

# YCA1 participates in the acetic acid induced yeast programmed cell death also in a manner unrelated to its caspase-like activity

Nicoletta Guaragnella<sup>a,b,1</sup>, Clara Pereira<sup>c,1</sup>, Maria João Sousa<sup>c</sup>, Lucia Antonacci<sup>a,b</sup>, Salvatore Passarella<sup>b</sup>, Manuela Côrte-Real<sup>c</sup>, Ersilia Marra<sup>a</sup>, Sergio Giannattasio<sup>a,\*</sup>

<sup>a</sup> CNR, Istituto di Biomembrane e Bioenergetica, I-70126 Bari, Italy

<sup>b</sup> Dipartimento di Scienze per la Salute, Università del Molise, I-86100 Campobasso, Italy

<sup>c</sup> Departamento de Biologia, Universidade do Minho, Braga 4719, Portugal

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**Abstract** Yeast cells lacking the metacaspase-encoding gene YCA1 (*Δyca1*) were compared with wild-type (WT) cells with respect to the occurrence, nature and time course of acetic-acid triggered death. We show that *Δyca1* cells undergo programmed cell death (PCD) with a rate lower than that of the WT and that PCD in WT cells is caused at least in part by the caspase activity of Yca1p. Since in *Δyca1* cells this effect is lost, but z-VAD-fmk does not prevent both WT and *Δyca1* cell death, PCD in WT cells occurs via a Yca1p caspase and a non-caspase route with similar characteristics.

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**Keywords:** PCD; Acetic acid; YCA1; Caspase; z-VAD-fmk; *Saccharomyces cerevisiae*

## 1. Introduction

In the last decade it has been shown that *Saccharomyces cerevisiae* may undergo a form of cell death similar to apoptosis with its own physiological significance [1–3]. In fact, in response to different stimuli *S. cerevisiae* commits to cell death showing typical hallmarks of metazoan apoptosis [4]. Although several yeast orthologues of key apoptotic regulators have already been identified [5–10], how they work in yeast programmed cell death (PCD) remains to be fully established.

In mammalian apoptosis, caspases play a major role [11–13], however the occurrence of a caspase-independent PCD has also been proposed [14,15]. In yeast, YCA1 codes for a protein belonging to the superfamily of caspase-related proteases, termed metacaspases [16]. YCA1 is involved in *S. cerevisiae* PCD triggered by different stimuli [2,6,17–20]. In addition, dis-

ruption of YCA1 attenuated the stimulation of apoptosis due to Aifp overexpression [10] or hydrogen peroxide [21]; however, yeast metacaspase-independent PCD has also been reported [22,23].

We have shown that *S. cerevisiae* commits to PCD upon treatment with acetic acid [24,25]. The role of YCA1 in acetic acid-induced PCD (AA-PCD) is still controversial although Yca1p has been indicated as an executor of AA-PCD in *S. cerevisiae* [6]. More recently, a minor role for YCA1 in AA-PCD was proposed [26]. Thus, comparison is made here between WT and YCA1-lacking (*Δyca1*) yeast cells with respect to the occurrence, nature and time course of the death process. We show that YCA1 contributes to AA-PCD but not as a conventional caspase.

## 2. Materials and methods

### 2.1. Strains, media, growth conditions and YCA1 gene deletion

The *Saccharomyces cerevisiae* strain used in this study was W303-1B (*MATα ade2 leu2 his3 trp1 ura3*) and cells were grown at 30 °C in rich medium (1% yeast extract and 2% Bacto-peptone) containing 2% dextrose (YPD). To delete the YCA1 gene, genomic DNA was isolated from BY4743 (*MATα/MATα leu2/leu2 his3/his3 ura3/ura3 lys2/LYS2 MET15/met15 yca1::kanMX4/YCA1*) cells, kindly provided by Prof. R. A. Butow, and amplified using the oligonucleotide couple 5'-TTA TTG GCC GAG TTG CGC T-3' and 5'-GGA AGA ACA GGA AGA GTC TG-3'. The *Δyca1::KanMX4* cassette-containing PCR product was purified from agarose gel and used to replace the YCA1 gene in W303-1B cells. Recombinant clones were selected for resistance to the antibiotic G418 disulfate (Sigma–Aldrich). Gene disruption was verified by PCR analysis. *Δyca1* cells were grown in YPD medium supplemented with 200 mg/l G418.

### 2.2. Acetic acid treatment

Exponential phase ( $OD_{600} = 0.5–0.8$ ) wild-type (WT) and *Δyca1* cells were grown in YPD medium at 26 °C, at 150 rpm and were suspended ( $10^7$  cells/ml) in fresh YPD medium. For acetic acid treatment this medium, containing 80 mM acetic acid, was adjusted to pH 3.0 (set with HCl). Cells were incubated for different times at 26 °C as described previously [24]. To induce hyperosmotic stress, wild-type cells were transferred to medium containing 60% (w/w) of glucose as described [18] and incubated for 300 min at 26 °C.

Cycloheximide (Sigma–Aldrich) dissolved in water or carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone (z-VAD-fmk) (Calbiochem) dissolved in dimethylsulfoxide, were added to yeast cell cultures grown to  $OD_{600} = 0.5–0.6$  at a final concentration of 100 μg/ml or 20 μM, respectively, and the cultures incubated for 30 min before acetic acid treatment. Cell viability was determined by measuring colony-forming units (cfu) after 2 days of growth on YPD plates at 30 °C.

\*Corresponding author. Fax: +39 0805443317.

E-mail address: s.giannattasio@ibbe.cnr.it (S. Giannattasio).

<sup>1</sup>Contributed equally to this paper.

**Abbreviations:** PCD, programmed cell death; AA-PCD, acetic acid-induced PCD; PI, propidium iodide; WT, wild-type; *Δyca1*, YCA1-lacking; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; z-VAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone; FITC-VAD-fmk, fluorescein isothiocyanate conjugate of z-VAD-fmk; D<sub>2</sub>R, (Asp)<sub>2</sub>-Rhodamine 110

### 2.3. Fluorescence microscopy analysis

Exponential cells were treated with acetic acid in YPD pH 3.0 medium as described above. At different times,  $10^8$  cells were collected and resuspended in water in the presence of 0.25  $\mu\text{g}/\text{ml}$  propidium iodide (PI) and 50  $\mu\text{g}/\text{ml}$  4,6-diamidino-2-phenylindole dihydrochloride (DAPI) at 37 °C for 15 min. Then, live cells were observed using an Axioplan 2 microscope (Zeiss) equipped with a UV HBO 50/AC lamp and a 100 $\times$  objective. Digital images were acquired with an Axiocam CCD camera using Axio Vision software.

### 2.4. Detection of caspase activation

Detection of caspase activation was performed using the “CaspACE, FITC-VAD-fmk In Situ Marker” (Promega). Briefly,  $1 \times 10^6$  cells were washed in PBS, suspended in 100  $\mu\text{l}$  staining solution containing 50  $\mu\text{M}$  of the fluorescein isothiocyanate conjugate of z-VAD-fmk (FITC-VAD-fmk) and incubated for 20 min at 30 °C in the dark. Then, cells were washed once and suspended in PBS. For double staining with PI, cells were subsequently incubated with 2  $\mu\text{g}/\text{ml}$  of PI for 10 min at room temperature. For the assays with the broad caspase inhibitor z-VAD-fmk, cells were prepared as described above, washed with PBS and incubated with 20  $\mu\text{M}$  z-VAD-fmk for 30 min at 30 °C before incubation with FITC-VAD-fmk.

Flow cytometric analysis was performed using an Epics<sup>®</sup> XL-MCL™ (Beckman Coulter) flow cytometer, equipped with an argon-ion laser emitting a 488-nm beam at 15 mW, and with biparametric detection of FITC fluorescence (488/525 nm; FL1; log units) and PI fluorescence (488/620 nm; FL3; log units). Signal compensation that eliminates optical overlap between the dyes was set at 30%. Twenty thousand events were acquired for each analysis. Data were analyzed using WinMDI 2.8 software.

## 3. Results

### 3.1. Deletion of the YCA1 gene decreases the rate of yeast programmed cell death triggered by acetic acid

To ascertain the role of the YCA1 gene in AA-PCD, comparison was made between WT and  $\Delta yca1$  W303-1B yeast cells in exponential phase with respect to their viability up to 200 min after PCD induction (Fig. 1A). In both cases the yeast cells died but the cell death patterns were different. After 30 min of acetic acid treatment, wild-type and  $\Delta yca1$  cells showed about 74% and 83% cell viability, respectively. This progressively decreased to 0% for WT as in [25] and 7% for  $\Delta yca1$  cells at 200 min.  $\Delta yca1$  cell viability was significantly higher ( $P < 0.001$ ) than that of WT cells from 60 to 120 min after acetic acid challenge with death rates of  $\mu_d = 0.015 \pm 0.0021 \text{ min}^{-1}$  and  $0.0074 \pm 0.0002 \text{ min}^{-1}$  for WT and  $\Delta yca1$  cells, respectively.

To determine whether death of  $\Delta yca1$  cells occurs via PCD, given that AA-PCD is dependent on *de novo* protein synthesis [24], the effect of cycloheximide on survival of  $\Delta yca1$  and WT cells was investigated. In both cases cycloheximide prevented cell death in a similar way (Fig. 1B). Chromatin condensation, another PCD hallmark, was also analyzed during AA-PCD of both WT and  $\Delta yca1$  cells, through analysis of nuclear morphology and plasma membrane integrity by co-staining cells with DAPI and PI (Fig. 2). With both cell types chromatin condensation along the nuclear envelope was detectable after 60, 120 and 200 min of acetic acid treatment in cells with an integral plasma membrane as shown by lack of staining with PI.

Thus, yeast cells lacking the metacaspase YCA1 gene undergo the process of AA-PCD in a manner similar to that of the WT cells but at a lower rate.

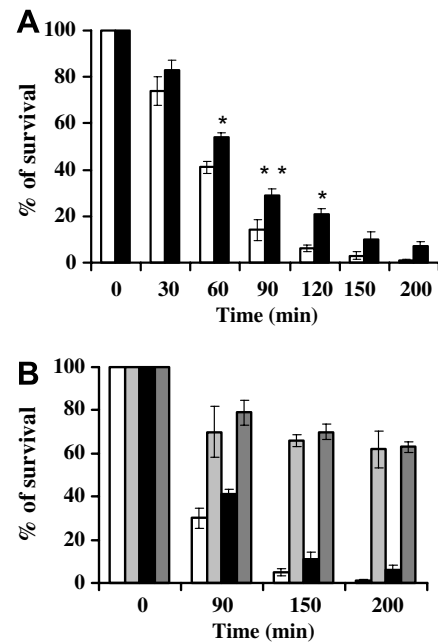


Fig. 1. Effect of YCA1 deletion and/or cycloheximide on viability of *Saccharomyces cerevisiae* W303-1B exponential cells exposed to acetic acid. WT (white bars) and  $\Delta yca1$  (black bars) cell death was induced with 80 mM acetic acid in the absence (A and B, white and black bars, respectively) or in the presence of cycloheximide (B, light and dark grey bars, respectively) and cell viability analyzed at indicated times. Cell survival (100%) corresponds to cfu at time zero. Reported values are the mean of three experiments with standard deviations. ANOVA and Bonferroni test: statistically significantly different with (\*)  $P < 0.001$ , (\*\*)  $P < 0.0001$  when comparing WT and  $\Delta yca1$  cells. Death rate  $\mu_d$  (see text) was calculated by GraFit 3.0 software as the slope of the linear part of the semilogarithmic plot of the number of cfu as a function of incubation time.

### 3.2. z-VAD-fmk does not prevent yeast AA-PCD even though it partially inhibits caspase-like activity

In the light of the above results, to ascertain whether and how caspase activity is involved in yeast AA-PCD, we monitored caspase-like activity by simultaneous staining of cells with FITC-VAD-fmk [6,18] and with PI to differentiate between FITC-VAD-fmk specific and unspecific staining [27] (Fig. 3A). Cells treated with 60% (w/w) of glucose, which die via PCD [18], were used as a control for positive staining with FITC-VAD-fmk. About 36.9% of WT cells displayed FITC-VAD-fmk positive and PI negative staining (specific staining); after acetic acid treatment the corresponding percentage was 11.8%. Pre-incubation with the pan-caspase inhibitor z-VAD-fmk before staining with FITC-VAD-fmk, caused a reduction in the percentage of FITC-VAD-fmk positive cells both in acetic acid and 60% glucose treated cells.

Staining with FITC-VAD-fmk was very low in WT cells held at pH 3 but without acetic acid (Fig. 3A) and did not increase with time (Fig. 3B). In WT cells treated with acetic acid the staining with FITC-VAD-fmk increased progressively up to about 20% at 200 min after induction of PCD. By contrast, with  $\Delta yca1$  cells the percentage of FITC-VAD-fmk staining remained virtually constant at about 5% between 60 and 200 min of PCD. These results are consistent with YCA1 acting as a z-VAD-fmk sensitive caspase-like protease and/or as a protease activator. In parallel, cell survival was monitored either in the

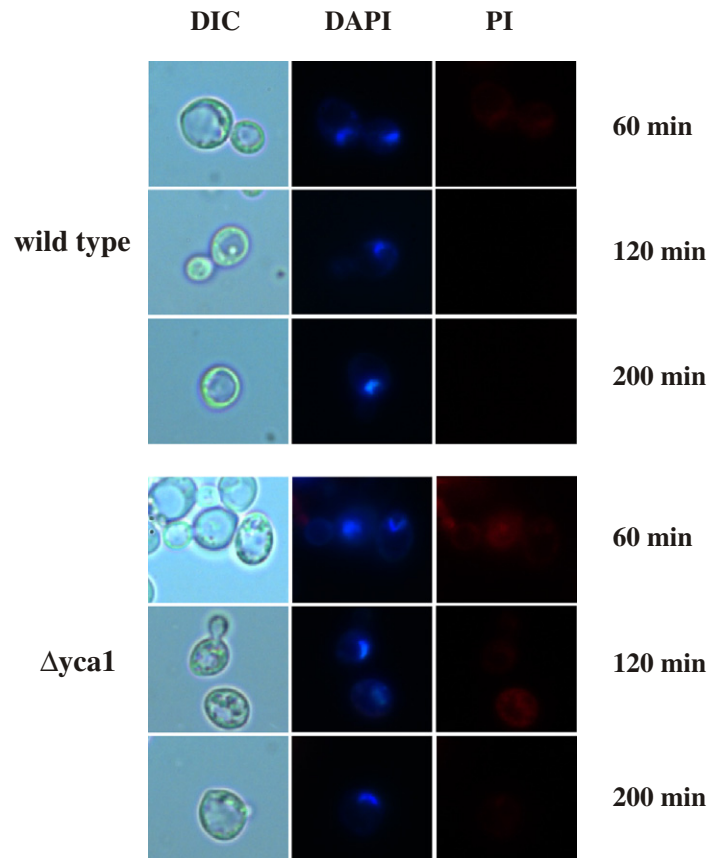


Fig. 2. DAPI/PI staining of WT and  $\Delta yca1$  *Saccharomyces cerevisiae* W303-1B exponential cells exposed to 80 mM acetic acid. WT and  $\Delta yca1$  yeast cells were exposed to 80 mM acetic acid. At indicated times cells were collected, stained with DAPI and PI and observed with a fluorescence microscope. Representative pictures of WT and  $\Delta yca1$  cells are shown. DIC, differential interference contrast. As a control, WT and mutant cells incubated in the same conditions, but in the absence of acetic acid, did not show either chromatin condensation or PI-positive cells at any time analyzed (not shown).

absence or presence of z-VAD-fmk. We found that either in the presence or in the absence of z-VAD-fmk there was no difference in AA-PCD time-course between WT and  $\Delta yca1$  cells (Fig. 3C).

We attempted to use the caspase substrate (Asp)<sub>2</sub>-Rhodamine 110 (D<sub>2</sub>R) to monitor caspase activity as in [27,28]. Unfortunately, D<sub>2</sub>R was not suitable for this purpose in cells committed to die in response to acetic acid because acetic acid interfered with the green fluorescence produced by the intracellular cleavage of the caspase substrate.

#### 4. Discussion

In this paper we investigate the role played by YCA1 in AA-PCD. We show that as with the WT,  $\Delta yca1$  cells die via PCD as shown by two criteria, i.e. cycloheximide sensitivity and the occurrence of chromatin condensation. Moreover, we show that AA-PCD in WT and  $\Delta yca1$  cells differ from one another essentially in the reduced rate of death in the latter case. That is, the characteristics of the death process are the same for both cell types except that YCA1 disruption increases cell survival.

We also used the pancaspase inhibitor z-VAD-fmk to investigate further the role of the yeast metacaspase. We showed

that in WT cells treated with acetic acid there is a time-dependent increase in caspase-like activity, as revealed by flow cytometry with FITC-VAD-fmk, which is inhibited by z-VAD-fmk. This does not occur in  $\Delta yca1$  cells, thus strongly suggesting a role for YCA1 in promoting caspase-like activity. Nonetheless, z-VAD-fmk addition did not result in death prevention (Fig. 3C). Given that this substance can inhibit caspase-like activity, as indicated by the experiments with flow cytometry, we conclude that yeast can die in a manner independent of caspase activity and consequently that YCA1 participates in AA-PCD in a manner unrelated to its putative caspase-like activity. In this regard, AA-PCD differs significantly from that due to defective N-glycosylation. In the latter case both FITC-VAD-fmk cell-staining activity as well as loss of cell viability have been shown to be inhibited by z-VAD-fmk and this caspase-like activity is independent of YCA1 [23].

At present the explanation of the reduced rate of death in yeast cells lacking YCA1 can only be speculative. In addition to the possibility that YCA1 is a caspase-like protein, as suggested by the fact that deletion of YCA1 inhibits FITC-VAD-fmk cell-staining, the possibility that YCA1 could be associated with other function different from caspase activity, perhaps protease activity must be taken into consideration. This is supported by recent findings which show that recombinant Yca1p exhibits arginine/lysine-specific cysteine endopepti-

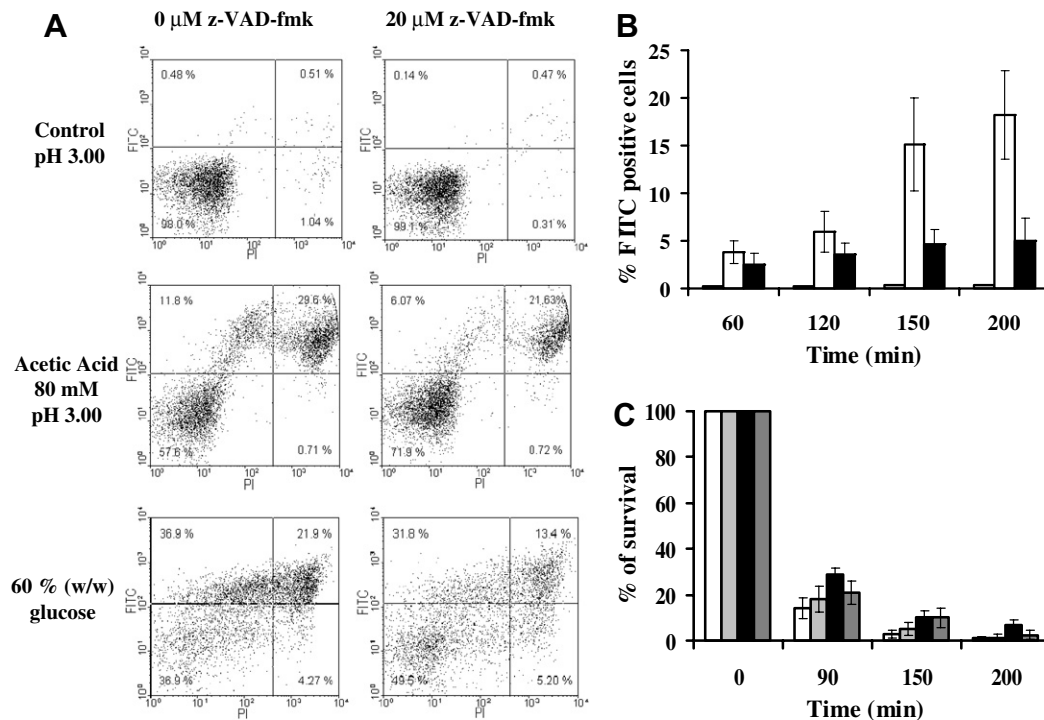


Fig. 3. Caspase-like activity in *Saccharomyces cerevisiae* W303-1B exponential cells upon treatment with acetic acid and effect of z-VAD-fmk on cell viability. (A) Flow cytometric analysis using bi-parametric detection of FITC fluorescence and PI fluorescence of: control non-treated cells with or without pre-incubation with 20  $\mu$ M z-VAD-fmk; cells treated with 80 mM acetic acid for 200 min with or without pre-incubation with 20  $\mu$ M z-VAD-fmk; cells treated with 60% (w/w) glucose for 300 min with or without pre-incubation with 20  $\mu$ M z-VAD-fmk. Percentages of cells exhibiting only FITC fluorescence (upper left quadrant), both FITC and PI fluorescences (upper right quadrant), only PI fluorescence (lower right quadrant) or absence of both fluorescences (lower left quadrant) are depicted in the respective quadrants. Data represent one of the three independent experiments. (B) Results presented are percentages of specifically FITC-VAD-fmk-stained wild-type cells incubated at pH 3.0 (grey bars), wild-type (white bars) and  $\Delta yca1$  (black bars) cells treated with 80 mM acetic acid as a function of time. A two-way ANOVA was employed to compare WT untreated versus acetic acid-treated cells and these latter versus acetic acid-treated  $\Delta yca1$  cells. In both cases the difference in the % of FITC positive cells was considered significant ( $P < 0.05$ ). Values are the mean of three independent experiments with 20000 cells counted (by flow cytometry) at each time point. (C) WT and  $\Delta yca1$  cell death was induced with 80 mM acetic acid in the absence (white and black bars, respectively) or in the presence of 20  $\mu$ M z-VAD-fmk (light and dark grey bars, respectively). Cell viability was analyzed at indicated times. 100% of cell survival corresponds to the cfu at time zero. Reported values are the mean of three experiments with standard deviations.

dase activity, which is not inhibited by z-VAD-fmk [29]. Thus, it cannot be excluded that Yca1p, acting as a protease, activates other caspase-like activities.

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