

Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia

Libuše Váchová¹ and Zdena Palková²

¹Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague-4, Czech Republic

²Department of Genetics and Microbiology, Charles University in Prague, 116 36 Prague-1, Czech Republic

The existence of programmed cell death (PCD) in yeast and its significance to simple unicellular organisms is still questioned. However, such doubts usually do not reflect the fact that microorganisms in nature exist predominantly within structured, multicellular communities capable of differentiation, in which a profit of individual cells is subordinated to a profit of populations. In this study, we show that some PCD features naturally appear during the development of multicellular *Saccharomyces cerevisiae* colonies. An ammonia signal emitted by aging colonies triggers metabolic changes that localize

yeast death only in the colony center. The remaining population can exploit the released nutrients and survives. In colonies defective in Sok2p transcription factor that are unable to produce ammonia (Váchová, L., F. Devaux, H. Kucerova, M. Ricicova, C. Jacq, and Z. Palková. 2004. *J. Biol. Chem.* 279:37973–37981), death is spread throughout the whole population, thus decreasing the lifetime of the colony. The absence of Mca1p metacaspase or Aif1p orthologue of mammalian apoptosis-inducing factor does not prevent regulated death in yeast colonies.

Introduction

Programmed cell death (PCD) in metazoa is essential for the development of differentiated tissues as well as for the harmless removal of aged or impaired cells. Several ways of dying, including apoptosis, apoptosis-like PCD, and necrosis-like PCD, as well as various signaling pathways triggering the PCD were described previously (Leist and Jaattela, 2001). These programs partially share some morphological characteristics and regulations, whereas some other features are specific for a particular mode of death. Usually, each of these processes results in safe cell removal and degradation of macromolecules to basic compounds, avoiding the release of toxic components. There are indications that at least some of the biochemical changes that are typical for mammalian PCDs exist in yeast. Markers of phosphatidylserine (PS) relocation, DNA breaks, and chromatin fragmentation were described in yeast that were exposed to different extracellular toxic compounds (e.g., acetic acid or H₂O₂; Madeo et al., 1999; Ludovico et al., 2001) or were producing some proapoptotic proteins of higher

metazoa (Shimizu et al., 2000). Such changes also appear both in replicatively (Laun et al., 2001) and chronologically (Herker et al., 2004) aged yeast cells growing in liquid cultures. Both Mca1p, which belongs to metacaspases (Madeo et al., 2002), and Aif1p, an orthologue of mammalian apoptosis-inducing factor (Wissing et al., 2004), were described as playing roles in yeast death. Despite these findings, two principle doubts must be dispelled before accepting mammalian-like apoptosis in yeast. The first one is linked to findings showing that even in metazoa, some of the “apoptotic” death features can also be found in other PCDs. It is also linked to the fact that the use of some reagents that were developed for studies of mammalian apoptosis could be problematic in yeast (e.g., detection of caspase by caspase inhibitors; Wysocki and Kron, 2004). The second doubt concerns the fact that, considering standard liquid yeast cultures as a population of unicellular individuals (Fannjiang et al., 2004), a reason for, and the importance of, a programmed mode of death is not clear.

In this study, we present evidence that regulated yeast cell death (YCD) exhibiting some PCD features plays an important role in the long-term development and survival of yeast multicellular colonies. In contrast to more or less homogeneous yeast cultures in liquids, populations within colonies growing on solid media have more possibilities to differentiate and to form specialized cell variants in favor of the whole population. Thus, colonies can be considered multicellular microorganisms

Libuše Váchová and Zdena Palková contributed equally to this work.

Correspondence to Zdena Palková: zdenap@natur.cuni.cz; or to Libuše Váchová: vachova@biomed.cas.cz

Abbreviations used in this paper: ASPase, protease cleaving after aspartate residue; BKP, bromocresol purple; DHE, dihydroethidium; GMA, glycerol medium agar; PCD, programmed cell death; PS, phosphatidylserine; ROS, reactive oxygen species; YCD, yeast cell death.

The online version of this article contains supplemental material.

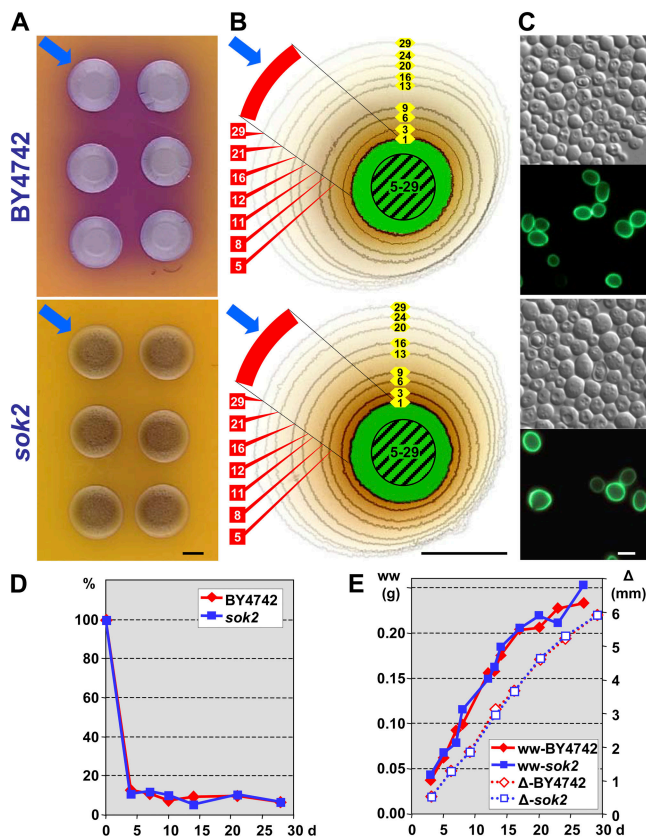


Figure 1. Growth properties of cells within BY4742 and *sok2* colonies. (A) Giant BY4742 colonies at the time of ammonia production (12 d old) and nonproducing *sok2* colonies of the same age; ammonia production is indicated by violet coloring of the pH indicator BKP. Bar, 5 mm. Blue arrows indicate positions of the colonies in B. (B) Timing of colony accrual when growing on GMA. Colony edges on particular days are marked by yellow hexagons. Colony positions on the plates (A) are indicated by blue arrows. Shaded circles indicate the regions of central samples (taken from the 5th to 29th day). Red annular ring sectors and respective red arrows with red boxes indicate size and position of outer samples on particular days (arrows mark outer boundary). Central and outer samples were used for the analyses described in the text and in Figs. 2–4. Green, area inoculated by Alexa-labeled cells. Bar, 5 mm. (C) Alexa-labeled cells in the sample picked up from the center of 10-d-old colonies. Bar, 5 μ m. (D) Decrease of the percentage of Alexa-labeled cells in the colony center. (E) Increase of wet biomass of the whole colony (ww) and accrual of outer margin radius (Δ).

(Palková, 2004) that are capable of simple differentiation and of processes attributed, so far, to metazoa.

Results and discussion

Aged and newly born cells are located at distinct colony areas

To analyze the timing of the appearance and localization of dying cells in yeast colonies, we grew giant colonies of *Saccharomyces cerevisiae* BY4742 strains (Fig. 1 A) on glycerol medium agar (GMA). At the indicated time intervals, we picked up cells from two distinct areas of a colony (the center and outer margin; Fig. 1 B) in order to check the occurrence of features that are characteristic of apoptosis-like YCD, as described previously (Madeo et al., 1997; see Figs. 2 and 3).

In initial experiments, we evaluated the basic characteristics of colony growth and cell distribution within colonies (Fig. 1, B–E) to be able to estimate the relative age and fate of cells analyzed in individual samples. First, we monitored colony biomass increments and occupancy areas in individual phases of colony development. The data revealed that the biomass of a colony increases linearly, at least during the first 16 d of cultivation. Also, the radius of expansion at the outer colony margin is almost linear over the entire estimated time interval of 29 d (Fig. 1, B and E). In parallel, we monitored relative age and the possible relocation of cells within a colony during its growth (Fig. 1, B–D). For this purpose, we inoculated giant colonies with cells vitally stained with AlexaFluor488 5-TFP, and, at the time intervals, we quantified the amount of stained cells in the center and in newly grown colony margins. After a quick decrease in the percentage of stained cells as a result of intensive cell growth during the first 4 d, the amount of stained cells in the colony center continued to decrease, but did so slowly (Fig. 1 D). None of the stained (i.e., primal and, thus, older) cells appeared in newly growing margins, even at very early developmental phases (unpublished data). This implies that, during their division, cells are not effectively pushed in a horizontal direction to other colony regions, but instead remain approximately at their original location. Therefore, the samples picked up from outer colony margins should contain substantial portions of relatively young infant cells, whereas the samples picked up from the center are mostly composed of older, chronologically aging cells.

During colony development, YCD is restricted to specific colony areas

Fig. 2 summarizes the time course of the stress and death features in colonies; i.e., the presence of reactive oxygen species (ROS), PS relocation, presence of a protease hydrolyzing (aspartate)₂-rhodamine (D₂R; a substrate designated for the detection of caspase activity in mammalian cells) (Hug et al., 1999; Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200410064/DC1>), DNA breaks, changes in chromatin condensation and integrity, and changes in cell morphology. Three relatively early markers (ROS, protease, and DNA breaks) are already perceptible in cells both in the center and at the outer margin of BY4742 colonies at the 5th day and culminate between the 8th and 12th day. Remaining evident in central cells, they later disappear from cells of the outer margin. PS relocation was found in central and outer margin cells between the 8th and 16th day (Fig. 2 F). However, exact quantification of this relocation was not possible because of inefficient protoplasting of aged cells. Advanced changes in chromatin (cell type 3; see Fig. 3) develop only in the colony center, and, starting at the 21st day, they are visible in a relatively high number of cells (Fig. 2 D). In addition to these features, the percentage of permeabilized (stained with bromocresol purple [BKP]) dead cells is already low at the outer margin ($2.5 \pm 0.7\%$) on the 21st day, whereas it is ~ 10 times higher in the colony center ($26.2 \pm 3.3\%$). Again, this documents the different fates of cells in the respective colony areas.

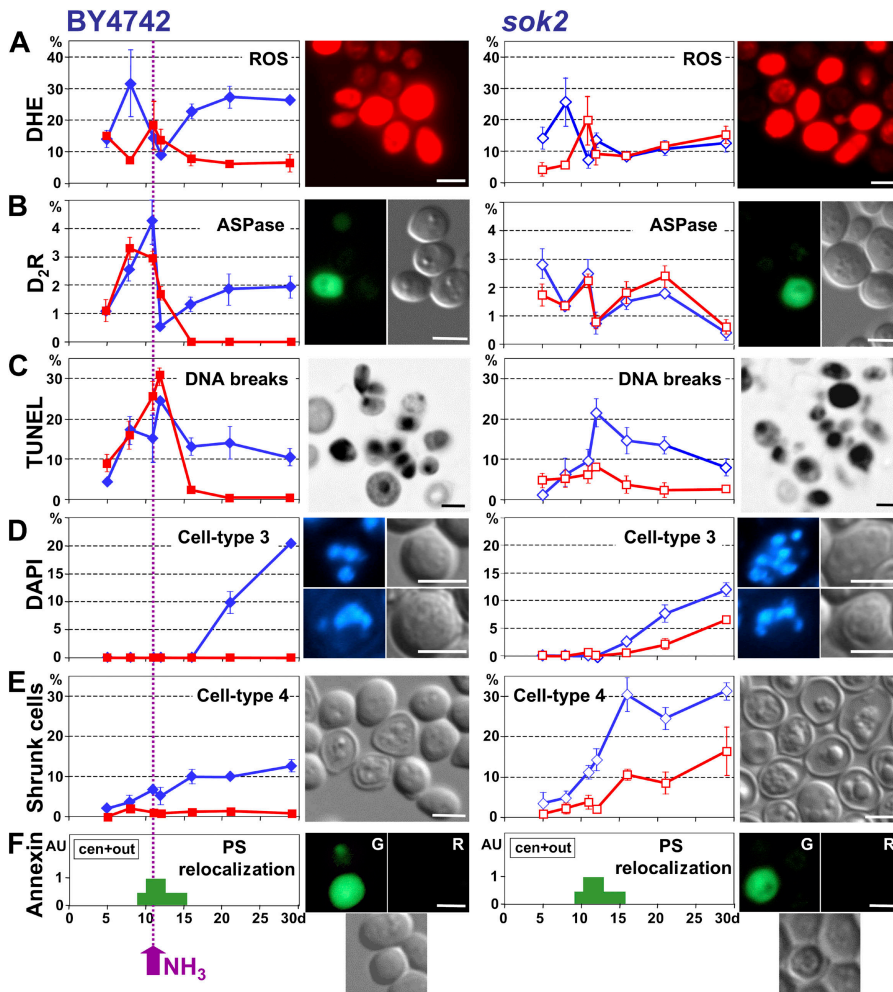


Figure 2. Time course distribution of stress and PCD-like markers in the center and in the outer margin of BY4742 and *sok2* colonies. Positions of the samples within colonies are marked in Fig. 1 B. Violet arrow indicates the beginning of ammonia production in BY4742 colonies. Changes in percentage of cells stained for (A) ROS with DHE; (B) ASPase with D₂R; (C) DNA cleavage with TUNEL assay; (D) chromatin with DAPI (shows proportion of cell type 3 as defined in Fig. 3); (E) PS externalization with Annexin V (filter for green, G); parallel propidium iodide staining distinguishes permeabilized cells (filter for red, R; green PS-positive cells are not permeabilized). Cen + out, same profile of staining in the center and outer samples. (E) Shrunken cells (cell type 4 in Fig. 3) visualized with Nomarski contrast. Blue lines represent changes in central cells; red lines represent changes in outer margin cells. Examples of cells picked up from the colony center at the 21st day are shown. To determine the frequencies of particular apoptotic markers, at least 500 cells were evaluated in each of the two independent experiments. Mean values with error bars showing differences between the experiments are plotted. The relative increase of Annexin V-positive cells is shown in arbitrary units (AU). Bars, 5 μ m.

Most typical cell nuclei changes (visualized by DAPI and TUNEL) and their appearance during BY4742 colony development are summarized in Fig. 3. Both in the center and at the outer margin of colonies, cell type 1 (having a V-shape or a slightly dispersed nucleus with DNA breaks detectable by TUNEL) appears early (already at the 5th day). Later, cell type 2, containing two or more distinctly demarcated pieces of DNA, starts to appear with relatively low frequency (1–5%). These cell types (1 and 2) almost completely disappear from the outer colony margin by the 16th day. In contrast, they remain evident in the central region, where cell types 3 and 4 also gradually appear and then dominate in aged colonies. Cell type 3 seems to be the penultimate stadium of death. It is characterized by the presence of diffuse fragmented (3a) or dispersed chromatin (3c) or by chromatin condensed near the nucleus periphery (3b, present with low frequency, 1–5%), and it is followed by the last visible stadium of YCD (“shrunken” cells, type 4; Figs. 2 and 3).

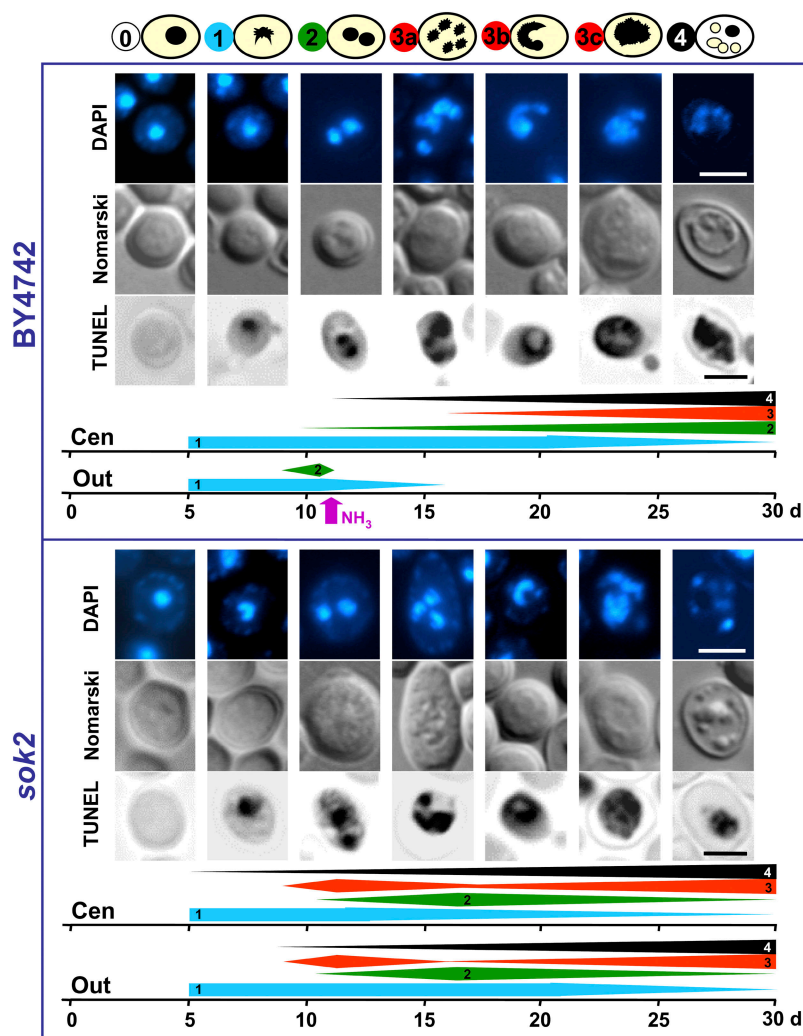
Area-specific YCD occurs within yeast colonies independently on yeast metacaspase *Mca1p* and *Aif1p*

It was shown recently that *Mca1p* metacaspase (Madeo et al., 2002) and yeast *Aif1p* (Wissing et al., 2004) can function as regulators of yeast apoptosis. Interestingly, the absence of either

MCA1 or *AIF1* genes has no impact on the normal development of colonies or on the localization of YCD features (unpublished data). Moreover, a fraction of living cells from colonies of the *mca1* strain was stained by D₂R, indicating the presence of another caspase-like (or another protease cleaving after aspartate residue [ASPase]) activity (Hug et al., 1999; Fig. 4). This agrees with the observation of Herker et al. (2004) that, in chronologically aged *mca1* cells, the caspase-like signal is restored. The number of cells that are stainable by D₂R is relatively low (up to 5%), but, after the 16th day, it reproducibly differs in the center (~2%) and at the outer margins (no cells) of the colony (Fig. 2 B). Additionally, larger numbers of cells in both BY4742 and *mca1* colonies seem to possess a nonactive ASPase, which can be activated by heat shock of cells picked up from colonies (Fig. 4 B). Thus, it cannot be excluded that, in colonies, an inactive precursor of ASPase is activated in cells in which some intracellular stress factors (e.g., ROS) reached detrimental levels.

The fluorochrome-labeled inhibitors of caspases, which were previously used for the monitoring of caspase activity in yeast (Madeo et al., 2002), appear to be unsuitable for the detection of potential caspase activity in colonies because they nonspecifically stain not only permeabilized cells, as described recently (Wysocki and Kron, 2004), but also stain intact cells

Figure 3. **Changes in cellular morphology and in the nuclei of BY4742 and *sok2* cells.** 0, normal nuclei; 1–3, chromatin condensation and fragmentation visualized by DAPI and TUNEL; 4, the last visible stage of YCD (shrunken cells). Morphology of cells stained by DAPI is shown by using Nomarski contrast. Cells picked up from the center of 8-d (cell types 0 and 1) or 28-d-old (cell types 2–4) colonies are shown. On the top, the scheme of particular cell types (chromatin in black and cytosol in yellow). Diagrams show the approximate timing of changes in a number of particular cell types in the center (cen) and at the outer margin (out). Color and numbering of individual diagrams correspond to the labeling in the top scheme. Violet arrow indicates the beginning of ammonia production in BY4742 colonies. Bars, 5 μ m.



that are probably still living (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200410064/DC1>).

Specific localization of YCD in colonies is dependent on ammonia signaling

The preceding data clearly indicate that both old cells in the center as well as newly grown cells at the outer margins exhibit stress and death features (e.g., ROS, ASPase, and DNA breaks) until the 11th or 12th day. Thereafter, the dying process continues only in the central cells, whereas the newly born outer cells appear to gain the ability to escape YCD. The timing of this turn nicely correlates with the developmental transition of colonies to the ammonia-producing phase (Figs. 2 and 3; Palková and Váchová, 2003) that is connected with the reprogramming of cellular metabolism, enabling an escape from oxidative stress (Palková et al., 2002). This finding provides interesting implications for a role of ammonia signaling in colony differentiation and in control of yeast death in particular colony areas. To test this hypothesis, we repeated all experiments with colonies formed by strains defective in Sok2p transcription factor that do not produce significant amounts of ammonia and that exhibit defects in long-term survival (Váchová et al., 2004). Although the growth and time course distributions of cells within *sok2*

colonies are similar to those in BY4742 colonies (Fig. 1, B–E), the profile of the appearance of dying markers significantly differs. Particularly, *sok2* cells located at the outer margin lack an ability to escape YCD at later developmental phases; i.e., almost all dying markers are perceptible in these cells throughout the entire investigated period (Figs. 2 and 3). Additionally, the proportion of cells with presumably intact cell walls but with reduced cellular content (shrunken cells) rapidly increases in the entire *sok2* colony after the 12th day (Fig. 2 E). Some of these cells are completely empty, whereas others contain pieces of cytosol, vacuoles (as indicated by yeast vacuole membrane marker MDY-64 staining; unpublished data), and fragments of DNA (stage 4; Fig. 3). The high proportion of these cells ($\sim 30\%$ after the 16th day) indicates that YCD is accelerated in *sok2* colonies as compared with BY4742 colonies (Fig. 2), thus explaining the overall lower percentage of *sok2* cells that occurs in less developed YCD stages (Fig. 2). A relatively moderate increase (two-fold) in the number of D₂R-stained *sok2* cells evoked by the heat shock (Fig. 4 B) suggests that *sok2* cells probably already passed over the stage of ASPase activation.

Faster *sok2* death is also documented by the fact that already at the 21st day, the *sok2* colony possesses only $38.5 \pm 6.2\%$ of cells that are able to form new progeny as compared with the

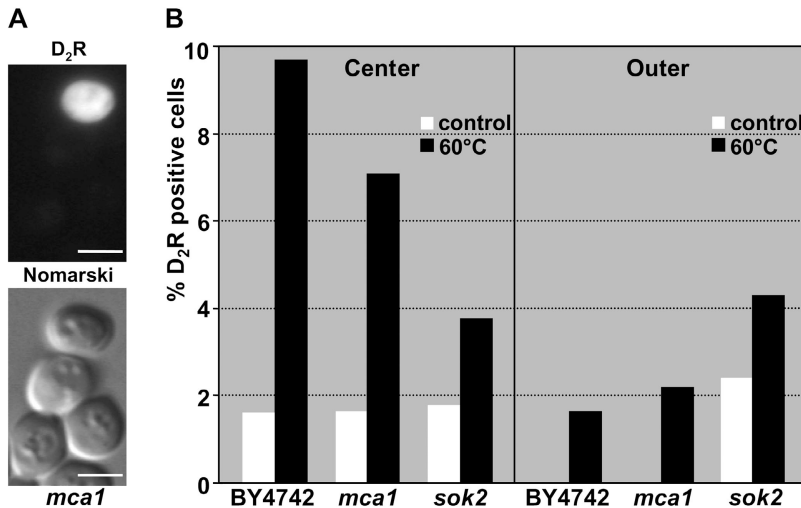


Figure 4. **ASPase activity in BY4742, *mca1*, and *sok2* cells.** (A) Presence of ASPase activity in cells from 18-d-old *mca1* colonies detected by D₂R. Bars, 5 μm. (B) Percentage of D₂R-positive cells in BY4742, *mca1*, and *sok2* cells picked up from either the colony center or from the outer margin of 18-d-old colonies (white bars). Increased number of D₂R-positive cells in the same samples after heat shock (black bars).

BY4742 colony of the same age. Also, there is about a six-time higher percentage of BKP-positive dead cells in *sok2* outer colony margins ($12.1 \pm 1\%$) than in that of BY4742 ($2.5 \pm 0.7\%$).

Model of YCD timing, its regulation, and importance for colony differentiation and growth

On the basis of the preceding data, we propose a model of events occurring within yeast colonies that appear to be crucial for colony development and long-term survival (Fig. 5 A). The progressively increasing level of oxidative stress during the first acidic phase (Palková et al., 2002) causes an induction of YCD throughout the whole colony. However, before stress reaches the detrimental level and before YCD proceeds

to later stages, cells in the colony dispatch an ammonia signal. Then, ammonia induces metabolic changes, enabling an escape from oxidative stress (Palková et al., 2002) and allowing at least some of the cells (e.g., newly born cells at the periphery) to escape YCD and to launch new, healthy generations. Conversely, a majority of cells located in the central, and probably more stressed, parts of the colony proceeds to die. However, even there, some newly born cells appear in aged colonies (starting at the 21st day of development; unpublished data). For cells in a colony that are unable to emit and accept ammonia signals (e.g., *sok2* cells), they are also not able to modify their metabolism (Váchová et al., 2004), and the stress gradually increases throughout the entire colony, including the newly grown regions. In such a case, there is no proper differ-

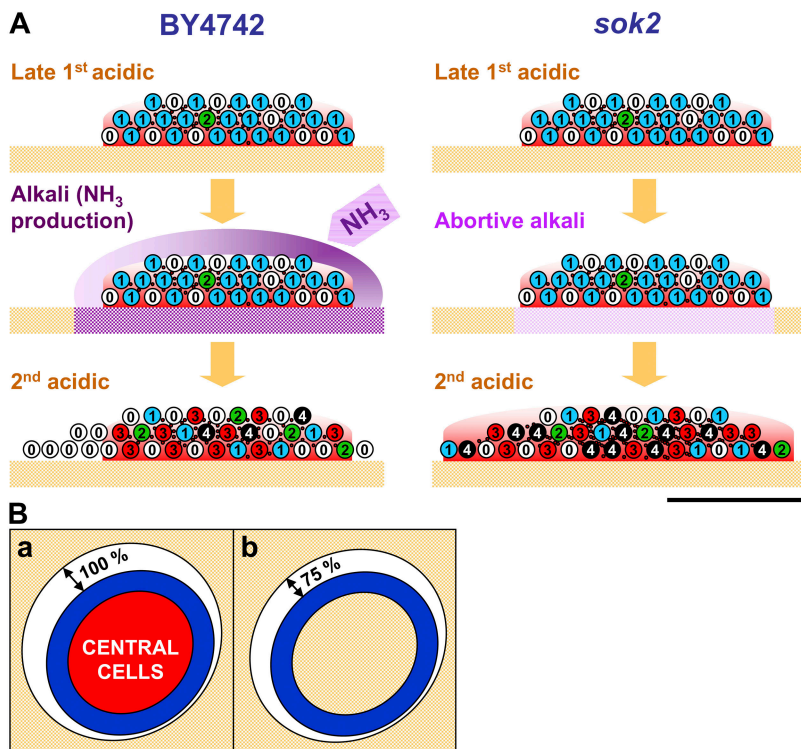


Figure 5. **Model of regulated cell death in yeast colonies.** (A) Model of regulated YCD changes during the development of BY4742 and *sok2* colonies. At the late first acidic phase, cell types 0–2 (Fig. 3) are present in colonies of both strains as a result of an increase in ROS (or other harmful compounds; red). To escape the perils, BY4742 colonies emit and sense an ammonia signal, triggering changes in the metabolism. Consequently, cells stop to produce additional ROS. This allows the healthy cells (type 0) to escape YCD and to form new cell generations at the second acidic phase. Their growth and reproduction can be sustained by cellular compounds released from late-stage YCD cells. Cells in *sok2* colonies are not able to change their metabolism, and death proceeds quickly to the final stages even in a high proportion of *sok2* cells located in “younger” colony areas. Colored circles represent particular cell types, as defined in Fig. 3. Bar, 5 mm. (B) The scheme of growth reduction at the outer margin (white) as a result of the preceding removal of central dying cells (red). The mean value is calculated from a margin accrual of 14 normal colonies (a) and from 14 colonies from which central dying cells were removed (b).

entiation, and death spreads throughout the whole colony, including the peripheral younger cells and their progeny (Fig. 5). Moreover, an increasing stress level, which is already perceptible in young *sok2* colonies (Váchová et al., 2004), can both accelerate the regulated YCD process and induce necrotic-like death in parallel. The first is indicated by a higher level of empty cells observed in *sok2* colonies (Fig. 2 E), and the second by an increased number of destabilized and disrupted cells in *sok2* colonies, as described previously (Váchová et al., 2004).

Contrary to uncontrolled cell lysis, regulated YCD should allow the safe elimination of cells injured by stress from central colony parts, thus preventing a release of toxic cellular components until they are degraded to “safe” simple compounds. These, consequently, could serve as important nutrient sources that enable the growth of healthy cells (e.g., those located at the periphery). To prove this, we compared the accrual of the outer margin of a colony, from which we removed its whole central part at the time when advanced YCD changes start to appear (16–19 d), with that of the normal colony (Fig. 5 B). The data clearly indicate that the removal of central cells reduces the accrual of the outer margin to $\sim 75.3 \pm 8.1\%$.

To summarize, the presented data indicate that yeast death exhibiting some features of higher eukaryotic PCD naturally occurs in *S. cerevisiae* colonies. This YCD is dependent on the time periods of colony development and on the position of cells within the multicellular colony. Cells in aging colonies reach YCD spontaneously, probably responding to cumulative intracellular stress. Contrary to apoptosis-like processes that were described previously (Herker et al., 2004), YCD within colonies is the first example of regulated yeast death that is not uniformly spread in a population, but instead is located at specific areas of a colony. This differentiation appears to be regulated by ammonia signaling, which is responsible for changes that locally prevent YCD progress. Regulated YCD can prevent a release of toxic cellular components, thus avoiding necrotic collapse of the colony, and can also provide nutrients for healthy cells. Therefore, regulated cell death in yeast colonies (and possibly in other multicellular communities of microorganisms) can be as important for their development as are apoptosis and related processes that occur within metazoa.

Materials and methods

Yeast strains and media

The wild-type strain *S. cerevisiae* BY4742 (*MAT α* , *his3 Δ 1*, *lev2 Δ 0*, *lys2 Δ 0*, and *ura3 Δ 0*) and the isogenic mutants *sok2*, *mca1*, and *aif1* are from the European *Saccharomyces cerevisiae* Archive for Functional Analysis collection. Colonies were grown on GMA (3% glycerol, 1% yeast extract, and 2% agar) or on GMA–BKP (GMA with 0.01% BKP) at 28°C (Palková and Forstová, 2000). In each experiment, a large number of parallel plates was inoculated, with six giant colonies per plate (Fig. 1 A). This always allowed us to analyze cells from a colony, the development of which was not influenced by preceding manipulation (e.g., by the previous removal of some cells).

Monitoring of a colony accrual during its growth on GMA

Colonies growing on GMA were photographed at specified time points using an integrating 3CCD color camera (model HV-C20; Hitachi Denshi), and the final picture was created by the overlaying of all photos after colony edge highlighting in Adobe Photoshop 7. A similar procedure was

used to determine the effect of the removal of central parts of the colony on subsequent outer margin expansion (Fig. 5 B). On seven plates with six colonies, all cells from the center of two corner colonies were carefully removed, and the remaining two untreated corner colonies were used as controls. The expansion was compared after 6 d.

Monitoring of relative age and possible relocation of cells within giant colonies

Giant colonies were inoculated with cells vitally stained with AlexaFluor488 5-TFP (AlexaFluor488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester amine-reactive probe; Molecular Probes). The labeling was performed according to the manufacturer's manual. Cells were picked up from colonies at various time intervals and were resuspended in 10 mM MES and 1 M sorbitol. The percentage of stained cells in the center and in newly grown outer colony margins was quantified using fluorescence microscopy.

Staining of YCD markers

Cells were picked up from the colony center and from outer colony margins (as indicated in Fig. 1 B) and were resuspended in 10 mM MES, pH 6, and 1 M sorbitol.

Staining for ROS with dihydroethidium (DHE; Sigma Aldrich), which preferentially stains superoxide radicals, was performed as previously described (Madeo et al., 1999). DHE was added to the final concentration of 5 μ g/ml, and samples were incubated for 10 min at room temperature.

ASPase was detected by staining with D₂R (CaspSCREEN Flow Cytometric Apoptosis Detection Kit; BioVision), the nonfluorescent substrate, which is cleaved to green fluorescent monosubstituted rhodamine 110 and free rhodamine (Fig. S4). Cells were incubated with D₂R at 30°C for 30 min. To monitor protease activation by heat shock, cells were picked up from colonies and heated for 5 min at 60°C in D₂R incubation buffer before ASPase detection.

Staining of DNA breaks with TUNEL assay was performed as described previously (Madeo et al., 1999), but in Eppendorf tubes. DNA ends were labeled using the in situ Cell Death Detection Kit, POD (Roche Diagnostics). All buffers and solutions were supplemented with 1.2 M sorbitol.

For the detection of chromatin changes, cells were stained with 0.25 μ g/ml DAPI as described previously (Hasek and Streiblova, 1996).

PS relocalization was visualized with Annexin V/propidium iodide (ApoAlert Annexin V and Apo2.7-PE kit; BD Biosciences) as described previously (Madeo et al., 1997). In brief, after cell protoplasting (5.5% glucosylase [Boehringer Mannheim] and 15 U/ml lyticase [Sigma Aldrich] for 2 h at 28°C), protoplasts were stained with Annexin V–FITC and propidium iodide for 20 min at room temperature. All solutions contain 1.2 M sorbitol.

Cells were viewed under a fluorescence microscope using filter N2.1 (DHE and propidium iodide red stainings), I3 filter (protease and Annexin V green stainings), and A filter (DAPI staining).

Determination of dead and clonogenic cells

Permeabilized dead cells were stained with BKP (entering only permeabilized cells) in colonies in situ during their growth on GMA–BKP. Cells were resuspended in 10 mM MES, pH 6, and 1 M sorbitol and were viewed under a fluorescence microscope using filter N2.1. At least 300 cells in five parallels were evaluated in each of the two independent experiments. For clonogenic cell determination, the whole colony was resuspended in water, the concentration of wet biomass was adjusted to 10 mg/ml ($\sim 10^8$ cells/ml), and cells were plated after dilution on YPG (1% yeast extract, 1% peptone, 2% glucose, and 2% agar).

Fluorescence microscopy and image acquisition

We used a fluorescence microscope (model DMR; Leica) equipped with a 100 \times /1.3 oil objective (model HCX PL fluorar; Leica), a high performance CCD camera (model 4912; Cohu), and Lucia 32 software version 4.50 (Laboratory Imaging).

Online supplemental material

Supplemental materials and methods describe the problems connected with the use of fluorochrome-labeled inhibitors of caspases for caspase detection in yeast and the data supporting the use of D₂R caspase substrate. Fig. S1 shows examples of caspase inhibitors. Table S1 shows staining of *S. cerevisiae* cells with different combinations of FLICA and FITC. Fig. S2 shows a comparison of staining with FLICA and FITC and counterstaining with PI. Fig. S3 shows staining of dead cells with FITC and FITC–VAD–FMK. Fig. S4 shows staining of active ASPase in cells from yeast colonies with D₂R substrate. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200410064/DC1>.

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