.2, 10 mM HEPES, pH 7.2, 1 mM EDTA), centrifuged at 100,000 g for 10 min, and total (lane 1), membrane (lanes 2, 4, 6, 8), and supernatant (lanes 3, 5, 7, 9) were analyzed by western blotting using an antibody against Flag. (D) Submitochondrial localization of Flag-Isc1p. Mitochondria purified from *isc1⊿+pYES/FLAG-ISC1* were treated without osmotic shock (lanes 1, 2; 250 mM sucrose, 10 mM HEPES, pH 7.2, 1 mM EDTA) or with osmotic shock (lanes 3, 4; 10 mM HEPES, pH 7.2, 1 mM EDTA) for 10 min on ice and with (lanes 2, 4) or without (lanes 1, 3) final 50 μg/ml proteinase K for 30 min on ice. 2 mM phenylmethylsulfonyl fluoride was added, mitochondria were centrifuged at 12,000 g for 5 min, washed once, and pelles were applied to western analysis with antibody against Flag, porin, or Cox3p.

A

B

C
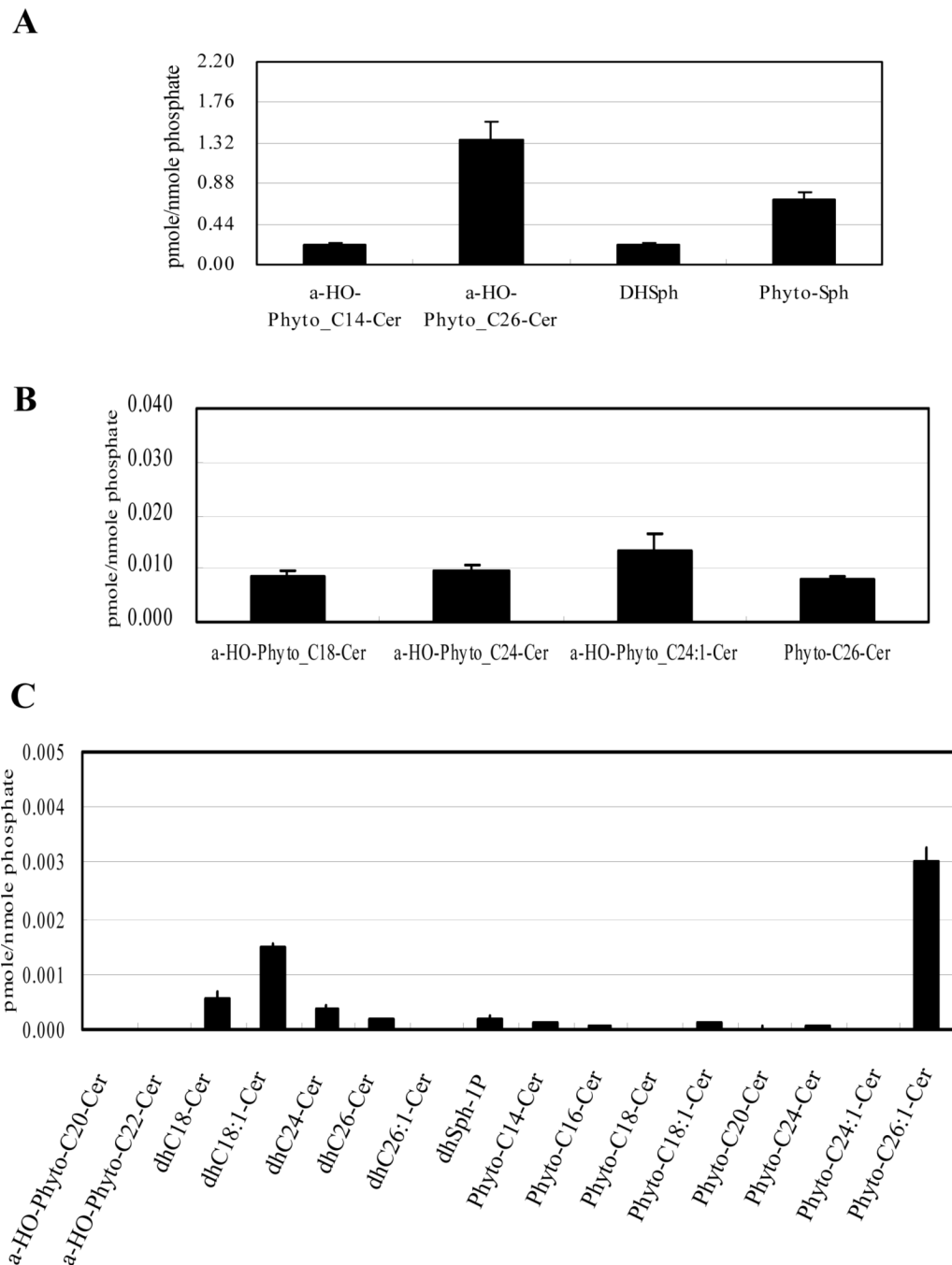
Fig. 2. Sphingolipid profile of WT cells
WT cells were incubated to the post-diauxic phase. Sphingolipids were analyzed by LC-MS and normalized by organic phosphates as described in Materials and Methods. (A) Species which are more than 0.040 pmol/nmol organic phosphate, (B) species which are more than 0.0050 pmol/nmol organic phosphate but less than 0.040 pmol/nmol organic phophate, and (C) species which are less than 0.0050 pmol/nmol organic phosphate of the whole cell of WT. The results are mean values +/− SEM of lipid measurements from three independent cultures.

A
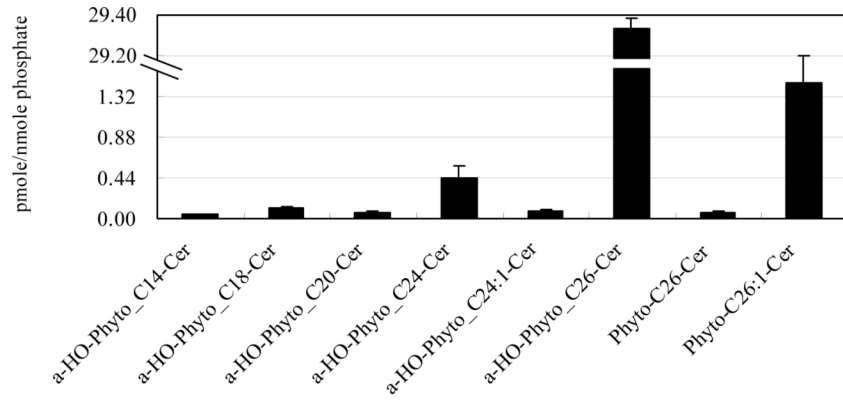
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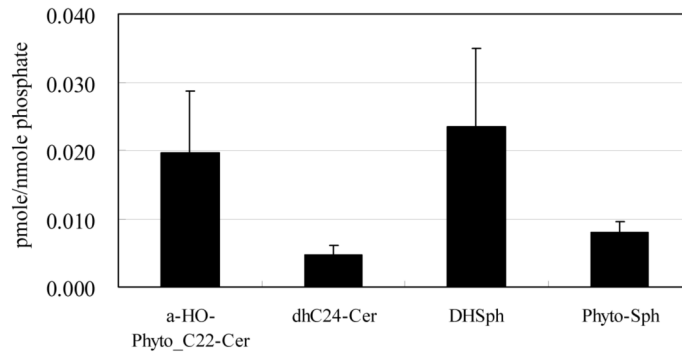
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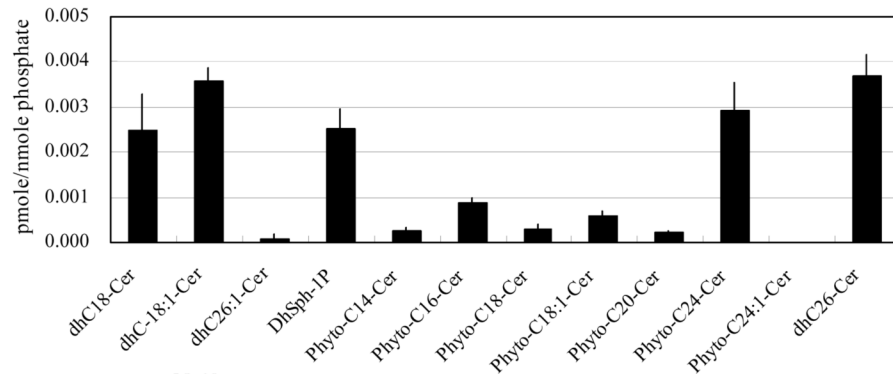
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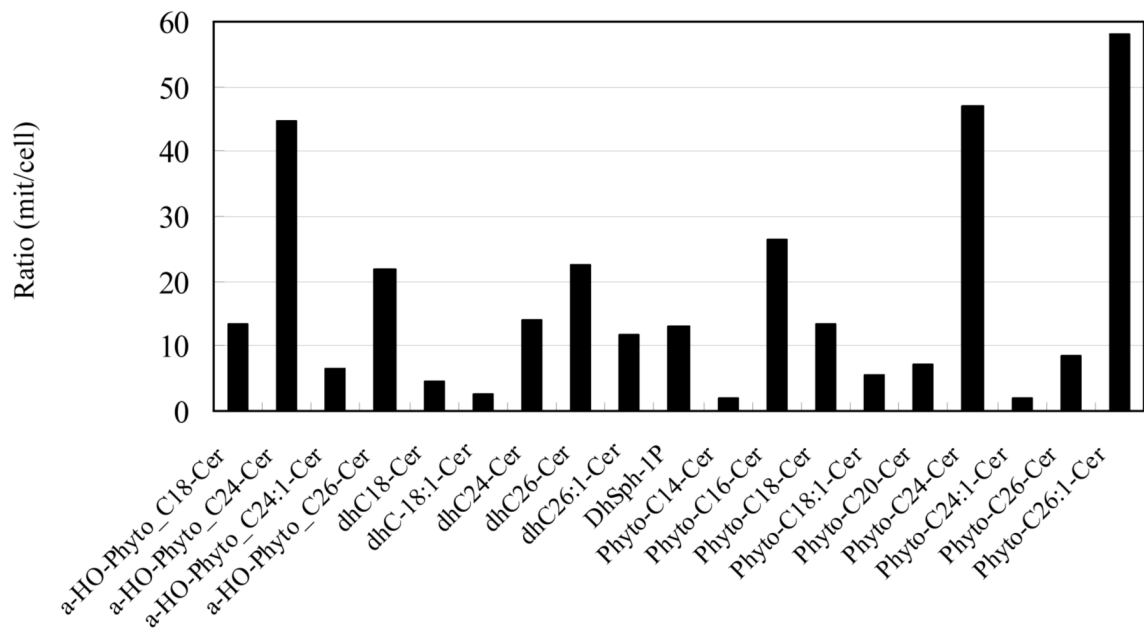
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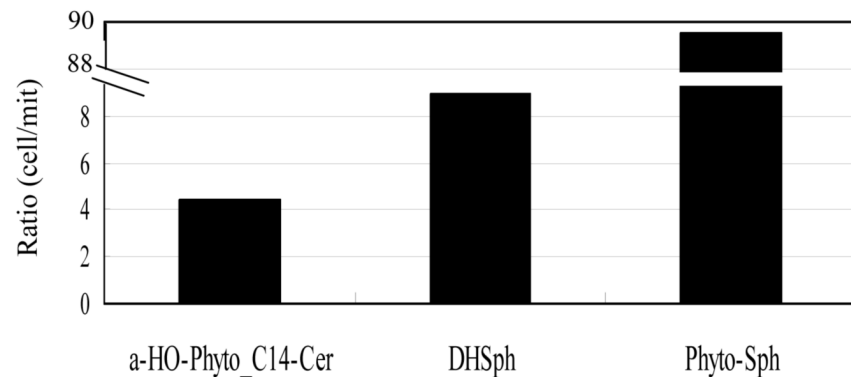
Fig. 3. Sphingolipid profile of WT mitochondria

WT cells were incubated to the post-diauxic phase, and mitochondria were purified as described in Materials and Methods. Sphingolipids were analyzed by LC-MS and normalized by organic phosphates as described in Materials and Methods. (A) Species which are more than 0.040 pmol/nmol organic phosphate, (B) species which are more than 0.0050 pmol/nmol organic phosphate but less than 0.040 pmol/nmol organic phophate, and (C) species which are less than 0.0050 pmol/nmol organic phosphate of the WT mitochondria. The results are mean values +/− SEM of three lipid measurements from two independent mitochondrial purifications. Mitochondrial sphingolipids were divided by the amount of sphingolipids in the whole cell analyzed in Fig. 2. (D) Ratio of mitochondrial sphingolipids to the sphingolipids of the whole

cell. Sphingolipids that were increased in mitochondria are shown. (E) Ratio of the sphingolipids in the whole cell to mitochondrial sphingolipids. Sphingolipids that were decreased in mitochondria are shown.

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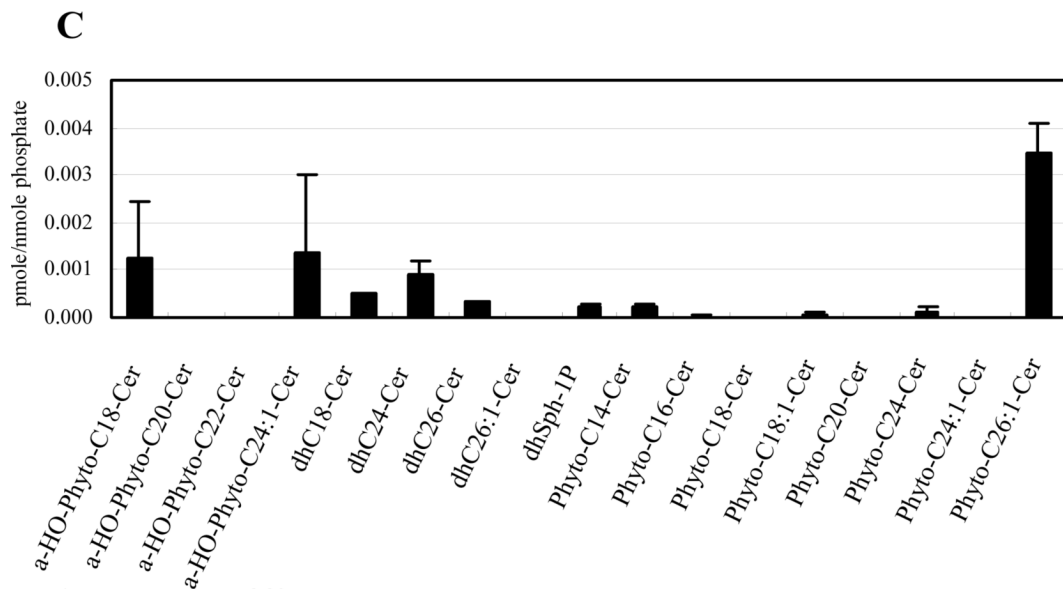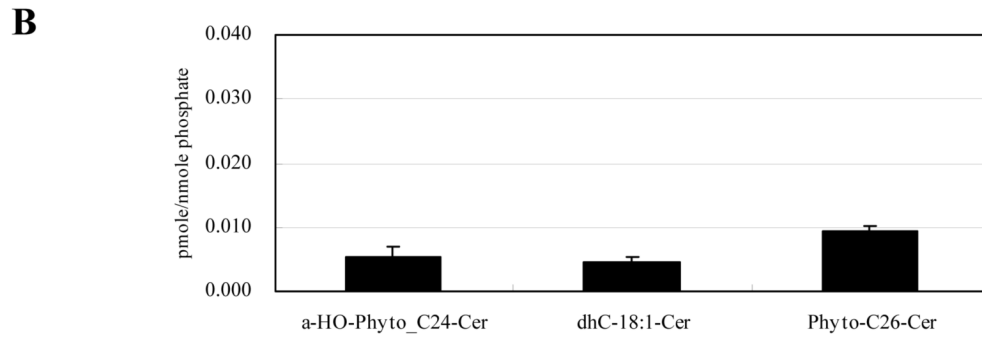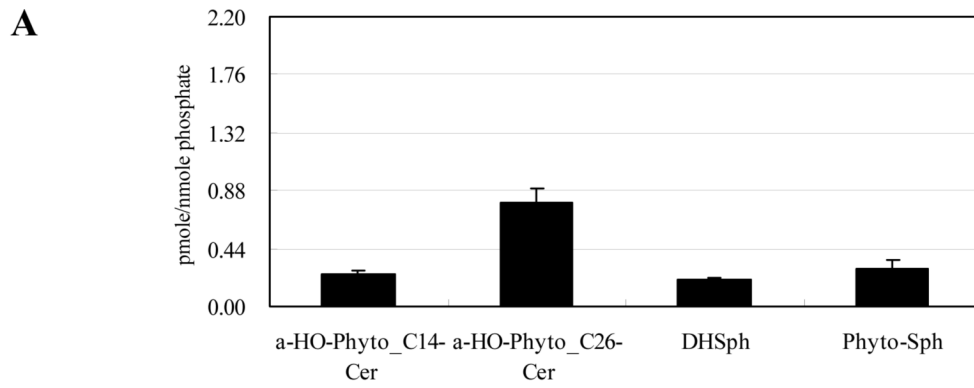
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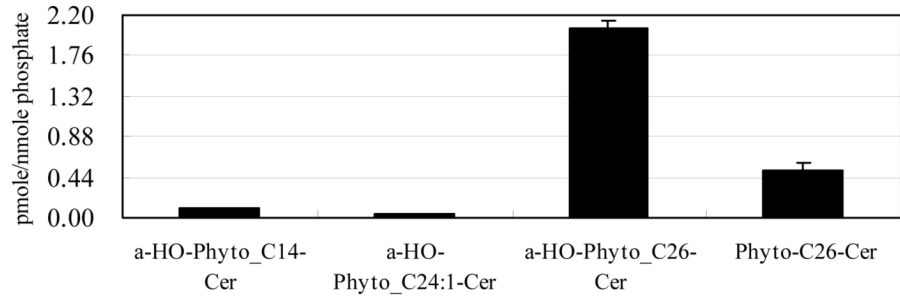
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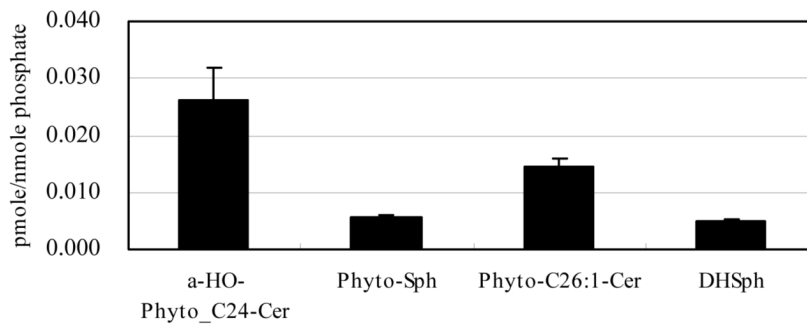
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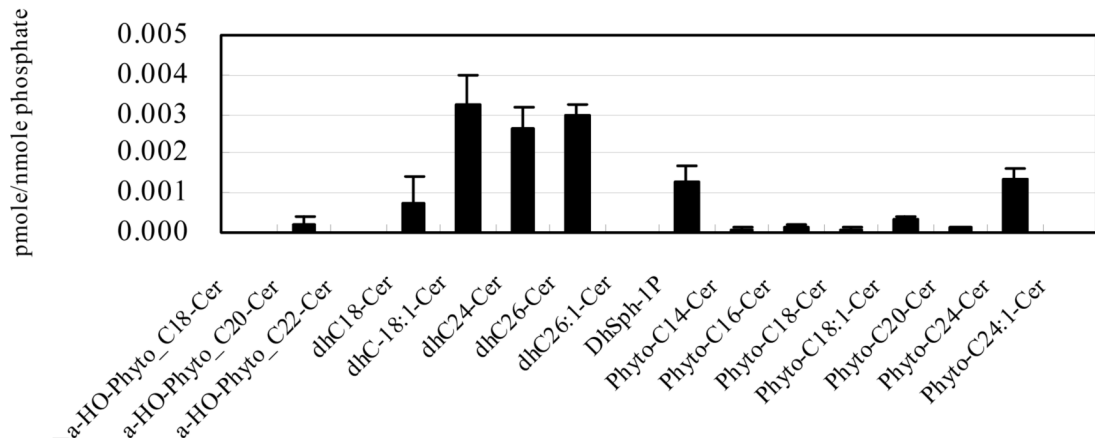
Fig. 4. Sphingolipid profile of *isc1Δ*

isc1Δ cells were incubated to the post-diauxic phase, and collected for whole cell analyses or for mitochondrial purification as described in Materials and Methods. Sphingolipids were analyzed by LC-MS as described in Materials and Methods, and normalized by organic phosphates. (A) Species which are more than 0.040 pmol/nmol organic phosphate, (B) species which are more than 0.0050 pmol/nmol organic phosphate but less than 0.040 pmol/nmol organic phophate, (C) species which are less than 0.0050 pmol/nmol organic phosphate of *isc1Δ* whole cell. (D) Species which are more than 0.040 pmol/nmol organic phosphate, (E) species which are more than 0.0050 pmol/nmol organic phosphate but less than 0.040 pmol/ nmol organic phophate, and (F) species which are less than 0.0050 pmol/nmol organic

phosphate of *isc1*⊿mitochondria. The results are mean values +/− SEM of lipid measurements from three independent cultures for the whole cell analysis, or, three lipid measurements from two independent mitochondrial purifications for mitochondrial analysis.
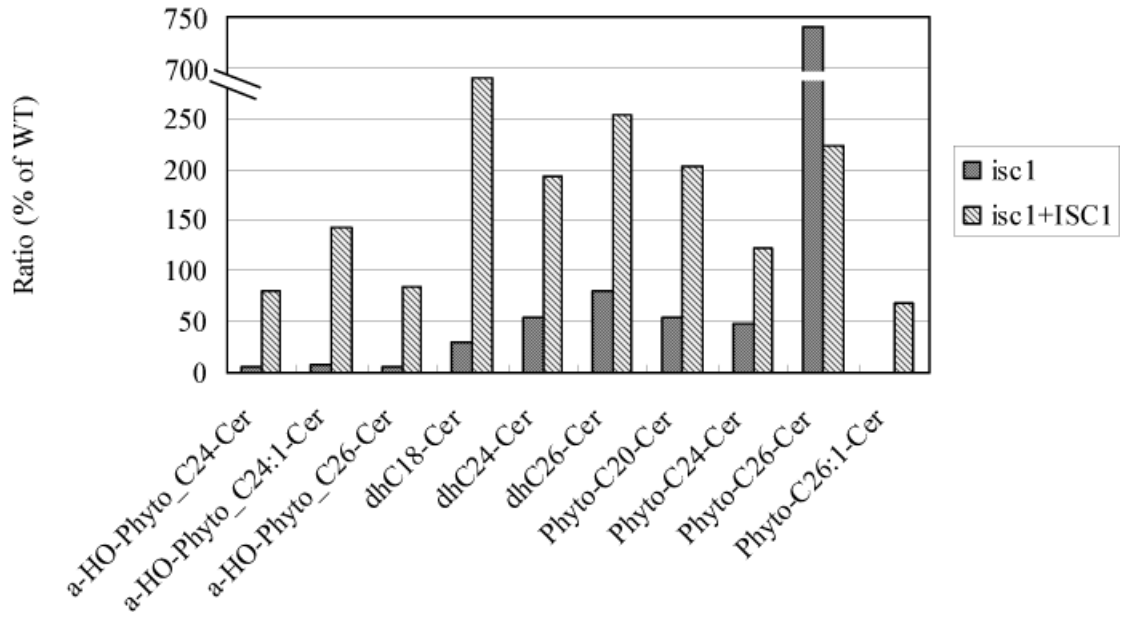
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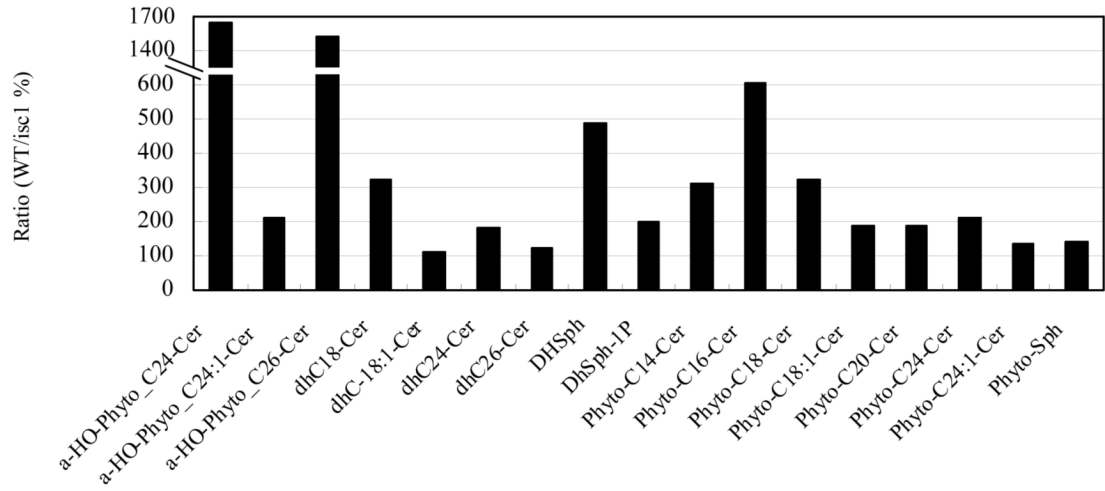
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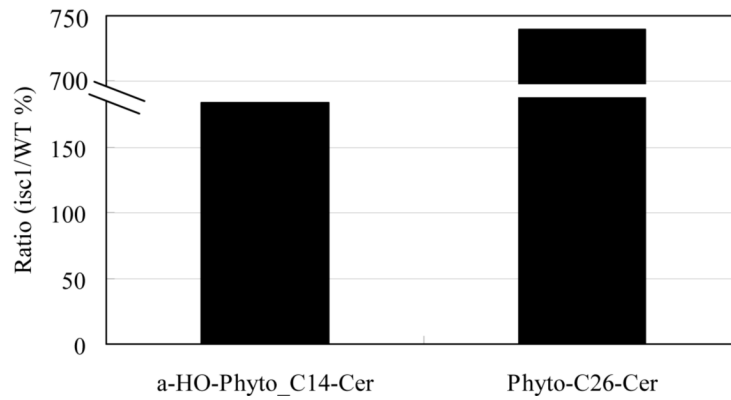
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Fig. 5. Comparison of mitochondrial sphingolipids of *isc1Δ* with those of WT
The relative amounts of individual mitochondrial sphingolipids of *isc1Δ* and *isc1Δ*+pYES/
FLAG-*ISC1* were compared with those of WT. (A) Ratio of the sphingolipids in WT relative
to those in *isc1Δ*. Sphingolipids that were decreased in *isc1Δ* are shown. (B) Ratio of the
sphingolipids in *isc1Δ* relative to those in WT. Sphingolipids that were increased in *isc1Δ* are
shown. (C) Ratio of the sphingolipids in *isc1Δ* and *isc1Δ*+pYES/FLAG-*ISC1* relative to those
in WT.

A

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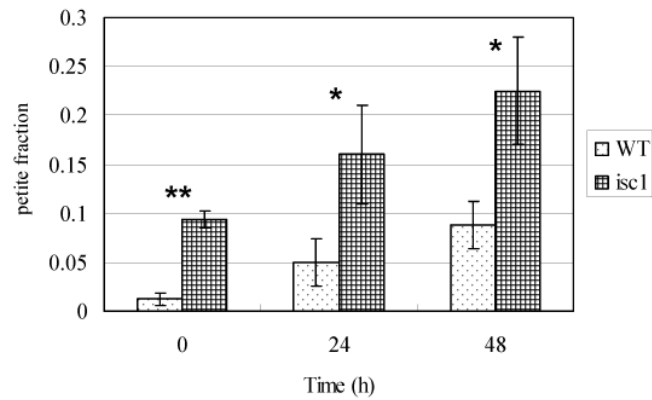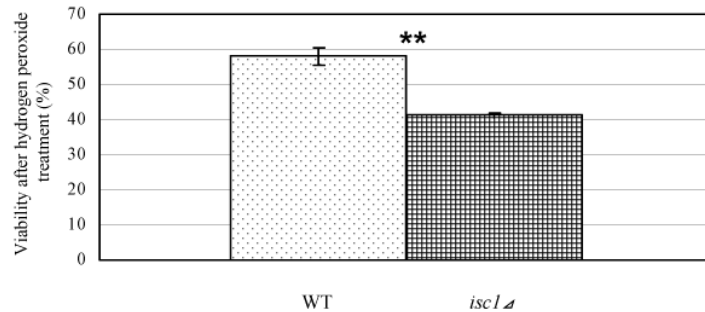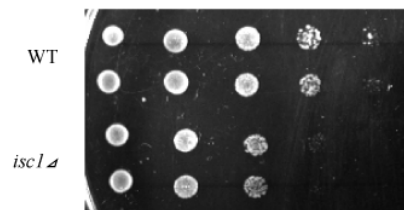
Fig. 6. *isc1∆* shows mitochondrial defects

(A) Petite ratio of *isc1∆*. WT and *isc1∆* cells were inoculated at OD_{600} of 0.1 in YPD medium, incubated at 39°C for the indicated times, and plated onto YPD plates. Small colonies that grew on YPD plates were replica plated onto YPGE plates to confirm the inability to grow on non-fermentable carbon medium, and the numbers of the colonies which grew on YPD but not on YPGE plates were counted. Statistical differences were evaluated by Student's unpaired *t* test and are indicated by asterisks (**p<0.01, *p<0.12). (B) Hydrogen peroxide sensitivity of *isc1∆*. WT and *isc1∆* were treated with 1 mM hydrogen peroxide for 45 min, plated onto YPD and the formed colonies were counted. Statistical difference was evaluated by Student's unpaired *t* test and is indicated by asterisks (**p<0.01). (C) Ethidium bromide sensitivity of

isc1Δ. WT and *isc1Δ* cells were treated with 50 μM ethidium bromide, and 5 μl of the suspension was spotted onto a YPD plate with serial dilutions at 10 fold.