Membrane-tethering of cytochrome c accelerates regulated cell death in yeast

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Abstract
Intrinsic apoptosis as a modality of regulated cell death is intimately linked to permeabilization of the outer mitochondrial membrane and subsequent release of the protein cytochrome c into the cytosol, where it can participate in caspase activation via apoptosome formation. Interestingly, cytochrome c release is an ancient feature of regulated cell death even in unicellular eukaryotes that do not contain an apoptosome. Therefore, it was speculated that cytochrome c release might have an additional, more fundamental role for cell death signalling, because its absence from mitochondria disrupts oxidative phosphorylation. Here, we permanently anchored cytochrome c with a transmembrane segment to the inner mitochondrial membrane of the yeast Saccharomyces cerevisiae, thereby inhibiting its release from mitochondria during regulated cell death. This cytochrome c retains respiratory growth and correct assembly of mitochondrial respiratory chain supercomplexes. However, membrane anchoring leads to a sensitisation to acetic acid-induced cell death and increased oxidative stress, a compensatory elevation of cellular oxygen-consumption in aged cells and a decreased chronological lifespan. We therefore conclude that loss of cytochrome c from mitochondria during regulated cell death and the subsequent disruption of oxidative phosphorylation is not required for efficient execution of cell death in yeast, and that mobility of cytochrome c within the mitochondrial intermembrane space confers a fitness advantage that overcomes a potential role in regulated cell death signalling in the absence of an apoptosome.

Introduction
Cytochrome c is an evolutionary highly conserved protein localized in the mitochondrial intermembrane space (IMS), which transfers electrons from cytochrome bc1 reductase (complex III) to cytochrome c oxidase (COX, complex IV), a reaction regarded as the rate-limiting step of mitochondrial respiration1. Cytochrome c is a water-soluble protein that can diffuse in three dimensions in the IMS, but also associates with the inner mitochondrial membrane (IMM)2,3. In the baker’s yeast Saccharomyces cerevisiae, cytochrome c is encoded by CYC1 and its paralog CYC7, with the first accounting for 95% of total cytochrome c content during aerobic growth, while the latter is expressed during hypoxia4. Beyond its crucial role in the mitochondrial respiratory chain, cytochrome c is a key player during intrinsic apoptosis, a form of regulated cell death associated with mitochondrial outer membrane permeabilization5. In higher eukaryotes, cytochrome c released from the IMS into the cytosol binds to apoptotic peptidase activating factor 1 (APAF1) and pro-caspase 9 to form the apoptosome, a supermolecular complex that initiates a caspase cascade, culminating in apoptotic cell death5,6.

Regulated cell death is not limited to multicellular organisms but also occurs in unicellular eukaryotes (including several yeast species) and even in some prokaryotes5.
In yeast and higher eukaryotes, programmed necrotic and apoptotic cell death subroutines have been described as regulated cell death modalities. While sharing key features and basic components of the molecular machinery executing regulated cell death in metazoan, yeast cells also display distinct differences. The yeast genome codes for several apoptosis-related proteins, including the metacaspase Yca1, the HtrA-like protease Nma111 and the mitochondrial pro-apoptotic proteins Apoptosis-inducing factor Aif1 and endonuclease G. Yeast apoptosis can be triggered by multiple stimuli, ranging from acetic acid, hydrogen peroxide, hypochlorous acid, UV radiation and pheromones to heterologous expression of human pro-apoptotic proteins. In addition, several physiological scenarios such as mating, antagonistic interaction between yeast species, colony formation as well as replicative and chronological ageing have been shown to trigger apoptotic death of uninfected cells within a yeast population. Although the release of cytochrome c can be detected in several of these scenarios, yeast cells do not contain an apoptosome, raising the intriguing question of why cytochrome c release occurs in this organism. Thus, the existence of cytochrome c release in yeast suggests that an alternative, potentially evolutionary ancient pathway for initiation of regulated cell death might exist. Indeed, when comparing the phylogenetically conserved role of cytochrome c in respiration and cell death between various species, it is particularly interesting that eukaryotic cells solely express soluble forms of cytochrome c, allowing for high mobility within the IMS. However, some bacterial species harbour membrane-bound cytochrome c variants, which mediate electron transport during respiratory growth.

The exclusive presence of soluble forms of cytochrome c in the mitochondrial IMS of eukaryotic cells suggests that this has evolved to allow this protein to additionally participate in apoptotic cell death. To test this hypothesis, we used baker’s yeast as a model, employing an evolutionary highly conserved, robust cell death pathway that is accompanied by the release of cytochrome c into the cytosol, but lacking an apoptosome. We engineered a yeast strain to contain exclusively membrane-anchored cytochrome c and analysed its impact on mitochondrial function, ageing and cell death. Membrane anchoring of cytochrome c retained proper respiratory growth and correct assembly of mitochondrial respiratory chain supercomplexes, but resulted in increased cellular respiration and elevated production of reactive oxygen species (ROS). Importantly, regulated cell death, including age-dependent as well as acetic acid-induced cell death, was accelerated in this strain, demonstrating that loss of cytochrome c from mitochondria does not contribute to the execution of cell death in evolutionary old regulated cell death regimes.

Material and methods
Construction of membrane-anchored cytochrome c
The CYC1 gene from Saccharomyces cerevisiae, with its own promoter and terminator sequences, was synthesized by GeneArt® Gene Synthesis (Thermo Fisher Scientific) and was provided in a pRS305 vector. The mitochondrial targeting segment and the transmembrane sequence from the yeast CYB2 (lactate dehydrogenase) gene were inserted upstream of the CYC1 coding sequence. Thereby, the transmembrane segment of Cyb2 served as membrane-anchoring part for cytochrome c. In addition, the linker sequence from the membrane-anchored cytochrome c-y of Rhodobacter sphaeroides, was inserted upstream of the CYC1 gene, which confers flexibility to the membrane-anchored cytochrome c in yeast.

This construct encoding the membrane-anchored form of cytochrome c (Cyc1MA), coding sequence of the whole construct is given in Table 1 was PCR-amplified (primer sequences: 5′- CTGTATGTATATAAATCTTTGGTTT TCTTC -3′ and 5′- AAAATAAATAGGG ACCTA GACCTAGGTTGTCTAATCC -3′), and transformed into a yeast strain (obtained from Euroscarf), lacking both membrane-anchoring part for cytochrome c and yeast CYC1 gene, under the control of the endogenous CYC1 promoter.

Media and culturing conditions
Cells were grown at 28 °C and 145 rpm in synthetic complete (SC) medium, containing 0.17% yeast nitrogen base (Difco, BD Biosciences), 0.5% (NH4)2SO4, 30 mg/l of all amino acids (except 80 mg/l histidine and 200 mg/l leucine), 30 mg/l adenine and 320 mg/l uracil with 2% D-glucose. Full media (YPEP) agar plates contained 1% yeast extract (Bacto, BD Biosciences), 2% peptone (Bacto, BD Biosciences), 4% D-glucose and 2% agar. All media were prepared with double-distilled water and were autoclaved for 25 min at 121 °C, 210 kPa. Amino acids were prepared as 10x stocks, separately sterilized and added to the media after autoclaving.

Overnight cultures were grown for 16–20 h in SC medium using glass eprouvettes and were applied for inoculation of 10 ml SC medium in baffled 100 ml Erlenmeyer flasks to an OD600 of 0.3. After 6 h of incubation, cells were used for experiments. Treatments with antimycin A were performed in Erlenmeyer flasks. Therefore, antimycin A was dissolved in ethanol and added to cells after 24 h with a final concentration of 50 µM. Equivalent amounts of ethanol were added to control cells. For acetic acid treatment, cells were transferred into 96-well deep-well plates (500 µl of culture per well) and acetic acid was added to a final concentration of...
120 or 160 mM. Strains were incubated for 1 h at 28 °C, 1000 rpm and subsequently applied for further analysis.

**Analysis of cellular growth**

Growth was analyzed with a Bioscreen C™ automated microbiology growth curve analysis system (Growth Curves USA). Cells were inoculated to an OD$_{600}$ of 0.1 in SC media with indicated carbon sources in the suppliers “honeycomb microplate” in a final volume of 250 µl media per well and OD$_{600}$ was measured automatically every 30 min at 28 °C and shaking on maximum level. Respective media without cells was used as blank. The doubling time was calculated from growth curves during logarithmic growth phase.

**Analysis of cell death**

Loss of membrane integrity as a marker of necrotic cell death was determined via propidium iodide (PI) staining as described previously. In brief, ~2 × 10$^6$ cells were collected by centrifugation in 96-well plates and resuspended in 250 µl phosphate buffered saline (PBS, 25 mM potassium phosphate; 0.9% NaCl; adjusted to pH 7.2) containing 100 µg/l PI. After incubation for 10 min at room temperature (RT) in the dark, the cells were washed once in PBS and 30,000 cells per sample were analysed via flow cytometry (BD LSR Fortessa; BD FACSDivia software).

For discrimination between necrotic and early/late apoptotic cell death phenotypes, AnnexinV/PI co-staining was performed according to refs. Therefore, ~2 × 10$^6$ cells were harvested, washed once in digestion buffer (35 mM K$_3$PO$_4$, 0.5 mM MgCl$_2$, 1.2 M sorbitol; adjusted to pH 6.8) and resuspended in 30 µl staining buffer (10 mM HEPES, 140 mM NaCl, 5 mM CaCl$_2$, 0.6 M sorbitol; adjusted to pH 7.4) containing 100 µg/l PI and 2 µl Annexin-V-FLUOS reagent (Roche). After 20 min incubation at RT in the dark, 100 µl staining buffer was added per sample and transferred into 96-well plates for subsequent analysis via flow cytometry.
were harvested in 96-well plates, resuspended in 250 µl PBS containing 2.5 mg/l DHE and incubated for 10 min at RT in the dark. Afterwards, cells were washed and analysed as described for PI staining above.

To investigate mitochondrial transmembrane potential ($\Delta \psi_m$), the protocol described in ref. 33 was slightly adapted. ~2 × 10^6 cells were resuspended in 250 µl PBS containing 5% glucose and 200 nM Mitotracker CMXRos. Cells were incubated, washed and analysed as described for DHE staining above.

Microscopy
Specimen were prepared on agar slides to immobilize yeast cells and investigated with a Leica SP5 confocal laser scanning microscope, equipped with a Leica HCX PL Apo 63× NA 1.4 oil immersion objective. Z-stacks were acquired using 64 × 64 × 12.6 (x/y/z) nm sampling and analysed as well as processed with the open-source software Fiji34. To that end, three-dimensional Gaussian filtering ($\sigma$ = yσ = zσ = 1), followed by background subtraction (rolling ball radius ~ 50 pixels) was applied and pictures were illustrated using the maximum-intensity projection method. Volume rendering to visualize mitochondrial morphology (“Projection” in Fig. 2b, d and f) was applied with the build-in Fiji Macro “Volume Viewer” by Kai Uwe Barthel (Mode: Volume; Interpolation: Trilinear; Sampling: 1.0). For micrographs presented in Fig. 3g, samples were prepared on agar slides and analysed with a Leica DM6B epifluorescence microscope, using a HC PL Apo 100× NA 1.4 oil immersion objective. The dynamic range of presented figures was adapted by using the “Brightness/contrast” tool of Fiji. All pictures within an experiment were captured and processed with the same settings.

Measurement of cellular oxygen consumption
Oxygen consumption of yeast was quantified with a Fire-Sting optical oxygen sensor system (Pyro Science) as described previously.35 In brief, 2 ml of culture were transferred into glass tubes, hermetically sealed and directly used for analysis. Oxygen concentration was measured for 1 min and the slope of the regression line was calculated and normalized to the number of PI negative (and thus viable) cells in the glass tube (evaluated by CASY cell counting and quantification of PI negative cells with flow cytometry as described above). Oxygen consumption per living cells is expressed as fold value normalized to wild type cells.

Isolation of mitochondria
Isolation of mitochondria from yeast cells was performed as described in ref. 35. In brief, cells grown to mid-logarithmic phase were harvested by centrifugation and resuspended in 2 ml/g cell wet weight MP1 buffer (0.1 M Tris-H2SO4, 10 mM dithiothreitol; adjusted to pH 9.4). After incubation for 10 min at 30 °C, samples were washed in 1.2 M sorbitol and resuspended in MP2 buffer (20 mM potassium phosphate, 0.6 M sorbitol; adjusted to pH 7.4), containing 3 mg/g of cell wet weight zymolyase 20 T. Spheroplasts were created by incubation for 1 h at 30 °C and harvested by centrifugation. Samples were carefully resuspended in 13.4 ml/g of cell wet weight in homogenization buffer (10 mM Tris, 0.6 M sorbitol, 1 mM EDTA, 1 mM PMSF; adjusted pH 7.4) and homogenized by 10 strokes with a Teflon plunger (Sartorius Stedim Biotech S.A.). Homogenates were centrifuged at 3000 g for 5 min at 4 °C and the resulting supernatants were subsequently centrifuged at 17,000 g for 12 min at 4 °C. Pelleted mitochondria were resuspended in isotonic buffer (20 mM HEPES, 0.6 M sorbitol; adjusted to pH 7.4) to a concentration of 10 mg/ml.

Immunoblot analysis
To obtain whole-cell extracts, cells were harvested by centrifugation at 18,400 g for 2 min and resuspended in 50 µL of Laemmli buffer (63 mM Tris, 2% SDS, 10% glycerol, 0.1% β-mercaptoethanol and 0.005% bromphenol blue; adjusted to pH 6.8). Samples were boiled for 3 min at 95 °C and 10 µl were applied for SDS-PAGE and immunoblotting following standard protocols.

For submitochondrial fractionation experiments, 100 µg of mitochondrial protein were treated with 0.2 M NaCl and five freeze-and-thaw cycles in liquid nitrogen were performed. After centrifugation at 100,000×g for 30 min at 4 °C, supernatants and pellet fractions were treated with trichloroacetic acid (12% final concentration) to precipitate proteins. Samples were incubated for 20 min at −20 °C and afterwards centrifuged at 28,000×g for 30 min at 4 °C. Pellet fractions were washed with acetone, followed by additional centrifugation at 28,000 rcf for 15 min at 4 °C. Of note, samples for total protein were left untreated. Finally, samples were boiled at 95 °C for 3 min, resuspended in Laemmli buffer and 10 µl of the samples were applied for SDS-PAGE and immunoblotting following standard protocols. Blots were probed with antibodies against cytochrome c (holo form), Tom70, Mdh1 and Aco1 as loading control, which were kindly provided by Nora Vögtle, University of Freiburg. Peroxidase-conjugated secondary anti-rabbit antibodies (BioRad, 1705046 and Sigma, A0545) were used for chemiluminescence detection.

Blue native electrophoresis
Isolated mitochondria were centrifuged at 16,000 g for 10 min at 4 °C and the pellet was subsequently resuspended in lysis buffer (50 mM Bis-Tris, 25 mM KCl, 2 mM Aminohexanoic acid, 12% glycerol, 1 mM PMSF, 2% digitonin and Complete Protease Inhibitor cocktail (Roche). 100 µg of mitochondrial protein was loaded on a
3–12% precast native gel (Invitrogen), which was subsequently stained with Coomassie.

**UV-VIS spectroscopy**

Optical spectra (350–700 nm) were recorded using Cary4000 UV-Vis spectrophotometer (Agilent Technologies). The concentration of a-type hemes was determined from the sodium dithionite-reduced minus potassium ferricyanide-oxidized difference spectra using the absorption coefficient $\varepsilon$ (630–605 nm) = 23.2 mM$^{-1}$ 36. Concentrations of $b$- and $c$-type hemes were measured simultaneously from difference spectra as described in ref. 37, using the following formula:

$$[\text{heme } b] \text{ (mM)} = (\Delta(A_{562} - A_{577}) \times 3.539 \times 10^{-2} - \Delta(A_{553} - A_{546})) \times 1.713 \times 10^{-3}$$

$$[\text{heme } c] \text{ (mM)} = (\Delta(A_{562} - A_{577}) \times 5.365 \times 10^{-2} - \Delta(A_{562} - A_{577})) \times 9.564 \times 10^{-3}$$

**Measurement of oxygen reduction rate in isolated mitochondria**

Isolated mitochondria were resuspended in HEPES buffer (20 mM HEPES, 250 mM sucrose, 50 mM KCl, 0.1 mM EDTA; adjusted to pH 7.4). Cytochrome $c$ oxidase activity (the oxygen reduction rate) was monitored using a Clark-type oxygen electrode (Hansatech) with 1 ml activity (the oxygen reduction rate) was monitored using a Clark-type oxygen electrode (Hansatech) with 1 ml chamber volume. 10 µM antimycin A (cytochrome $bc_1$ inhibitor) and 0.5 µM FCCP (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, uncoupling agent) were added to the reaction chamber. Sodium ascorbate (5 mM) was used as electron donor, 0.5 mM TMPD (N,N,N',N'-Tetramethyl-p-Phenylenediamine) as electron mediator. Addition of mitochondria (at a final concentration in the range of 2–5 nM cytochrome $c$ oxidase) started the reaction. Coupled cytochrome $bc_1$ / cytochrome $c$ oxidase activity was measured upon addition of 0.12 mM decylubiquinone (DBH$_2$) as electron donor. Baseline oxygen-consumption (auto-oxidation of DBH$_2$) was recorded before addition of the mitochondria. Oxygen consumption was blocked by addition of KCN, verifying that the observed oxygen reduction was due to the activity of cytochrome $c$ oxidase. Mitochondria devoid of the mitochondrial outer membrane (OMM) were prepared as described above. Where needed, 5 µM yeast cytochrome $c$ (Sigma, C2436) was added to the mixture before addition of DBH$_2$. Decylubiquinone (Sigma, D7911) was reduced to DBH$_2$ by addition of a small crystal of potassium borohydride to 50 µl of 60 mM decylubiquinone in DMSO. 5 µl aliquots of 0.1 M HCl were added with gentle mixing until the yellow solution became colorless. DBH$_2$ was transferred to a fresh tube, avoiding borohydride crystals. Final DBH$_2$ concentration was determined from the $A_{277}$ nm using $\varepsilon_{277} = 16$ mM$^{-1}$.

Coupled activity values were normalized to the concentration of cytochrome $c$ oxidase ($a$-type heme).

**Statistics and data representation**

Results are presented as line graphs or bar charts, indicating mean ± standard error of the mean (s.e.m.), or dot plots with mean (square) ± s.e.m. and median (centre line), as well as single data points. Exact sample sizes are given in the figure legends and represent biological replicates, except for Fig. 4e, where technical replicates were used. The sample size was thereby chosen according to empirical values that are standard in the field. Visualized data were taken from representative experiments that were replicated at least two times. Randomization was not performed in our study and investigators were not blinded. Outliers were identified using the 2.2-fold interquartile range labelling rule (outlier data points are highlighted in turquoise) and data was transformed upon the presence of outliers (detailed description of the method used in Supplementary Table 1; no data was excluded from the analysis). Normality of data was evaluated with a Shapiro–Wilk’s test and homogeneity of variances was examined with a Levene’s test (both analysed with Origin Pro 2018). A detailed description of the procedure upon violation of respective assumptions is given Supplementary Table 1. In brief, means of two groups were compared with a two-sample $t$-Test (with Welch correction upon the presence of significantly different variances). The means of three or more groups were compared upon the presence of one independent variable (genotype) with a One-way Analysis of Variance (ANOVA) followed by a Bonferroni post hoc test (calculated with Origin Pro 2018) or a Welch’s ANOVA with a Games-Howell post hoc test in case of significantly heterogenous variances (analysed with IBM SPSS Statistics, Version 25). To compare the means of groups upon the presence of two independent variables (genotype and treatment), a two-way ANOVA followed by a Bonferroni post hoc test was conducted with Origin Pro 2018. Analysis of cell death over time was statistically evaluated with a two-way ANOVA mixed design (strain as between-subject and time as within-subject factor) with a Bonferroni post hoc test using Origin Pro 2018. Significances for analyses with one independent variable are indicated with asterisks ($***P < 0.001$, **$P < 0.01$, *$P < 0.05$, n.s. $P > 0.05$), and for two independent variables main effects are displayed with diamonds ($###P < 0.001$, ###$P < 0.01$, ##$P < 0.05$, n.s. $P > 0.05$), simple main effects are depicted as asterisks ($**P < 0.001$, **$P < 0.01$, *$P < 0.05$, n.s. $P > 0.05$). Calculated $p$-values are presented in Supplementary Table 1. All figures were created with Origin Pro 2018 and further processed with Adobe Illustrator CS6.

**Results**

**Generation and characterization of a yeast model with membrane-tethered cytochrome $c$**

To test the significance of cytochrome $c$ loss from mitochondria for rudimentary cell death regimes, we
To genetically engineer baker’s yeast to exclusively express a membrane-anchored form of the wild type cytochrome c protein (Cyc1), which we termed Cyc1\textsuperscript{MA}. In brief, we deleted the two cytochrome c genes CYC1 and CYC7 and chromosomally integrated CYC1\textsuperscript{MA} into the original locus of CYC1. Thereby, CYC1\textsuperscript{MA} consists of the mitochondrial localization sequence and transmembrane domain of cytochrome b2 (CYB2) from S. cerevisiae at the N-terminus, connected to the linker region of membrane-anchored cytochrome c-γ from R. sphaeroideus followed by the coding sequence of wild type CYC1 (Fig. 1A; please see material and method section, as well as Table 1 for official journal of the Cell Death Differentiation Association
Fig. 1 Generation of a yeast model with membrane-anchored cytochrome c. a Scheme of respiratory supercomplexes in yeast, with wild type cytochrome c (left panel) and genetically engineered membrane-anchored cytochrome c (Cyc1MA, right panel). For construction of Cyc1MA, the mitochondrial localization sequence (MLS) and the transmembrane domain (TMD) of cytochrome b2 (Cyb2) from Saccharomyces cerevisiae was chromosomally integrated at the N-terminus of the CYC1 gene together with the linker sequence of Rhodobacter sphaeroides cytochrome c5. b Immunoblot analysis of submitochondrial fractionation with lysates from isolated mitochondria of wild type (WT) and Cyc1MA cells. Total lysates (T) were separated via ultracentrifugation and the supernatant (SN) and pellet (P) fractions were applied for immunoblotting. Blots were probed with antibodies against aconitase (Aco1), Cyc1 and Tom70. The asterisk indicates a cross reaction of the Cyc1 antibody. c Immunoblot analysis of isolated mitochondria from WT and Cyc1MA strains. Blots were probed with antibodies against Cyc1 and the outer mitochondrial membrane protein Tom70. d–f Reduced-minus-oxidized difference spectra of WT and Cyc1MA strains. Spectra were normalized to the maxima of the a band at 605 nm. d Direct comparison of spectra from WT and Cyc1MA strains. e Spectra from WT mitochondria before (black) and after (grey) removal of the outer mitochondrial membrane (OMM), as well as from respective wash fraction (red). f Spectra from Cyc1MA mitochondria before (black) and after (grey) removal of the outer mitochondrial membrane (OMM), as well as from respective wash fraction (red). g Measurement of decylubiquinol (DBH2)-driven coupled bc1 complex and cytochrome c oxidase activity in isolated mitochondria devoid of the outer mitochondrial membrane (OMM) of WT as well as CYC7 deletion strains harbouring a membrane-anchored form of Cyc1 (Cyc1MA). Where indicated, yeast cytochrome c (cyt c) was added. The oxygen reduction rate was calculated as electrons per second (e–/s) per cytochrome c oxidase. Mean (square) ± s.e.m., median (centre line) and single data points (n = 3) are depicted. *P < 0.05; **P < 0.01.

Loss of cytochrome c mobility causes cell death during exponential growth

Next, we evaluated how anchoring of Cyc1 to the IMM and thus lack of its release from mitochondria affects the ability of yeast cells to undergo cell death. Determination of clonogenic survival demonstrated that Cyc1MA expressing cells displayed slightly decreased viability during exponential growth compared to wild type or CYC7 deleted cells (Fig. 3a). Additional flow cytometric quantification of PI staining, indicating loss of plasma membrane integrity and thus necrotic cell death, showed that membrane-anchoring of cytochrome c caused a small increase in cell death (Fig. 3b). However, clonogenic death was generally higher compared to the loss of membrane integrity, suggesting a significant proportion of non-necrotic cell death in all analysed strains. Thus, we evaluated oxidative stress, a common pre-requisite of regulated cell death, by monitoring the ROS-driven conversion of non-fluorescent DHE to fluorescent ethidium (Eth). This revealed that the Cyc1MA mutant accumulated increased levels of ROS (Fig. 3c, d), which apparently originate from mitochondria as indicated by confocal microscopy (Fig. 3e). In turn, mitochondrial transmembrane potential (ΔΨm) was reduced in the Cyc1MA strain compared to control cells (Fig. 3f–h), an event associated with late stages of regulated cell death in yeast20. Together, these results suggest that the reduction of cytochrome c mobility leads to slightly increased cell death.
death in exponentially growing cells, revealing phenotypes of mitochondria-dependent regulated cell death.

**Mobility of mitochondrial c is important to maintain yeast lifespan and resistance against regulated cell death stimuli**

Due to these surprising effects of Cyc1MA on cell death and oxidative stress during normal growth, we next investigated the response to stimuli triggering regulated cell death. Treatment with acetic acid is a regime frequently applied to induce this cell death subroutine in yeast\textsuperscript{11–13} and results in a dose-dependent reduction of viability. Anchoring of Cyc1 to the IMM caused a clear increase in clonogenic cell death (Fig. 4a). To test for phosphatidylserine externalisation, an early apoptotic event, we performed AnnexinV/PI co-staining, which allows discrimination between early apoptotic (AnnexinV pos.), late apoptotic/secondary necrotic (AnnexinV/PI pos.) and primary necrotic (PI pos.) cell death phenotypes\textsuperscript{7,29}. The Cyc1MA mutant showed significantly increased rates of late apoptotic/secondary necrotic cell death phenotypes (Fig. 4b). High levels of ROS upon acetic acid treatment were detectable in the Cyc1MA mutant (Fig. 4c, d), likely explaining higher sensitivity of this strain against regulated cell death stimuli. Dead cells randomly accumulating the dye due to a loss of membrane integrity have been excluded from the analysis (Fig. 4d). To confirm the loss of cytochrome c from wild type mitochondria upon acetic acid treatment, as well as the absence of this process in the Cyc1MA mutant, cytochrome c protein levels were evaluated in isolated mitochondria of each strain, comparing cells that received acetic acid treatment 1 h prior to mitochondrial preparation and respective untreated controls. While the mitochondrial levels of wild type Cyc1 were prominently reduced in treated cells, levels of Cyc1MA were not changed (Fig. 4e, f), owing to its membrane anchoring (Fig. 1b, f). Furthermore, determination of chronological lifespan revealed premature death of the Cyc1MA during ageing (Fig. 4g). This was at least in part due to an induction of apoptosis, as we observed a prominent increase in phosphatidylserine externalisation in the Cyc1MA mutant after 48 h (Fig. 4h, i). Together, these results demonstrate that membrane-anchoring of cytochrome c increases sensitivity to cell death stimuli, elevates age-dependent regulated cell death and hence reduces the chronological lifespan of yeast cells. This suggests that the loss of cytochrome c from mitochondria is not required for cell death signalling in cells lacking an apoptosome. In contrast, anchoring cytochrome c as an integral inner membrane protein decreases cellular stress resistance.

**Alterations of cytochrome c mobility dysregulates mitochondrial function**

Prior to entering stationary phase, yeast cells undergo physiological changes during the diauxic shift, in which the metabolism changes from fermentation to respiration, resulting in increased mitochondrial abundance\textsuperscript{39}. To test whether changes in mitochondrial functionality precede the premature death of Cyc1MA cells during chronological ageing, we analysed the impact of Cyc1 anchoring on
Fig. 3 (See legend on next page.)
mitochondrial transmembrane potential, morphology and oxidative stress in stationary phase. Compared to exponentially growing cells (Fig. 3c–e), oxidative stress further increased after 24 h in the Cyc1MA strain (Fig. 5a). Microscopic analysis revealed mitochondria as the main source of ROS and, in addition, unveiled a diminished branching of the mitochondrial network compared to wild type cells (Fig. 5b). Even though ΔΨm was stabilized in stationary phase after 24 h (Fig. 5c, d) and 48 h (Supplementary Fig. 1), alterations in mitochondrial morphology could be confirmed with this staining approach, indicating lower branching and partially circularization of mitochondria (Fig. 5d). While ROS production further increased after 48 h in the Cyc1MA strain compared to control cells (Fig. 5e, f), the mitochondrial morphology appeared quite heterogenous in the Cyc1MA strain, with circular mitochondria showing the highest ROS levels and mitochondria similar to that in wild type cells, however, still displaying accumulation of ROS (Fig. 5f). In sum, our data demonstrates increased oxidative stress in chronologically aged Cyc1MA cells, most likely being causative for the observed increase of regulated cell death and resulting decreased chronological lifespan.

Enhanced cellular respiration is a compensatory mechanism to counteract toxicity caused by reduced respiratory chain activity

Finally, we investigated if alterations in the mitochondrial respiratory chain might be causative for the observed increase in oxidative stress. Since we detected normal assembly of respiratory chain supercomplexes in the Cyc1MA strain (Fig. 2a), we analysed enzymatic activity of their components. COX activity was measured by supplying ascorbate (Asc) and N,N,N′,N′-tetramethyl-p-phenylenediamine hydrochloride (TMPD), as well as carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) as uncoupling agent to isolated mitochondria, and was significantly higher in wild type cells compared to the Cyc1MA strain (Fig. 6a). Also coupled bc1 complex / COX activity (assessed with decylubiquinol; DBH2) was reduced in the Cyc1MA strain (Fig. 6a), presumably both due to the somewhat reduced levels of hemylated cytochrome c in the membrane-anchored variant. Since ΔΨm was only reduced in exponential but not in stationary Cyc1MA cells (Fig. 3f–h and 5c, d and Supplementary Fig. 1), we hypothesized that yeast cells compensate this reduced respiratory chain activity during ageing. Hence, we analysed cellular oxygen consumption in chronologically aged yeast cell cultures and observed a massive upregulation of respiration in the Cyc1MA strain compared to control cells after 48 h (Fig. 6b). To evaluate if this respiratory upregulation was a cytoprotective mechanism to compensate decreased respiratory chain activity, we inhibited this response by administration of antimycin A to stationary cultures after 24 h40, and again measured cellular oxygen consumption after 48 h, confirming inhibition of respiration by this treatment (Fig. 6c).

While the antimycin A-induced inhibition of respiration reduced the lifespan of all strains tested (Fig. 6d), this effect was most prominent in Cyc1MA cells. Disrupting respiration drastically increased the premature death of cells harbouring membrane-tethered cytochrome c, clearly indicating that the viability of these cells depends on the observed upregulation of respiratory activity (Fig. 6d). In addition, we observed increased accumulation of ROS upon antimycin A treatment. However, no difference in oxidative stress between Cyc1MA cells and respective controls were detectable upon antimycin A treatment, while we again observed increased ROS levels in the untreated Cyc1MA mutant compared to wild type cells (Fig. 6e). Thus, antimycin A induced oxidative stress in all strains, but selectively triggered cell death of Cyc1MA cells during early days of ageing (Fig. 6d, e). Hence, antimycin A does not reduce the lifespan of Cyc1MA cells via an additional burst of oxidative stress, but rather by inhibiting respiration in these cells.

In aggregate, our results demonstrate that diminished cytochrome c mobility results in reduced respiratory chain activity, leading to an upregulation of cellular respiration that sustains viability.
Fig. 4 (See legend on next page.)
Discussion

Beyond its biological function as an electron carrier in respiratory chains, cytochrome c is a key regulator of apoptotic cell death. Its release into the cytosol and the subsequent formation of an apoptosome triggers the activation of a caspase cascade\(^6\). Release of cytochrome c from mitochondria also occurs during regulated cell death in yeast\(^{12,41-43}\), an organism lacking an orthologous apoptosome. This evolutionary conserved cytochrome c release therefore could reflect a rudimentary form of signalling that could drive regulated cell death signalling in largely diverse eukaryotes. Possible scenarios are that either the depletion of cytochrome c from mitochondria, which subsequently impairs oxidative phosphorylation, or its presence in the cytosol modulates cell death. Likewise, complete lack of active cytochrome c, either via deletion of the genes coding for the two cytochrome c isoforms or via deletion of CYC3, encoding the cytochrome c heme lyase, has been shown to confer tolerance to distinct regulated cell death stimuli, including acetic acid\(^{15}\), manganese\(^{44}\) or human lactoferrin\(^{45}\) in yeast. Here, we report that anchoring cytochrome c as an integral membrane protein to the IMM and thus preventing its loss from mitochondria does not inhibit regulated cell death. These data therefore conclusively demonstrate that reduction of cytochrome c in mitochondria is not a causal event of regulated cell death signalling in cases where an apoptosome is missing. On the contrary, cells harbouring membrane-anchored cytochrome c were sensitised to regulated cell death stimuli with phenotypes of mitochondria-related cell death\(^{20}\) showing altered mitochondrial morphology, increased respiration, transient impairment of the $\Delta \Psi_m$ and increased ROS production. These cellular changes ultimately result in early cell death with apoptotic phenotypes and a reduced chronological lifespan.

How could permanent membrane tethering of cytochrome c decrease cellular fitness? Despite a mild reduction in coupled $bc_1$ complex/COX activity in the Cyc1\(^{MA}\) mutant, we observed increased cellular respiration of this strain, indicating a compensatory mechanism. This increase potentially elevates ROS levels, which we detected in the Cyc1\(^{MA}\) variant. Nevertheless, an inhibition of this response via antimycin A treatment drastically decreased the lifespan of Cyc1\(^{MA}\) strains compared to wild type cells. This was not simply due to a further accumulation of ROS, as we did not observe any additional accumulation of oxidative stress in Cyc1\(^{MA}\) cells treated with antimycin A compared to wild type cells receiving the same treatment. Instead, our data demonstrate that this upregulation of respiration in the Cyc1\(^{MA}\) strain represents a compensatory mechanism that sustains viability during ageing.

The molecular mechanism resulting in ROS production in Cyc1\(^{MA}\) strains remains to be further investigated. However, it was suggested in both mammalian\(^{46}\) and yeast cells\(^{47}\) that the release of cytochrome c into the cytosol might participate in a defence mechanism against oxidative stress by acting as a ROS scavenger. Hence, inhibition of this release from mitochondria might increase oxidative stress, resulting in cytochrome c-release-independent cell death, a form of regulated cell death described for yeast cells before\(^{48}\). Interestingly, during initial stages of apoptosis, cytochrome c has been shown to interact with and to selectively peroxidize cardiolipin in the IMM\(^{49,50}\). This peroxidation weakens the binding of cytochrome c to cardiolipin and facilitates its detachment from the IMM and subsequent release into the cytosol\(^{50}\). The peroxidase activity of cytochrome c is highly dependent on distinct surface charges facilitating interaction and structural arrangements upon binding to cardiolipin\(^{51,52}\). Thus, it is likely that anchoring cytochrome c to the IMM favours its peroxidase activity, leading to a progressive
increase of peroxidized cardiolipin that likely sensitizes cells towards cell death induction. A high degree of cardiolipin peroxidation not only facilitates the release of mitochondrial apoptogenic factors besides cytochrome c\(^50\), but has also been shown to reduce cytochrome c oxidase activity\(^53\). In this line, we find a modest decrease
in coupled \(bc_1\) complex/COX activity, which might be explained by potential alterations of cytochrome \(c\) peroxidase activity upon its membrane anchoring, subsequently affecting COX enzymatic activity.

The W65S mutation in yeast cytochrome \(c\) was demonstrated to abolish both its release into the cytosol and regulated cell death\(^54\), which might contradict our study. However, the authors of this study state that this mutation impairs electron transfer to COX and prevents respiratory growth. The \(\text{Cyc1}^{\text{MA}}\) strain showed a slight decrease in coupled \(bc_1\) complex/COX activity, which might be caused by alterations of cytochrome \(c\) peroxidase activity as described above or by the mild reduction of hemylated cytochrome \(c\) in these cells. Importantly, this effect was very mild compared to the W65S mutations, since no alteration of growth was observed in \(\text{Cyc1}^{\text{MA}}\).
strains, neither on fermentable nor on non-fermentable carbon sources. These differences are further emphasized by the fact that the W65S mutation caused decreased oxidative stress\(^{24}\), while the membraneANCHORing of cytochrome c elevated ROS levels.

While mitochondrial cytochrome c is a soluble mobile electron carrier, it occurs as both a soluble (Cyt c-2) or a membrane bound (Cyt c-γ) form of cytochrome c\(^{26}\) in alpha-proteobacteria, the evolutionary ancestor of mitochondria. Certain alpha-proteobacteria can carry out both aerobic and anaerobic respiration as well as photosynthesis. For example, Rhodobacter sphaeroides employs the soluble Cyt c-2 in photosynthesis, while the membrane bound form operates in respiratory electron transfer\(^{26}\). The adverse effects of membrane-tethered cytochrome c as revealed in this study suggests that soluble cytochrome c variants have been favoured during evolution, so that membrane tethered cytochrome c variants no longer operate as mobile electron carriers in mitochondria.

In sum, we show that preventing the loss of cytochrome c from mitochondria by tethering it to the IMM increases sensitivity towards acetic acid and exacerbates regulated cell death during ageing, indicating that the release of cytochrome c is not required for the induction of regulated cell death in yeast. Hence, a scenario where cytochrome c loss from mitochondria and the subsequent inhibition of oxidative phosphorylation is essential for regulated cell death appears to be unlikely. It is tempting to speculate that control of regulated cell death via cytochrome c mobility in the mitochondrial IMS and potentially cardiolipin peroxidation as an early event in regulated cell death is a phylogenetically older mode of this cell death routine, which was subsequently complemented by apoptosome-dependent regimes in higher eukaryotes to execute apoptosis. The strategy employed here to tether cytochrome c to the IMM could be used to investigate the consequences of absence of cytochrome c release in mammalian apoptosis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary Information

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