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Reliable Method for Detection of Programmed Cell Death in Yeast

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Summary

Accumulating evidence suggests that yeasts are capable of undergoing programmed cell death (PCD) to benefit long-term survival of the species, and that yeast and mammals may share at least partially conserved PCD pathways. In our experience, mammalian apoptosis assays have not been readily applicable to yeast. Therefore, to take advantage of yeast as a genetic tool to study PCD, we developed a yeast cell death assay that can reliably reveal viability differences between wild-type strains and strains lacking the mitochondrial fission genes *DNM1*/Drp1 and *FIS1*, orthologs of mammalian cell death regulators. Cell viability following treatment with acetic acid is quantified by colony formation and vital dye (FUN1) staining to reproducibly detect dose-dependent, genetically programmed yeast cell death.

Keywords

Yeast; Programmed cell death; Apoptosis; Fis1; Dnm1; Acetic acid; Colony forming assay; FUN1; Mitochondria; Fission

1. Introduction

The debate over the existence of programmed cell death (PCD) machinery in yeast stems in part from uncertainty about the benefit of a cell suicide program in unicellular species and the mechanisms for selecting such a program during evolution. However, purposeful death of a subset of yeast cells in the population has been suggested to benefit the population as a whole during nutrient limitation (1), failed mating attempts (2), and exposure to killer viruses (3). Furthermore, deliberate cell death was suggested to be a critical consequence of cell-to-cell communication by colonial organisms, resulting in the elimination of older members to benefit younger members (4). The controversy over yeast PCD also stems from conflicting terminologies. The yeast community often uses the term “apoptosis” to mean programmed death, while mammalian apoptosis is usually, but not always, reserved for caspase-dependent cell death to distinguish this pathway from other emerging pathways. Perhaps a more legitimate application of the term apoptosis for yeast is based on the evidence that dying yeast exhibit apoptosis-like features, fitting the original morphological definition of the term (5).

Although yeast appear to lack obvious orthologs of Bcl-2 family proteins that regulate apoptosis in metazoans, mammalian Bcl-2 proteins can retain their anti- and pro-death functions when exogenously expressed in yeast (6–10). While it is not known if the biochemical mechanisms of Bcl-2 family proteins in yeast and mammalian cells are the same or distinct, these observations by many laboratories are consistent with the existence of conserved PCD pathways between yeast and mammals. In contrast to Bcl-2 proteins, yeasts

encode homologs of many other mammalian cell death regulators, and these have been suggested to fulfill a similar role in yeast, such as Mca1/Yca1, which is designated a metacaspase based on sequence similarity to mammalian caspases (11), the dynamin-like mitochondrial fission protein Dnm1 (human Drp1) (9), Bir1, which contains two N-terminal BIR repeats found in baculovirus and cellular IAPs (inhibitor of apoptosis proteins) (12), the serine protease Nma111, which is homologous to mammalian HtrA2/Omi (13), and an ortholog of human AIF (14). Yeast knockouts and protein overexpression models have demonstrated that these factors influence cell death in yeast, but evidence that these factors arose during evolution in part to serve as cell suicide factors in yeast is experimentally challenging to demonstrate. Nevertheless, we anticipate that further analysis of their cell death functions in yeast will be revealing about mammalian processes. However, we have found it difficult to adapt published protocols to quantify cell death phenotypes in yeast with satisfying reproducibility.

Mammalian cell death assays that have been applied to yeast include the exposure of phosphatidylserine on the outer plasma membrane leaflet (Annexin V staining), nuclear DNA fragmentation (TUNEL labeling), activation of caspase-like activity (FITC-VAD-FMK cleavage), and release of cytochrome *c* from mitochondria (15). Questions have been raised about whether or not these markers in yeast truly reflect a purposeful death pathway analogous to caspase-dependent apoptosis or any other death pathway in mammalian systems (15,16). For example, hundreds of caspase substrates have been identified in mammals, and the roles for a subset of these in facilitating apoptosis have been partially delineated, but similar evidence in yeast is largely lacking. While acetic acid (17), pheromones (2), and exogenous Bax (7) induce cytochrome *c* release from yeast mitochondria, the physiological role of cytochrome *c* release in yeast, or in any nonmammalian species, remains in question.

Given the difficulties in defining PCD in yeast, we have turned to a genetic definition, and then applied this definition to the development of a reliable assay to detect yeast cell death/survival following treatment with acetic acid. Our definition of a condition that induces yeast PCD is one that can detect the increased survival of yeast lacking the pro-death factor Dnm1/ Drp1, a dynamin-like GTPase with pro-death function that is conserved in mammals, flies, and worms (18–21). Conversely, the increased sensitivity to cell death by yeast *FIS1* knockout strains from the YKO collection defines the opposite phenotype (21). Therefore, experimental conditions that distinguish the viabilities of these two knockout strains from the wild-type control strain are deemed to be reflective of PCD in yeast (9). We have now further developed our earlier acetic acid assay so that other investigators can readily apply this strategy with high reproducibility.

Several cell death stimuli have been suggested to induce yeast PCD, including hydrogen peroxide (22), acetic acid (23,24), high salt (25), and others, but regardless of the specific death stimulus applied, the dose is critical. Any death stimulus can induce non-PCD in yeast or mammalian cells when administered in high doses. Analogous to a clonogenic assay in mammalian models, the yeast community generally defines cell survival as the ability to form a colony on solid medium. This is a stringent test of cell viability in mammalian models, as cultured mammalian cells often exhibit only delayed cell death, such as with Bcl-2 overexpression following a variety of death stimuli. In cases such as this, vital dyes or related strategies are required to reliably quantify cell death (26,27). A comparable assay for yeast is FUN1 staining to identify metabolic viability, a convenient alternative assay to quantify yeast viability.

2. Materials

2.1. Yeast Cultures and Acetic Acid Treatment

1. Yeast strains:
Wild-type BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*)
Δfis1 (MATa *fis1::kanMX4 Δhis3Δ1 leu2Δ0 met15Δ0 ura3Δ0*)
Δdnm1 (MATa *dnm1::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*).
2. YPD liquid medium: 2% peptone, 1% yeast extract, 2% glucose.
3. Roller drum (TC-7, New Brunswick Scientific).
4. Glass yeast culture tubes (18 × 150 mm) with loose fitting caps.
5. Acetic acid stock solution (17.3 M) (J. T. Baker).

2.2. Colony Forming Assay

1. 96-well microplates (200 μL).
2. Omni trays.
3. YPD agar plates (YPD + 2% agar).
4. 12-place multichannel pipette.

2.3. FUN1 Staining Assay

1. Live/Dead Yeast Viability Kit (Molecular Probes) containing FUN1 cell stain [10 mM solution in anhydrous dimethylsulfoxide (DMSO)] and Calcofluor White M2R (5 mM solution in water). Stored at −20°C.
2. FUN1 staining solution: 2% glucose/dextrose and 10 mM Na-HEPES (pH 7.2), sterilized with 0.2-μm filter and stored at room temperature.
3. Fluorescence microscope equipped with multipass filter sets appropriate for viewing DAPI, fluorescein, and rhodamine.
4. Microslides (25 × 75 × 1 mm) and micro cover glass (18 × 18 mm) (VWR scientific).

3. Methods

3.1. Culture Growth and Acetic Acid Treatment

1. WT and mutant yeast strains are streaked out from frozen −80°C glycerol stock (without thawing) onto YPD agar plates and incubated at 30°C for 2 days.
2. Single colonies are picked from each plate, inoculated into 2-mL YPD in yeast culture tubes, and incubated at 30°C on a roller drum overnight (~35 rotations/min).
3. Overnight cultures are diluted to OD₆₀₀ = 0.2 in 5-mL YPD (or at least 1 mL more than the amount required) and further incubated at 30°C until mid-log phase, OD₆₀₀ = ≤0.5. Before treatment, all cultures are adjusted to the same OD₆₀₀ (for example the OD of the least dense culture). For best results, adjust the OD₆₀₀ 30 min before reaching 0.5 by diluting the slightly overgrown samples in YPD, then continue incubation until OD₆₀₀ = ~0.5. Further adjustments may be necessary to make all samples equal. If OD₆₀₀ exceeds 0.6, results may be less reliable (*see* Note 1).

4. Dispense 2 mL of each mid-log phase culture into each of the two new tubes for treatment with two doses of acetic acid and the remainder is held as the untreated control. Addition of 23- μ L and 28- μ L acetic acid stock (17.3 M) to 2-mL cultures yields a final concentration of 199 mM and 242 mM, respectively. Vortex immediately after addition and incubate at 30°C for 4 h on rotator (*see* Note 2).

3.2. Viability Measured as Colony Forming Units (cfu)

1. For the untreated control, transfer 100 μ L of culture to a 96-well plate and perform five 1:5 serial dilutions (20- μ L culture is added to 80- μ L ddH₂O and mixed well by pipetting 5–6 times). Then with clean tips plate 5 μ L from each dilution (highest to lowest) onto YPD agar plates.
2. For samples treated for 4 h with acetic acid, perform serial dilutions and plating the same as in **step 1** (*see* Note 3).
3. Incubate agar plates at 30°C for 2 days to read the results (Fig. 1). Note, the difference between WT and Δ *fis1* is greater at 199 mM, while the difference between WT and Δ *dnm1* is greater at 242 mM acetic acid (*see* Note 4).

3.3. Viability Measures by FUN1 Staining Assay

1. After 4 h of acetic acid treatment, 400 μ L of 199 mM acetic acid-treated cultures are mixed with 1-mL of sterile FUN1 staining solution in a 1.5-mL microcentrifuge tube and cells are pelleted at 6000 $\times$ g for 5 min. Cultures treated with 242 mM acetic acid are not usually suitable for FUN1 staining as too many cells are killed to yield reliable viable cell counts.
2. Discard the supernatant and resuspend the cell pellet in 1-mL sterile FUN1 staining solution. Add 0.5 μ L FUN1 cell stain and 5 μ L Calcofluor White M2R to the yeast suspension with final concentrations of 5 μ M and 25 μ M, respectively. Mix thoroughly.
3. Incubate at 30°C in the dark for 30 min.
4. Spin down the yeast pellet and remove all of the supernatant except for ~50 μ L. Resuspend the yeast pellet and trap 1.5- μ L stained yeast suspension between a slide and cover glass and observe on a fluorescence microscope.

¹Not only are equal cell numbers between samples important for calculating cell survival/death, but the metabolic state of the yeast culture to be treated is also critical. We have found that yeast cells in postdiauxic phase are more resistant to death stimuli than log phase cells (not shown). Therefore, all the samples must be grown under the same conditions, diluted from overnight cultures and recovered in fresh media for the same period. When performing a series of experiments, it is recommended to use the same batch of medium, as different lots can produce different growth characteristics. Because individual strains can contain genetic variants, it is also advisable to test several different colonies.

²Although this protocol was developed for BY4741 strains and for YPD medium, other strains and media can be used successfully with some adjustments to the protocol. For synthetic dropout medium (0.67% yeast nitrogen base, 30 mg/L amino acids mixture, 2% glucose/dextrose), the yeast cells will grow somewhat slower and will require a longer time to reach mid-log phase following dilution from overnight cultures. The same strains grown in synthetic medium are somewhat more sensitive to the death stimulus, and usually require a lower dose of acetic acid (e.g., 130–180 mM).

³Serial dilutions used here are a convenient method to reveal large viability differences, but may fail to detect real differences that are less than fivefold (for example, our standard for mammalian cells is only a 20% difference in survival for at least one time-point posttreatment with appropriate reproducibility), though smaller differences are frequently reported. For yeast strains with smaller differences in viability, colony numbers can be counted by plating larger volumes on 60-mm Petri dishes, by plating 1:2 dilutions in the critical range, or by adjusting the dose of acetic acid (Fig. 1).

⁴Other death stimuli can also be used successfully to distinguish these three strains of yeast. Assays conditions that fail to distinguish the survival of *FIS1* and *DNM1* knockouts from their wild-type control may not yield reliable results. Other factors can affect cell death susceptibility, such as age, ploidy, and background strain. For example, older haploid strains are more sensitive than newly sporulated haploid strains.

5. Dead cells stain diffusely green, while healthy cells stain blue and contain a red bar inside (9). Count at least 200 cells from representative fields.

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Fig. 1. Colony formation assay of WT, $\Delta fis1$, and $\Delta dnm1$ strains treated with the indicated doses of acetic acid and plated in 5-fold serial dilutions (left to right).