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Research Article

Ydc1p ceramidase triggers organelle fragmentation, apoptosis and accelerated ageing in yeast

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Abstract. *Saccharomyces cerevisiae* dihydroceramidase Ydc1p hydrolyzes ceramide, resulting in accumulation of free long-chain bases and their phosphates. Yeast mutants lacking *YDC1* are characterized by increased chronological lifespan. Moreover, we found *YDC1* up-regulated in a yeast mutant displaying reduced chronological lifespan. These data suggest an important role for Ydc1p in chronological lifespan determination in yeast. Mitochondria are known to play an important role in chronological lifespan and apoptosis. In this study we demonstrated that overexpression of *YDC1* results in reduced chronological lifespan and increased apoptotic cell death. We found *YDC1* overexpression to result in mitochondrial fragmentation and dysfunction. Interestingly, vacuoles also appeared to be fragmented and dysfunctional upon *YDC1* overexpressing. Exogenous addition of ceramide to *YDC1*-overexpressing cultures increased chronological lifespan and restored organelle function. In conclusion, this study describes a direct link between ceramide metabolism in yeast and mitochondrial and vacuolar fragmentation and function, with consequences for chronological lifespan in yeast.

Keywords. Yeast, ceramide, organelle fragmentation, apoptosis, lifespan.

Introduction

Sphingolipids were initially identified as important structural components of eukaryotic cell membranes. However, a number of recent studies revealed that intermediates in sphingolipid biosynthesis play important roles as signaling molecules and growth regulators [1, 2]. Among these bioactive sphingolipid intermediates, ceramide has been extensively studied in mammals and identified as a bioactive lipid with critical roles in apoptosis and stress responses [1, 2]. Its concentration is controlled by *de novo* synthesis, by breakdown of complex sphingolipids, and by ceramidases that hydrolyze the amide bond between sphingosine and the fatty acid [3].

In the yeast *Saccharomyces cerevisiae* two ceramidases have been identified, yeast dihydroceramidase Ydc1p [4] and yeast phytoceramidase Ypc1p [5]. Yeast mutants overexpressing either *YDC1* or *YPC1*

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are characterized by an increased breakdown of ceramide, resulting in accumulation of free long-chain bases and their phosphates [4]. Deletion of either *YPC1* or *YDC1* causes an increase in complex sphingolipids due to decreased breakdown of ceramides [4]. It was previously suggested that the two enzymes have distinct physiological functions since cells lacking Ydc1p activity display increased sensitivity to heat stress, whereas cells lacking Ypc1p activity show no such phenotype [4]. However, the biological role of these enzymes remains largely unknown. Since most research in yeast is done with log phase cells, these ceramidases may influence processes during other growth phases including stationary phase (such as determination of chronological lifespan), spore formation or germination or filamentous growth [3]. In this respect, yeast mutants lacking *YDC1*, but not *YPC1*, are characterized by increased chronological lifespan according to the ranking of Powers and coworkers [6]. In addition, we recently found *YDC1,* but not *YPC1,* to be up-regulated in a yeast mutant displaying reduced chronological lifespan ([7] and B. Smets, unpublished data). These data suggest an important role for Ydc1p in the regulation of chronological lifespan in yeast. With respect to *YPC1* no data indicate such an involvement in chronological ageing.

In this study, we investigated the role of the yeast ceramidase Ydc1p in chronological lifespan and associated phenotypes including apoptosis and oxidative stress sensitivity. We found that *YDC1* overexpression leads to reduced chronological lifespan and increased oxidative stress sensitivity in yeast. Analysis of DNA fragmentation, phosphatidylserine flip-flop and caspase activity showed that *YDC1* overexpression increases apoptotic cell death in yeast during chronological ageing. Our results further demonstrated increased fragmentation and dysfunction of mitochondria and vacuoles upon *YDC1* overexpression.

Materials and methods

Materials and microorganisms. C_2-, C_6- and C_8-dihydroceramides were purchased from Sigma-Aldrich (St. Louis, MO, USA). The caspase inhibitor Z-VAD-FMK was from Promega (Madison, WI, USA). Yeast strains used in this study are *S. cerevisiae* wild-type strain BY4741 (Invitrogen, Carlsbad, CA, USA). The *YDC1* overexpression plasmid pYES2-*YDC1* [4] and empty pYES2 were transformed to BY4741. In all assays described below, BY4741 yeast transformants containing pYES2 or pYES2-*YDC1* were grown overnight in SC-ura medium [0.8 g/L complete amino acid supplement mixture minus uracil (CSM-ura), Bio 101 Systems; 6.5 g/L yeast nitrogen base (YNB); 20 g/L glucose]. Expression of Ydc1p was induced in SC-ura containing 2% galactose [SC-ura (Gal)].

Ceramide analysis. Overnight yeast cultures, grown in SC-ura, were washed three times with sterilized water before being diluted in fresh SC-ura (Gal) at $OD_{600} = 0.2$. Ceramides in overnight yeast cultures in SC-ura (Gal) were fortified with internal standards (ISs) and extracted twice. For extraction, iso-propanol:water:ethyl acetate (30:10:60; v:v:v) was added, and samples were vortexed, sonicated and centrifuged for 10 min at 4000 rpm. Supernatants were combined and analyzed by electrospray ionization (ESI)-tandem mass spectroscopy (MS/MS), which was performed on a ThermoFinnigan TSQ 7000 triple quadrupole mass spectrometer, operating in a Multiple Reaction Monitoring (MRM)-positive ionization mode as described [8]. Levels of ceramides were normalized to total phosphate, which was determined as described [9].

Chronological lifespan of yeast. Overnight yeast cultures, grown in SC-ura, were diluted in fresh SC-ura (Gal) at $OD_{600} = 0.025$. Viability of the yeast cultures was analyzed by counting the number of colony-forming units (CFU)/mL on SC-ura agar plates [7]. Day 0 was set as the day with the highest CFU/mL and % survival was determined relative to this time point.

Apoptosis in yeast. Apoptotic markers, comprising reactive oxygen species (ROS) levels, phosphatidylserine externalization and DNA fragmentation, of the above-described chronologically ageing yeast cultures were quantified using flow cytometry after staining with dihydroethidium (DHE), FITC-labeled annexin V in combination with propidium iodide and terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) staining, respectively [10, 11]. For each staining, 30 000 yeast cells were evaluated (in four independent experiments which were pooled) using flow cytometry and BD FACSDiva software. Caspase activation of yeast cultures was analyzed by incubating the chronologically aged yeast cultures with the CaspACE™ FITC-VAD-FMK In Situ Marker (Promega), which allows the detection of cells with activated caspases *via* fluorescence microscopy [12]. The antagonizing effect of inhibiting caspase activity on apoptosis was analyzed by treatment of the yeast cultures during chronological ageing with the caspase inhibitor z-VAD-FMK (Promega) and counting the number of CFU/mL on SC-ura agar plates.

Table 1. Ceramide content of membranes of *YDC1*-overexpressing yeast population (pYES2-*YDC1*) and control population (pYES2). Data represent mean ± SEM.

	pmol ceramide/micromole P_i						
	$dhC_{18:1}$-Cer	dhC_{12}-Cer	dhC_{24}-Cer	$dhC_{24:1}$-Cer	Phyto-C_{24}-Cer	Phyto-C_{26}-Cer	Phyto-$C_{28:1}$-Cer
pYES2	0.611 ± 0.02	0.959 ± 0.002	1.788 ± 0.14	0.662 ± 0.013	20.74 ± 2.65	13.74 ± 2.27	2.03 ± 0.21
pYES2-*YDC1*	0.370 ± 0.057	0.451 ± 0.072	1.017 ± 0.028	0.38 ± 0.015	13.03 ± 2.18	8.79 ± 0.65	0.41 ± 0.038

Visualization of mitochondrial and vacuolar morphology. Yeast precultures were grown overnight in SC-ura and *YDC1* expression was induced by growing the yeast in SC-ura (Gal), starting at $OD_{600} = 0.025$. At $OD_{600} = 1$, 1 mL of cells was washed with fresh medium and resuspended in PBS. Mitochondria were visualized using 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; Acros Organics, Geel, Belgium) at 175 nM (100 ng/mL) (standard concentration) or 1.75 μM (1 μg/mL; tenfold excess concentration). Cells were mixed and visualized immediately under OptiPhot fluorescence microscope using 470/520LP filter set (Nikon, Japan). Alternatively, BY4741 containing pYES2-*YDC1* or empty plasmid were transformed with pYESH-mtGFP, containing mtGFP fused to the first 69 amino acids of the subunit 9 of the F0 ATPase from *Neurospora crassa,* under control of the inducible GAL1 promoter [13]. pYESH-mtGFP was obtained by exchanging URA3 marker of the pYES-mtGFP with HIS3 marker from pYX022 plasmid (R&D Systems) using *Nhe*I and *Ahd*I restriction enzymes. Transformants were grown on SC-ura-his and *YDC1* expression was induced under the same conditions as described above. Cells were visualized with a Leica DM4000B fluorescence microscope (Leica Microsystems, Switzerland) using a BP 470/BP 525 filter set. For visualization of vacuoles, yeast strains were grown in 50 mL SC-ura (Gal) to $OD_{600} = 1$. Cells were harvested, washed with fresh medium and stained with either 18 μM *N*-(3-triethylammoniumpropyl)-4-{6-[4-(diethylamino)phenyl]-hexatrienyl}pyridinium dibromide (FM4–64 dye; Molecular Probes) [14] or 100 μM CellTracker Blue 7-amino-4-chloromethylcoumarin (CMAC; Molecular Probes) [15] in 10 mM HEPES buffer (pH 7.4) containing 2 % galactose [HEPES(Gal)]. Cells were washed with HEPES(Gal) and vacuoles were visualized using a OptiPhot fluorescence microscope with a 525/590LP or a 350/470 filter set (Nikon, Japan), respectively. Pictures were captured using a Nikon DS Camera Head DS-5M and Control Unit DS-L1 and Eclipse Net software (Laboratory Imageing, Praha, Czech Republic).

Oxidative stress, HCl and NaOH sensitivity assay. Fifteen microliters of 250 mM H2O2, 5 μL 5 M HCl or 5 μL 5 M NaOH were spotted on SC-ura (Gal), which was inoculated with 1:50 dilution of an overnight yeast culture. After 2 days of incubation at 30°C, diameters of the inhibitory halos were measured. To assess the effect of ceramides on H_2O_2, HCl and NaOH hypersensitivity of yeast cultures, the minimal inhibitory concentration (MIC) was determined for H_2O_2, HCl and NaOH, using a twofold dilution series in SC-ura (Gal) and 10^5/mL yeast inoculum of an overnight culture in SC-ura, in the presence of 0–100 μg/mL of various ceramide species.

Results

***YDC1* overexpression leads to decreased ceramide levels.** The yeast Ydc1p was previously characterized as an alkaline ceramidase [4]. We confirmed this further by analyzing ceramide levels in membranes of a *YDC1*-overexpressing yeast population and the control population (BY4741 containing the empty plasmid pYES2) in SC-ura (Gal) using ESI-MS/MS. We found the levels of various phyto- and dihydroceramides [namely dihydro (dh) $C_{18:1}$-Ceramide (Cer), dhC_{12}-Cer, dhC_{24}-Cer, $dhC_{24:1}$-Cer, Phyto-C_{24}-Cer, Phyto-C_{26}-Cer and Phyto-$C_{28:1}$-Cer] to be decreased by 35–80 % upon *YDC1* overexpression (Table 1), demonstrating the ceramidase activity of Ydc1p.

***YDC1* overexpression shortens chronological lifespan and increases apoptosis.** To get more insight in the role of ceramidases in stationary phase processes in yeast, such as determination of its chronological lifespan, we analyzed the effect of overexpression of *YDC1* on chronological lifespan. We found that the chronological lifespan of a *YDC1*-overexpressing yeast population in SC-ura (Gal) was reduced as compared to the control population: the maximal chronological lifespan (CL_{max}) of the *YDC1*-overexpressing yeast population, *i.e.*, the time point corresponding to 0.1 % survival of the yeast population, was 8 days, whereas CL_{max} of the control population was more than 14 days (Fig. 1). The survival of chronologically ageing *YDC1*-overexpressing cultures drastically dropped after 3 days of incubation. Since a link between chronological lifespan and apoptosis in

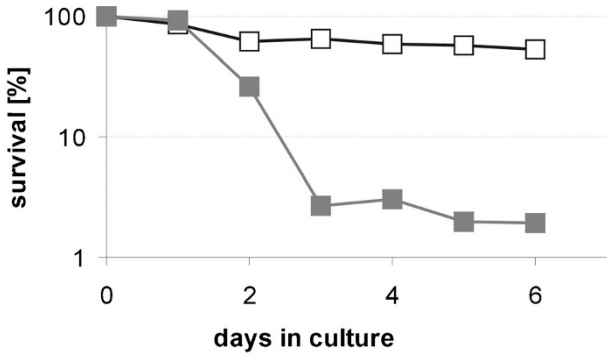

Figure 1. Yeast transformants overexpressing *YDC1* are characterized by reduced chronological lifespan. Chronological lifespan of wild-type strain BY4741 containing empty pYES2 (open squares) and BY4741 overexpressing *YDC1* (black squares). Yeast cultures were grown in SC-ura (Gal) and viability of these cultures was analyzed by counting the number of CFU/mL. This figure is a representative of five experiments.

yeast exists [16], we assessed the apoptotic features of a chronologically ageing *YDC1*-overexpressing yeast population as compared to the control population.

After 3 days of chronological ageing, coinciding with the survival drop of *YDC1*-overexpressing yeast cells, this population exhibited increased apoptotic features as compared to the control population (Fig. 2): its endogenous ROS levels, as visualized by DHE, were significantly increased (68.5 ± 1.85% of the *YDC1*-overexpressing yeast population was DHE positive) as compared to the control population (43.1 ± 1.05% of the population was DHE positive) (Fig. 2A). In addition, 10.2 ± 1.8% of the *YDC1*-overexpressing yeast population was TUNEL positive, visualizing chromosome fragmentation, *versus* 4.8 ± 0.7% of the yeast control population (Fig. 2B). Furthermore, 19.9 ± 1.0% of the *YDC1*-overexpressing yeast population showed phosphatidylserine externalization (annexin V-positive, but propidium iodide-negative cells) *versus* 9.6 ± 0.5%% of the control population (Fig. 2C). All the data above were quantified by FACS analysis [11]. Chronological ageing in yeast was previously shown to depend on caspase activation [12, 16]. At the above indicated time point, we found 89±4% of the *YDC1*-overexpressing yeast cells characterized by active caspases *versus* 75±2% of the control population, pointing to caspase-dependent accelerated chronological ageing upon *YDC1* overexpression. Moreover, in the presence of 60 μM caspase inhibitor Z-VAD-FMK, we found CL_{max} of *YDC1*-overexpressing yeast population to increase by 12±2.5% as compared to *YDC1*-overexpressing yeast population treated with DMSO. Hence, inhibition of caspases can increase the chronological lifespan of *YDC1*-overexpressing yeast cultures. All the above data point to accelerated caspase-dependent

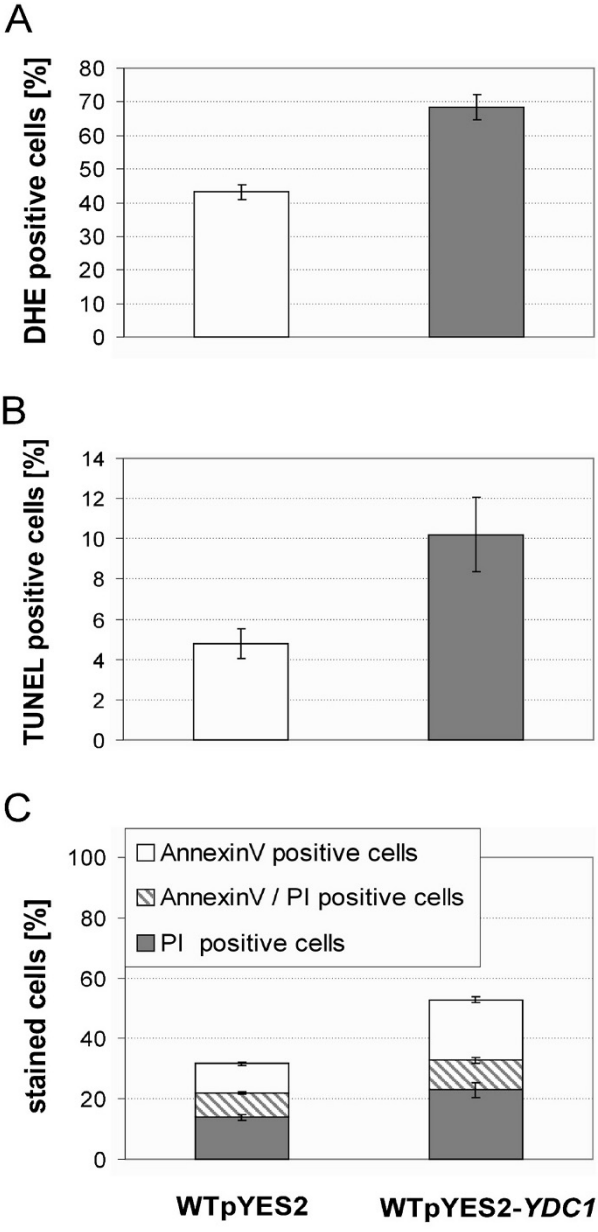

Figure 2. Yeast transformants overexpressing *YDC1* are characterized by accelerated apoptosis. Apoptotic features of the yeast cultures [containing empty pYES2 (left panels) or overexpressing *YDC1* (right panels)] after 3 days in SC-ura (Gal) were assessed by determining (*A*) endogenous ROS levels *via* DHE staining, (*B*) DNA fragmentation *via* TUNEL staining, and (*C*) phosphatidylserine externalization and membrane integrity *via* annexinV/propidium iodide co-staining. In each experiment, 30 000 cells were evaluated using flow cytometry. Data represent mean ± SEM.

apoptosis upon *YDC1* overexpression in yeast, resulting in reduced chronological lifespan.

The differences concerning apoptosis between the *YDC1*-overexpressing yeast population and the control population during chronological ageing are not that severe, although statistically significant and in line with previous reports comparing apoptosis be-

tween yeast mutant populations during chronological ageing [16, 17].

***YDC1* overexpression sensitizes to oxidative stress.** Reduced chronological lifespan and mitochondrial dysfunction, which can be assessed by hypersensitivity to H_2O_2 [18], have been linked in yeast [19, 20]. Functional mitochondria can dispose of excess ROS, such as H_2O_2, mainly *via* the superoxide dismutases (SODs): Sod1p, which localizes to the cytoplasm and the mitochondrial intermembrane space, and Sod2p, a mitochondrial matrix protein [20]. Therefore, we assessed the sensitivity to oxidative stress induced by H_2O_2 of *YDC1*-overexpressing yeast transformants as compared to yeast control transformants. *YDC1*-overexpressing yeast transformants were characterized by increased sensitivity to H_2O_2 as compared to control transformants (Fig. 3A and B), indicative for dysfunctional mitochondria.

***YDC1* overexpression results in fragmentation of mitochondria and the vacuole.** To get more insight in the dysfunctional character of the mitochondria in *YDC1*-overexpressing yeast transformants, we opted to visualize mitochondria of *YDC1*-overexpressing yeast transformants and yeast control transformants using the potential-dependent fluorescent dye $DiOC_6$. At a concentration of 20–100 ng/mL, this dye accumulates specifically at mitochondrial membranes in response to the electrochemical potential across the inner membrane and can be observed by fluorescence microscopy [21, 22]. However, cells that have low mitochondrial membrane potential will fail to accumulate $DiOC_6$ at the indicated concentration. At 100 ng/mL $DiOC_6$, staining was greatly reduced in *YDC1*-overexpressing yeast transformants as compared to control transformants (Fig. 3C and D), pointing to a reduced mitochondrial membrane potential in *YDC1*-overexpressing yeast transformants. In the next series of experiments, we used tenfold higher $DiOC_6$ concentrations for visualization of mitochondrial membranes in *YDC1*-overexpressing yeast transformants and control transformants. Balanced fusion and fission of mitochondria results in tubular mitochondrial morphology, as is the case for control transformants (Fig. 3C and E). In *YDC1*-overexpressing yeast transformants, however, excessive fission of tubular mitochondria into short punctate structures, also referred to as mitochondrial fragmentation [23], was observed (Fig. 3F). Alternatively, for visualization of mitochondrial networks, we transformed *YDC1*-overexpressing yeast transformants and control transformants with pYESH-mtGFP, encoding the GFP protein targeted to the mitochondrial matrix [13]. The latter experiment confirmed the

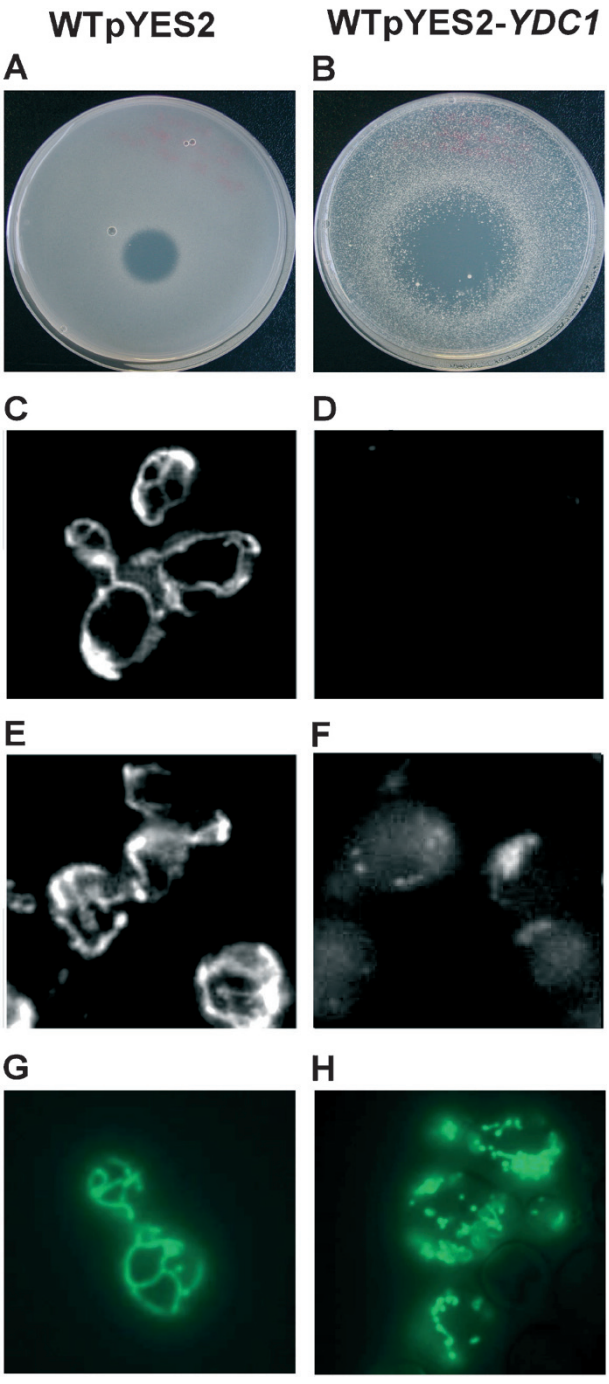

Figure 3. Mitochondria of yeast transformants overexpressing *YDC1* are fragmented and dysfunctional. (*A*, *B*) Sensitivity of wild-type strain BY4741 containing empty pYES2 (WTpYES2, left panel) and BY4741 overexpressing *YDC1* (WTpYES2-*YDC1*, right panel) to 250 mM H_2O_2 was assessed *via* growth inhibitory halo assay in SC-ura (Gal) agar plates. (*C*–*F*) $DiOC_6$ mitochondrial membrane straining of wild-type strain BY4741 containing empty pYES2 (WTpYES2, left panels) and BY4741 overexpressing *YDC1* (WTpYES2-*YDC1*, right panels) at (*C*, *D*) standard $DiOC_6$ concentration (100 ng/mL) and (*E*, *F*) tenfold increased $DiOC_6$ concentration (1 µg/mL). (*G*, *H*) Visualization of mitochondrial networks of wild-type strain BY4741 containing empty pYES2 (WTpYES2, left panel) and BY4741 overexpressing *YDC1* (WTpYES2-*YDC1*, right panel) using mtGFP marker on pYESH plasmid.

mitochondrial fragmentation upon *YDC1* overexpression (Fig. 3G and H). This altered mitochondrial morphology of *YDC1*-overexpressing yeast transformants probably results in mitochondrial dysfunction, as *YDC1*-overexpressing yeast transformants were hypersensitive to oxidative stress as compared to yeast control transformants (Fig. 3A and B) and were characterized by a decreased mitochondrial membrane potential (Fig. 3C and D).

Faergeman and coworkers [24] previously reported that yeast mutants with impaired ceramide synthesis show fragmented vacuoles. Therefore, we assessed vacuolar morphology in yeast upon overexpression of *YDC1*, using the dyes FM4–64 and Blue CMAC, which stain the membrane and the lumen of the vacuole, respectively. We found the vacuolar morphology to be affected when overexpressing *YDC1* in yeast as the vacuoles of *YDC1*-overexpressing yeast transformants were characterized by excessive fragmentation visualized by FM4–64 (Fig. 4A and B). A similar vacuolar fragmentation upon *YDC1* overexpression was visualized using Blue CMAC staining (data not shown). Moreover, *YDC1*-overexpressing yeast transformants were hypersensitive towards HCl (Fig. 4C and D) and NaOH (Fig. 4E and F), as compared to yeast control transformants, which is characteristic for yeast strains with dysfunctional vacuoles [25].

Interestingly, fragmentation of the mitochondria and the vacuole of *YDC1*-overexpressing yeast cells was already observed in the exponential phase (after 10–16 h of growth), while at this time point no differences in ROS accumulation and caspase activation was observed between *YDC1*-overexpressing yeast cultures and control cultures (data not shown). These data point to the fact that the mitochondrial and vacuolar fragmentation precedes the accelerated apoptosis in *YDC1*-overexpressing yeast cultures.

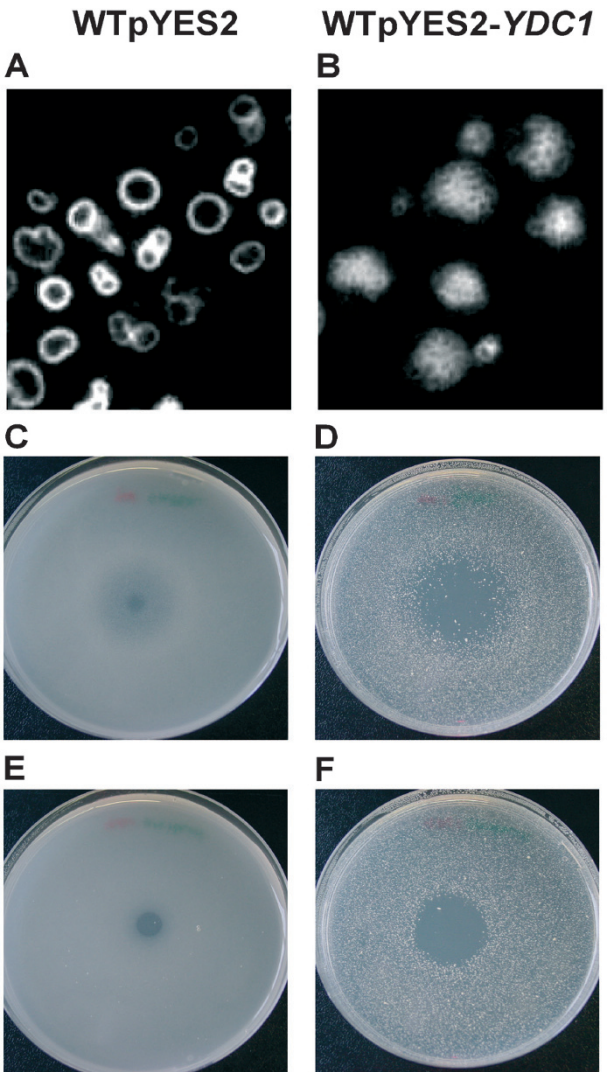

Figure 4. Vacuoles of yeast transformants overexpressing *YDC1* are fragmented and dysfunctional. (*A*, *B*) FM4–64 vacuolar membrane staining of wild-type strain BY4741 containing empty pYES2 (WTpYES2, left panel) and BY4741 overexpressing *YDC1* (WTpYES2-*YDC1*, right panel). (*C*–*F*) Sensitivity of wild-type strain BY4741 containing empty pYES2 (WTpYES2, left panel) and BY4741 overexpressing *YDC1* (WTpYES2-*YDC1*, right panel) to (*C*, *D*) 5 M HCl and (E, F) 5 M NaOH, was assessed *via* growth inhibitory halo assay in SC-ura (Gal) agar plates.

Exogenous addition of C_6-dihydroceramide reduces organelle dysfunction upon *YDC1* overexpression. To link the mitochondrial and vacuolar dysfunction upon overexpression of *YDC1* with ceramide metabolism, we assessed whether exogenous addition of ceramide could reduce the observed hypersensitivity to H_2O_2, HCl and NaOH, as well as increase the chronological lifespan of *YDC1*-overexpressing transformants. We first determined the MIC of H_2O_2 for *YDC1*-overexpressing yeast transformants in the absence and presence of C_2-, C_6-, C_8-dihydroceramide. Apparently, exogenous addition of 10 μg/mL C_6-dihydroceramide (added as lipids dissolved in DMSO) was most effective in reducing the H_2O_2 hypersensitivity of *YDC1*-overexpressing yeast transformants: MIC of H_2O_2 was 220 ± 60 μM, whereas MIC of H_2O_2 in the presence of 10 μg/mL C_6-dihydroceramide was 530 ± 130 μM (Table 2). Next, we assessed whether C_6-dihydroceramide could reduce the NaOH and HCl hypersensitivity of *YDC1*-overexpressing yeast transformants as well as increase their chronological lifespan. Indeed, MIC of NaOH and HCl were 3.6 ± 0.5 mM and 940 ± 150 μM respectively, whereas addition of C_6-dihydroceramide increased these MICs to 7.3 ± 1.0 mM and 1.88 ± 0.25mM, respectively (Table 2). Moreover, addition of 0.5 μg/mL C_6-dihydroceramide (added as lipids dissolved in DMSO) increased CL_{max} of *YDC1*-overexpressing yeast trans-

Table 2. Effect of exogenous addition of 10 μg/mL C_6-dihydroceramide dissolved in DMSO on mitochondrial and vacuolar dysfunction upon *YDC1* overexpression. Data represent mean ± SEM.

	MIC[a]		
	H_2O_2	NaOH	HCl
0 μg/mL C_6-dihydroceramide	220 ± 60 μM	3.6 ± 0.5 mM	940 ± 150 μM
10 μg/mL C_6-dihydroceramide	530 ± 130 μM	7.3 ± 1.0 mM	1.88 mM ± 0.25 mM

[a] MIC values are the minimal concentrations required to inhibit the growth of *YDC1*-overepressing transformants by 100%.

formants by 9 ± 2.5% as compared to *YDC1*-overexpressing yeast transformants treated with DMSO (Fig. 5). Hence, exogenous addition of C_6-dihydroceramide can reduce the mitochondrial and vacuolar dysfunction upon *YDC1* overexpression.

Discussion

To investigate the role of the yeast ceramidase Ydc1p in chronological lifespan, the alkaline ceramidase encoded by *YDC1* was overexpressed [4], which resulted in membranes with low ceramide levels ([4] and this study). We demonstrated that *YDC1* overexpression results in reduced chronological lifespan and an accompanying accelerated caspase-dependent apoptosis in yeast. Mitochondria are known to play an important role in determination of chronological lifespan and apoptosis in yeast. We found that *YDC1* overexpression resulted in increased mitochondrial and vacuolar fragmentation and, moreover, in mitochondrial and vacuolar dysfunction, as assessed by (i) reduced mitochondrial membrane potential and H_2O_2 hypersensitivity, and (ii) hypersensitivity to HCl and NaOH. Addition of exogenous C_6-dihydroceramide to a *YDC1*-overexpressing yeast culture increased the chronological lifespan and reduced mitochondrial and vacuolar dysfunction, pointing to the direct link between ceramide metabolism and organelle function.

Figure 5. Exogenous addition of C_6-dihydroceramide suppresses reduced chronological lifespan upon *YDC1* overexpression. Chronological lifespan of BY4741 overexpressing *YDC1* with (black squares) and without (open squares) exogenous addition of 0.5 μg/mL C_6-dihydroceramide dissolved in DMSO in the growth medium. Yeast cultures were grown in SC-ura (Gal) and viability of these cultures was analyzed by counting the number of CFU/mL. This figure is a representative of three experiments.

There is only one other report describing a link between increased ceramidase activity, *via* high ectopic expression of human ceramidase (haCER2) in human cells, and fragmentation of an organelle, namely Golgi complex [26]. Low ceramide levels in membranes can, apart from the above-described increased ceramidase activity, also be the result of reduced hydrolysis of complex sphingolipids or low ceramide synthase activity. In this respect, disruption of *ISC1* in yeast, resulting in reduced hydrolysis of inositol phosphosphingolipids and reduced ceramide content [27, 28], was shown to result in dysfunctional mitochondria [17, 28] and decreased chronological lifespan [17]. Whether organelle morphology of an *isc1*-mutant is affected has not been reported. Moreover, Faergeman and coworkers [24] demonstrated that yeast mutants affected in ceramide synthases, such as the *lag1-lac1*-mutant, were characterized by low ceramide content and fragmented vacuoles. Apparently, yeast mutants characterized by membranes with low ceramide content have previously been reported to be characterized by either increased organelle fragmentation or organelle dysfunction. In this study, we report on both mitochondrial and vacuolar fragmentation and dysfunction upon overexpression of the alkaline ceramidase Ydc1p in yeast, resulting in membranes with low ceramide content, and on the ability of exogenously added ceramide to restore the organelle dysfunction induced by *YDC1* overexpression. These data point to the direct link between ceramide content of membranes and organelle function.

There are several reports on the membrane-stabilizing role of ceramide [29, 30]. Apart from a possible direct effect of ceramide on membrane stability, it has been described that ceramide-enriched macrodomains in membranes are important recruiting platforms for proteins involved in fusion machinery of organelles [31, 32]. Hence, membranes with low ceramide

content could either become instable, or alternatively, be devoid of organizing platforms for the membrane fusion machinery, resulting in fragmented and dysfunctional organelles.

Moreover, in this study, we demonstrate a link between mitochondrial morphology, i.e., increased mitochondrial fragmentation, and dysfunction in yeast, and both reduced chronological lifespan and accelerated caspase-dependent apoptosis during chronological ageing. Some of these different aspects have previously been linked, corroborating our findings: (i) the correlation between decreased mitochondrial fragmentation, increased chronological lifespan and resistance to apoptosis. Apparently, deletion of *DNM1*, encoding a mitochondrial fission protein, in the filamentous ascomycete *Podospora anserina*, results in extreme elongation of mitochondria, and reduced mitochondrial fragmentation, and in increased chronological lifespan because of increased resistance to apoptosis [33]. In yeast, deletion of *DNM1* was shown to result in large networks of mitochondria and resistance to apoptotic stimuli [34]. (ii) Long-living yeast mutants were found to accumulate less ROS and had a delayed initiation of apoptosis compared with wild-type cells [16, 35, 36]. (iii) A link between dysfunctional mitochondria and reduced chronological lifespan was previously reported. Yeast cells with impaired mitochondrial functions, as a result of deletion of prohibitin, catalase, or SOD, or defective mitochondrial gene expression, phosphorylation or respiration, were characterized by reduced chronological lifespan and increased endogenous ROS levels [20, 37, 38]. Functional mitochondria can dispose of excess ROS mainly *via* the SODs (encoded by *SOD1* or *SOD2*). Reduction of ROS, *via* overexpression of SOD, was shown to extend the chronological lifespan of various of the above described yeast mutants [20].

Interestingly, we found that *YDC1*-overexpressing yeast cells displayed fragmented mitochondria and vacuoles already in the exponential phase (after 10–16 h of growth), while the increased ROS and caspase induction were not yet initiated at this time point. This observation indicates that mitochondrial fragmentation precedes ROS accumulation and caspase activation (and apoptosis) upon *YDC1* overexpression, and not *vice versa* as previously demonstrated, *e.g.*, for mating pheromone and amiodarone administration or for yeast *lsm4* mutants that have increased mRNA stability [39, 40, 41].

Apart from Ydc1p, the yeast *S. cerevisiae* harbors another ceramidase, yeast phytoceramidase Ypc1p [5]. It was suggested that the two enzymes have distinct physiological functions since cells lacking Ydc1p activity display increased sensitivity to heat stress and increased chronological lifespan, whereas cells lacking Ypc1p activity show no such phenotypes [4, 6]. In this study, we demonstrated that overexpression of *YDC1* leads to reduced chronological lifespan, oxidative stress hypersensitivity and increased apoptosis, which may all result from the fragmentation of the mitochondria upon *YDC1* overexpression. Whether Ypc1p activity has also a role in chronological lifespan, oxidative stress sensitivity, apoptosis and organelle membrane integrity still needs to be investigated. Interestingly, it was recently reported that *YPC1* is differentially regulated during replicative ageing (i.e., the number of divisions undertaken by an individual yeast cell) [42]. Moreover, overexpression of *YPC1* was previously demonstrated to be much more effective in rescuing replicative lifespan of a *lag1lac1*-double deletion mutant as compared to *YDC1* [43]. This may suggest that Ypc1p is involved in replicative ageing rather than in chronologically ageing.

In conclusion, this is the first report on the link between ceramide metabolism on one hand and organelle morphology and function on the other, with consequences for chronological lifespan and apoptosis in yeast.

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