Chapter 13

Modeling Metazoan Apoptotic Pathways in Yeast

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Abstract

This chapter describes techniques for characterizing metazoan apoptotic pathways using *Saccharomyces cerevisiae*. Active forms of the major apoptotic effectors—caspases, Bax and Bak—are all lethal to yeast. Using this lethality as a readout of caspase/Bax/Bak activity, proteins and small molecules that directly or indirectly regulate the activity of these effectors can be investigated in yeast, and apoptotic inhibitors can be identified using functional yeast-based screens. Caspase activity can also be monitored in yeast by cleavage-dependent liberation of a transcription factor from the plasma membrane, enabling it to activate the lacZ reporter gene. This system can be used to define the sequences that can be efficiently cleaved by particular caspases.

Key words *Saccharomyces cerevisiae*, Yeast, Apoptosis, Caspase, Bcl-2, IAP, Bax, Programmed cell death, BH3-mimetic

1 Introduction

1.1 Researching Cell Death Pathways Using Yeast

For decades, the budding yeast *Saccharomyces cerevisiae* has been one of the most widely used genetic model organisms and a popular molecular biology workhorse. The plethora of established yeast tools and techniques enable metazoan apoptotic pathways to be modeled using this species. Yeast are well suited for researching metazoan apoptotic regulators because they are eukaryotic: in particular their subcellular organelles resemble those of mammals, insect, and nematodes (the organisms whose apoptotic pathways we have usually sought to model). Some researchers have proposed that yeast can undergo a form of cell death that in some ways resembles apoptosis [1]. However, proteins proposed to mediate yeast cell death did not affect the activity of exogenous apoptotic effector proteins from mammals, nematodes, or insects when expressed in yeast [2]; thus yeast can be considered a naïve yet eukaryotic cellular environment in which to reconstitute and study the regulation of metazoan apoptotic pathways.

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This chapter describes two methods we have employed to identify and characterize apoptotic regulators. The first method exploits the observation that yeast die following expression of active forms of the major apoptotic effectors: caspases, Bax and Bak (Fig. 1). Activators of these pro-apoptotic proteins can be

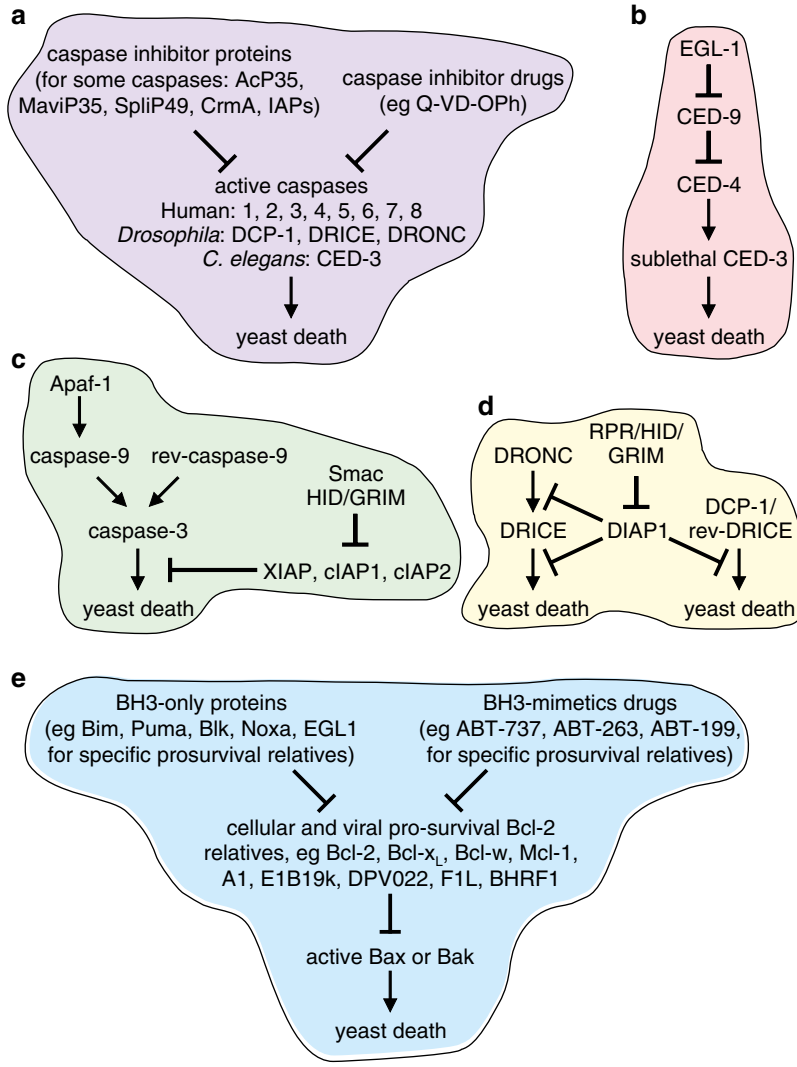


Fig. 1 Reconstitution of apoptotic pathways in yeast. Yeast can be killed by expression of many active caspases, Bax or Bak. This lethality can be exploited to assemble pathways to model the function of upstream regulators of caspase/Bax/Bak activity. (**a**) DCP-1 and human caspases-1, 5, 8 auto-activate in yeast when expressed at low to moderate levels, and these caspases have appropriate specificities to kill yeast. The native forms of other caspases are inactive or weakly active when expressed in yeast. The lethality of caspases-2, 4, DRONC, CED-3 can be enhanced by transforming yeast with multiple expression plasmids bearing different nutritional selection markers. Auto-activating forms of caspases-2, 3, 6, 9 and DRICE can be expressed by reversing the order of the subunits. Auto-activating caspase-3 can also be produced by fusing the caspase to β-galactosidase. Removing an amino-terminal sequence from caspase-7 enhances its lethality. Proteins that inhibit particular

identified by virtue of their ability to activate caspase zymogens or Bax or Bak expressed at sublethal levels. Functional screens of cDNA expression libraries can be carried out to identify inhibitors of caspases, Bax or Bak (or their activators), and antagonists of such inhibitors can also be modeled in this system. The second method was designed to define caspase specificity. Caspase cleavage within the linker of a fusion protein liberates a transcription factor from its membrane tether, so activation of a transcriptional reporter provides evidence that the caspase has the specificity required to cleave that particular linker sequence (Fig. 2).

1.2 Pathways Controlling Caspase-Dependent Yeast Death

Very high level expression of some caspases, such as that achieved using the GAL1/10 promoter, led to their autoactivation and lethality in yeast. Caspase-mediated yeast lethality manifested as loss of plasma and nuclear membrane integrity, mitochondrial function, and clonogenic potential, but did not lead to detectable DNA damage [2]. The GAL1/10 promoter has the useful feature of being induced by galactose and repressed by glucose, so plasmids encoding lethal proteins such as caspases can be transformed into yeast without provoking any toxicity, as long as glucose is supplied (Fig. 3). Raffinose can be used as a "neutral" sugar, allowing immediate induction of genes controlled by the GAL1/10 promoter after addition of galactose, rather than delayed induction following replacement of glucose with galactose. We created a truncated GAL1/10 promoter, which is also sugar-regulated but did not produce as high level transgene expression as the intact promoter [3] (Fig. 3). Other promoters which can be useful in particular contexts include copper-inducible (CUP1) [3], methionine-repressible (MET), and constitutive (ADH) promoters [4] (Fig. 3).

Galactose-inducible expression of mammalian caspases-1, 5, 8, or *Drosophila* DRONC or DCP-1, was lethal to yeast. Caspases-2, -4, -7 and *C. elegans* CED-3 were weakly toxic, but their lethality

Fig. 1 (continued) caspases can protect yeast from the death associated with their expression, as can incubation with the pan-caspase inhibitor drug Q-VD-OPh. (**b**) Sublethal levels of CED-3 can be activated by CED-4 in yeast, triggering death. This lethality can be inhibited by CED-9, unless EGL-1 is also expressed. (**c**) Caspase-9 can be activated in yeast by Apaf-1 or by reversing the order of its subunits ("rev-caspase-9"). A truncated version of Apaf-1 lacking the WD40 domain (Apaf-11-530) activated pro-caspase-9 in vitro without cytochrome-c or dATP [12]. This mutant cooperates efficiently with pro-caspase-9 and -3 to kill yeast; the full-length Apaf-1 protein is less active. Active caspase-9 lacks the specificity to kill yeast, but can proteolytically activate caspase-3 in yeast, leading to yeast death. Caspase-3 activity and death can be inhibited by XIAP and less potently by c-IAP1 and c-IAP2. The protection conferred by these IAPs can be antagonized by Smac/DIABLO or its insect counterparts HID or GRIM. (**d**) The corresponding core insect apoptotic machinery can be reconstituted in yeast, exploiting the observation that low levels of DRONC are insufficient to kill yeast but can activate DRICE, which is highly lethal once activated. (**e**) Yeast can also be killed by expression of Bax or Bak. Pro-survival Bcl-2 family members confer protection from this lethality, and that protection can be neutralized by co-expression of BH3-only proteins or by incubation with BH3-mimetic drugs

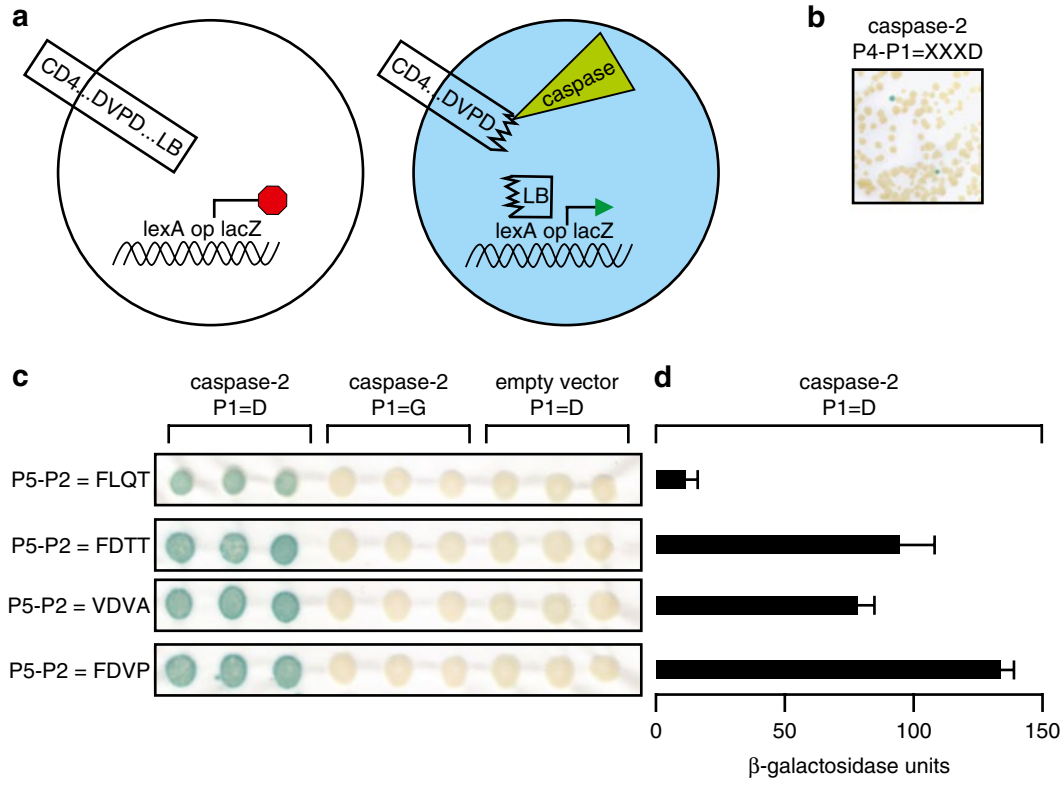


Fig. 2 Analysis of caspase specificity using the transcriptional reporter system. (**a**) A model describing the system. Yeast express a fusion protein in which the LexAB42 ("LB") transcription factor is separated from a membrane anchor by a sequence that may be susceptible to proteolysis by a caspase. If the yeast express an active caspase capable of cleaving this sequence, the transcription factor is liberated from the membrane and can induce expression of the reporter gene lacZ. (**b**) EGY48 yeast bearing the lacZ reporter gene plasmid were transformed with a caspase-2 expression plasmid and a plasmid encoding a fusion protein library with random P4-P2 residues. Induced yeast were stained with Xgal to identify colonies expressing fusion proteins containing caspase-2-sensitive cleavage sites. (**c**, **d**) EGY48 yeast bearing the lacZ reporter gene plasmid were transformed with a caspase-2 expression plasmid and a plasmid encoding a fusion proteins bearing the specified cleavage sites. Suspensions were made of three transformants expressing fusion proteins containing each of the specified P4-P2 residues. (**c**) Five μl of each suspension was spotted on repressing plates and grown for a day then filter-lifted onto inducing agar and stained with Xgal. (**d**) The same clones were grown in liquid inducing medium, lysed by freeze-thawing and β-galactosidase activity was quantitated by ONPG, as a measure of caspase-mediated cleavage at the cleavage sites contained within the fusion proteins (mean +/− SEM from three clones of each type)

could be boosted by increasing the copy number of the plasmids bearing these genes. We favor centromeric yeast expression plasmids bearing the auxotrophic markers *LEU2*, *TRP1*, *HIS3*, or *URA3*. These plasmids confer the ability to survive on media lacking leucine, tryptophan, histidine, or uracil respectively, and they are maintained in yeast at a relatively stable copy number of two to five copies per cell [5]. Transformation of yeast with two or more plasmids bearing with different selectable markers, all encoding the

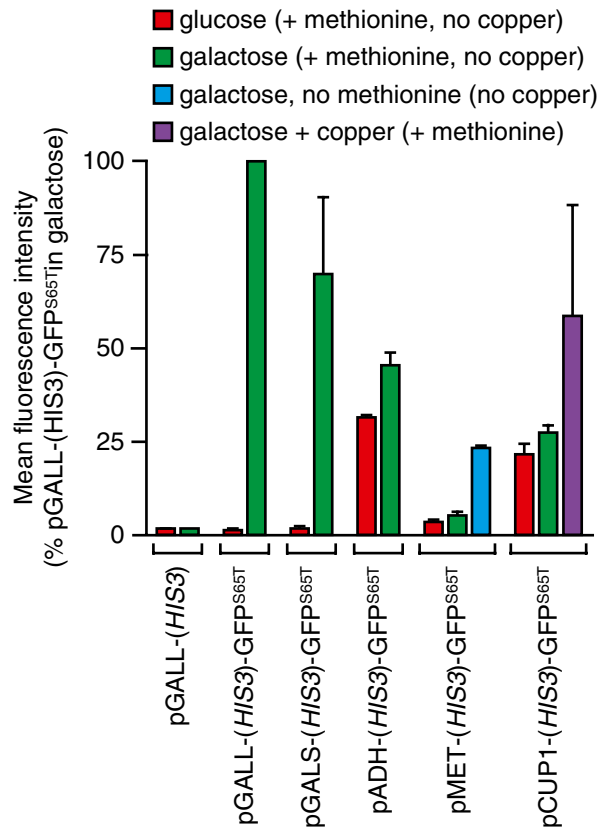


Fig. 3 Vectors for controlling transgene expression in yeast. W303α yeast were transformed with an empty vector or histidine-selectable plasmids engineered to direct expression of GFP^{S65T} under the control of various promoters: an intact GAL1/10 galactose-inducible/glucose-repressing promoter (GALL), a truncated, weaker GAL1/10 promoter (GALS), a constitutive promoter (ADH), a methionine-repressible promoter (MET), or a copper-inducible promoter (CUP1). Transformants were grown in glucose/methionine-containing medium overnight, then washed and grown for 8 h in selective media containing either 2 % glucose or 2 % galactose and containing or lacking 300 μM methionine and/or 100 μM copper sulfate. The fluorescence of each sample was then measured by flow cytometry. Data show the means and SEM from three independent transformants of each type

same weakly lethal caspase, yielded robust caspase-mediated yeast death. Alternatively, higher but more variable plasmid copy numbers (~15–34), and enhanced lethality, could be achieved by expressing caspases using vectors incorporating the "2 μ" origin of replication [6, 7].

Some executioner caspases seemed to possess specificities that enable them to kill yeast once activated, but failed to auto-activate even when expressed at extremely high levels. It was, however, possible to activate these proteases and other weakly lethal caspases, either by providing an upstream activator (e.g., sublethal levels of DRONC activated DRICE to kill yeast) or by reversing

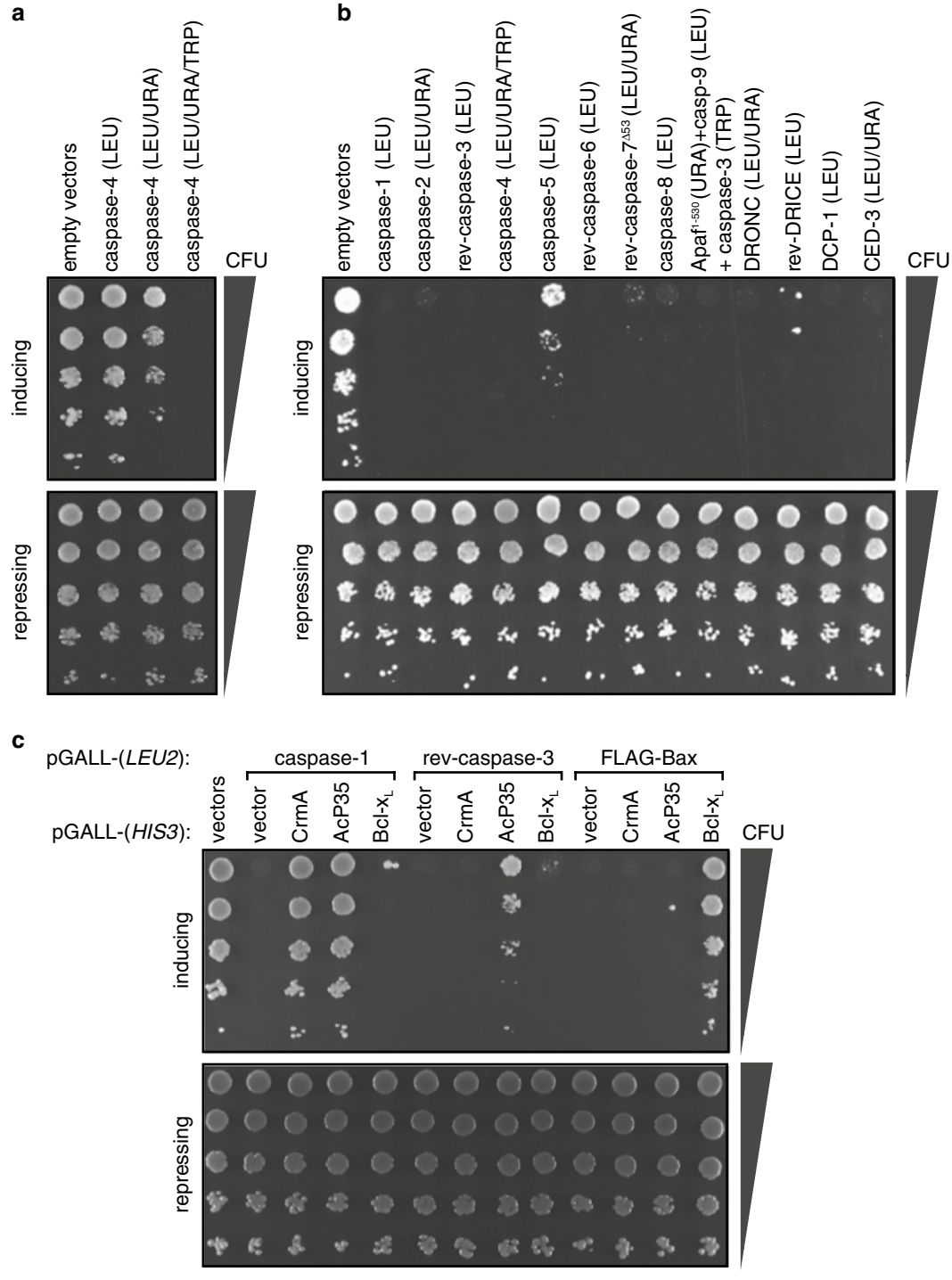


Fig. 4 Active caspases kill yeast. W303α yeast were transformed with centromeric plasmids bearing the specified selectable markers and encoding the specified genes under the control of the GAL1/10 promoter. Transformants were grown in repressing (glucose-containing) selective media, washed, and resuspended in TE at an A_{620} corresponding to 1250 CFU per μl. The suspensions were serially diluted 1:4 in TE and 5 μl of each dilution of each clone was pipetted onto repressing (glucose) and inducing (galactose) plates. Growth was

the order of the caspase subunits. Emad Alnemri pioneered this approach to caspase activation, showing that rearranged caspases in which the small subunit is amino terminal to the large subunit (separated by a cleavage site) mimicked the structure of the active enzyme [8]. We have used this trick to produce auto-activating versions of caspases-2, 3, 6, 7, 9, DRONC and DRICE in yeast [4, 9, 10]. Caspase-3 could also be expressed in an active lethal form by fusing it to β-galactosidase [4]; presumably this promotes aggregation and hence autoactivation.

Human caspase-9 is unusual. It could be forced to auto-activate in yeast by swapping the order of its subunits, or by co-expression of its natural activator Apaf-1, but active caspase-9 did not kill yeast [3, 4], presumably because its restricted P2 specificity [11] prevented it from efficiently cleaving essential yeast proteins. Active caspase-9 could, however, activate pro-caspase-3 or -7 in yeast, leading to death. In this way, it was possible to reconstitute the mammalian apoptosome in yeast. A truncation mutant of Apaf-1 (Apaf-$1^{1\text{-}530}$) that lacks the C-terminal repressive WD40 domain [12] cooperated strongly with caspases-9 and -3 to kill yeast [3, 4]. Presumably the truncation bypasses the need for derepression performed by cytosolic cytochrome-*c* in mammalian cells undergoing apoptosis. Surprisingly, we found that full-length Apaf-1 could also promote yeast death upon co-expression with caspases-3 and -9, although less efficiently than the truncation mutant (data not shown). *S. cerevisiae* cytochrome-*c* was reportedly unable to promote apoptosome activation [13], so it is unlikely that this activity is due to small amounts of cytochrome-*c* leaking from viable yeast mitochondria. Instead, we suspect that a small but evidently sufficient proportion of overexpressed full-length Apaf-1 adopts a conformation that facilitates activation of enough caspase-9 molecules to cleave and activate executioner caspases. The approaches outlined above can be exploited to establish yeast-based assays for monitoring the activity of a large number of caspases from mammals, nematodes, and insects (Fig. 4).

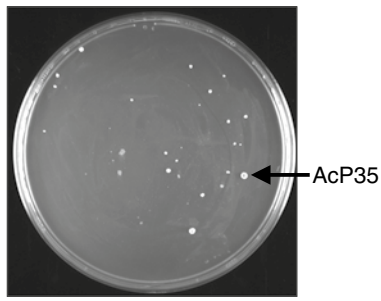
Fig. 4 (continued) photographed after 3 days (galactose plates) or 2 days (glucose plates). Growth on inducing plates indicates survival and proliferation of yeast expressing the transgenes. (**a**) Caspase-4-mediated toxicity is more potent when the protease is expressed from multiple centromeric plasmids. (**b**) Many caspases can be used to kill yeast. The indicated caspases were expressed either in their natural configuration or with the order of the subunits swapped ("rev"). Caspases (and Apaf-1) were expressed from one or more plasmids bearing leucine (LEU), uracil (URA), or tryptophan (TRP) selectable markers. Empty vectors were included in transformations as required to enable all clones to grow on media lacking all three nutrients. (**c**) Yeast death induced by caspases or Bax can be prevented by co-expression of inhibitors. Yeast bearing leucine-selectable plasmids directing galactose-inducible expression of either empty vector, caspase-1, rev-caspase-3, or FLAG-Bax (or empty vector) were transformed with histidine-selectable, galactose-inducible plasmids encoding either the caspase-1/8 inhibitor CrmA, the broad spectrum caspase inhibitor AcP35, or the Bax/Bak inhibitor Bcl-x_L

Active lethal caspases could be prevented from killing yeast by co-expression of cellular or viral proteins that can inhibit their enzymatic activity. We have used this approach to test the activity and specificity of candidate caspase inhibitors, including p35 family members from insect viruses [14–16] (Fig. 4). This technique can be adapted to screen cDNA expression libraries to identify proteins that inhibit particular caspases, allowing the yeast to survive and form colonies despite expressing the caspase ([3], Fig. 5). It can also be used to visualize caspase inhibition by small molecules such as Q-VD-OPh [17].

a

Plasmid(s) encoding pro-apoptotic proteins	RNA source for library	Inhibitor identity, accession number (verified in vitro?)
pGALL-(*LEU2*)-DCP-1	*Drosophila* embryo	DIAP1 (yes)
pYX143 KAS caspase-3-lacZ	human glioma	c-IAP1 (yes)
pGALL-(*URA3*)-DRONC + pGALS-(*LEU2*)-DRONC + pGALL-(*TRP1*)-DRICE	*Ac*MNPV-infected Sf21 cells	AcP35 (yes) 63% identical to XP_004926257.1 (no) EIF3p40, BAM17757.1 (no)
pGALL-(*URA3*)-DRONC + pGALS-(*LEU2*)-DRONC + pGALL-(*TRP1*)-DRICE	*Ld*MNPV-infected Sf21 cells	none (only 45% library screened)
pGALL-(*LEU2*)-CED-3 validated versus pGALS-(*LEU2*)-CED-3 + pMET1-(*URA3*)-CED-4	*C. elegans*	F49E8.2, NP_001255303.1 (no) BRP-1, NP_001255152.1 (no)

b



inducing

Fig. 5 Isolation of caspase inhibitors by functional screening of cDNA expression libraries in yeast. (**a**) RNA extracted from the specified sources was used to create galactose-inducible yeast expression libraries. W303α yeast bearing the specified caspase expression plasmids were transformed with these libraries and plated onto inducing media. Only yeast that acquired a library plasmid that encoded a caspase inhibitor would be expected to survive and form colonies. Library plasmids were isolated from these colonies, amplified in bacteria, and re-transformed into yeast to verify that they protected yeast from caspase-dependent death. The right column shows the identities of these "yeast-validated" candidate inhibitors. Those that have been confirmed to inhibit caspases in vitro are noted. (**b**) The AcMNPV/Sf21 library was screened for DRONC/DRICE inhibitors. A transformation plate is shown: the indicated colony bore a library plasmid encoding the pan-caspase inhibitor AcP35. The authors thank Rollie Clem for the RNA that was used to make the AcMNPV and LdMNPV libraries and Michael Hengartner for the RNA to make the *C. elegans* library.

1.3 Pathways Controlling Bax- or Bak-Dependent Yeast Death

We have not investigated Bax/Bak-mediated yeast death in as much detail as caspase-dependent killing, but others have reported that expression in yeast of active forms of Bax and Bak leads to loss of mitochondrial potential and release of cytochrome-*c* (reviewed by [18]). We observed that addition of amino-terminal tags to Bax and Bak, particularly the human orthologues, significantly boosted their lethality (Fig. 6). This probably reflects the tag's ability to encourage externalization of the amino-terminal α-helix, bypassing an early Bax/Bak activation step [19]. Co-expression of pro-survival proteins inhibited Bax or Bak from killing yeast (Figs. 4 and 7), and this protection could be alleviated by co-expression of BH3-only proteins, or exposure to BH3-mimetic drugs [17] (Fig. 7). Direct activation of Bax or Bak by BH3-only proteins could also be modeled, by expressing sublethal levels of Bax or Bak in the presence or absence of the potential direct activator. In this way, we observed that Puma enhanced killing by sublethal levels of Bax expressed from the methionine-repressible promoter (data not shown).

1.4 Transcriptional Reporter System for Testing Caspase Specificity

We developed a yeast-based system for detecting protease-mediated cleavage of an engineered protein substrate [3]. The substrate has three parts: a portion of the human CD4 protein encompassing the transmembrane domain, a linker region containing potential cleavage site(s), and a transcription factor (LexA-B42). When intact, the transcription factor is tethered to the plasma membrane, and cannot stimulate expression of a reporter gene like lacZ.

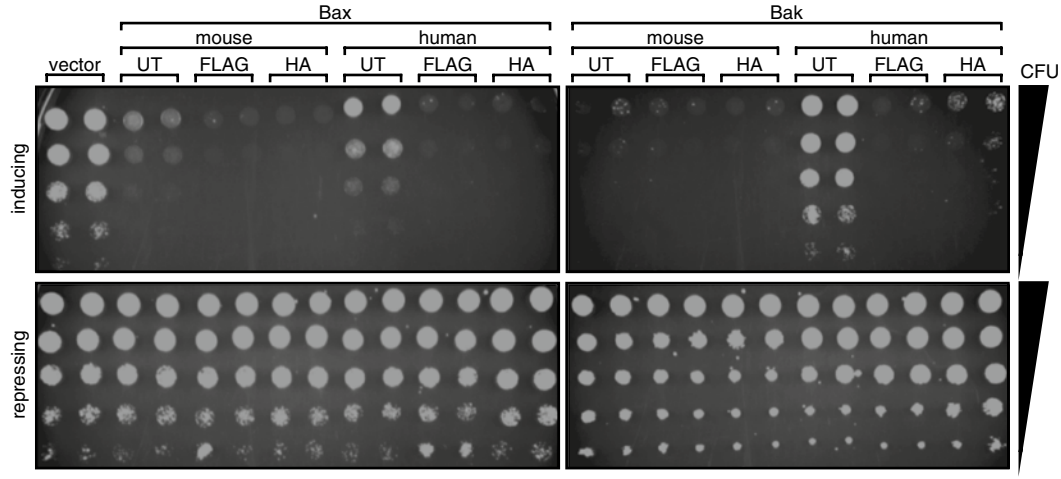


Fig. 6 Yeast growth is impaired by expression of Bax or Bak, and lethality is enhanced by N-terminal tags. W303α yeast were transformed with an empty vector or galactose-inducible plasmids encoding murine or human Bax or Bak; either untagged (UT) or tagged at the amino terminus with FLAG or HA epitopes. Spotting was performed as detailed in the legend to Fig. 4

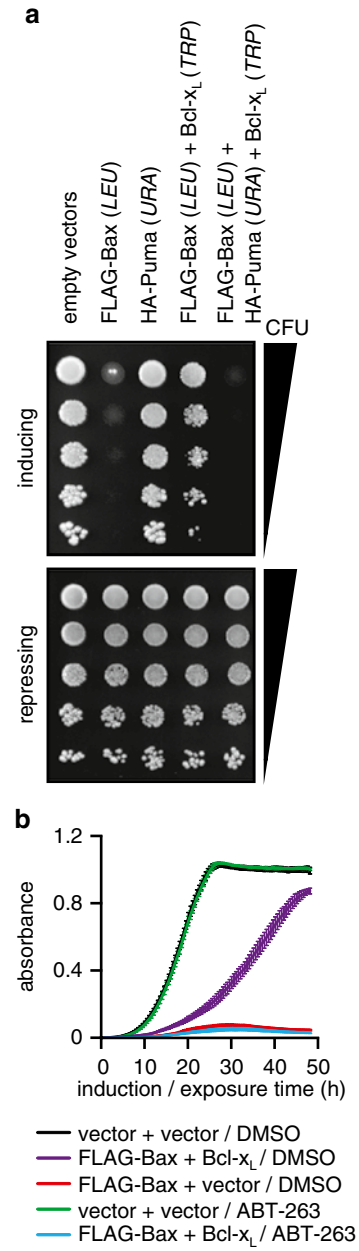


Fig. 7 Antagonism of pro-survival Bcl-2 proteins by BH3-only proteins and BH3-mimetics can be modeled in yeast. (**a**) FY1679-28C yeast were transformed with plasmids encoding the listed Bcl-2 family members. Spotting onto inducing and repressing media was performed as described in the legend to Fig. 4. (**b**) Three independent transformants bearing galactose-inducible plasmids encoding the specified proteins were grown in raffinose-containing selective medium then transferred into medium containing galactose and either 10 μM ABT-263 in DMSO or the equivalent concentration of DMSO. The absorbance of each culture was measured every 0.5 h for 48 h. Error bars indicate standard errors of the means from analyses of three independent clones of each type

However, if a caspase is co-expressed, and the linker region of the chimeric protein contains a sequence amenable to proteolysis by the caspase, cleavage liberates the transcription factor from the membrane, allowing it to access the nucleus and trigger expression of the reporter gene (Fig. 2). This system could be employed to screen expression libraries for proteases with particular specificities, but we have mainly used it to define the minimal specificity of selected caspases [20, 21]. For this application, we generated fusion protein libraries bearing random residues in the linker separating the transmembrane domain from the transcription factor. Yeast bearing an expression plasmid encoding the caspase of interest are transformed with such a library, and the resulting transformants are assayed (using X-gal or ONPG) for expression of the reporter gene (lacZ), which indicates that the fusion protein encoded by the library plasmid can be efficiently cleaved by the caspase. Sequencing the linker region of genes encoding the library fusion proteins from blue ("positive") and white ("negative") clones defines cleavable and noncleavable sites for that caspase.

As explained above, many caspases are toxic to yeast, so in this system the caspase is expressed in a galactose-dependent manner using the GAL1/10 promoter described above. Transformants are initially grown on media containing glucose to repress expression, then transferred to a filter (recording the orientation of the filter relative to the agar plate). The filter is then incubated on a plate containing galactose, to induce expression, then stained with Xgal to identify the clones that contain a caspase and fusion protein with a cleavage-sensitive site. The corresponding transformants can be recovered from the glucose-containing plate for further characterization. To more quantitatively analyze reporter gene expression, indicating cleavage efficiency, the β-galactosidase substrate ONPG can be used.

2 Materials

2.1 Yeast Strains

1. Experiments involving caspase or Bax/Bak-dependent death have predominantly used the yeast strain W303α (MATα; can1-100; leu2-3,-112; his3-11,-15; trp1-1; ura3-1; ade2-1).
2. The following strains and their derivatives have also been used for particular studies exploring the impact of mutations in genes associated with yeast cell death or drug transport on caspase/Bax/Bak lethality: BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3 Δ0); FY1679-28C (MATa, ura3-52, trp1Δ63, leu2Δ1, his3Δ200, GAL2+).
3. The transcriptional reporter gene assays for detecting caspase cleavage employ EGY48 (MATa, ura3, trp1, his3, LexAop6-LEU2).

2.2 Plasmids

1. pSH18-34 (Invitrogen). This is a uracil-selectable plasmid in which the lacZ gene is located downstream of eight lexA operator sequences, enabling lexA-dependent β-galactosidase expression.
2. The methods described below involve expressing apoptotic regulators in yeast using centromeric, galactose-inducible yeast plasmids bearing nutritional markers: pGALL-(*LEU2*), pGALL-(*TRP1*), pGALL-(*HIS3*), and pGALL-(*URA3*). These constructs were derived from the pRS31X series of plasmids [5] by introduction of the GAL1/10 promoter, a polylinker, and the actin terminator [3]. As mentioned above, similar vectors have also been created containing different promoters. All plasmids are available upon request.

2.3 Media

1. 10× glucose: 20 % (w/v) glucose. Dissolve 100 g of glucose in 300 ml of water, add water to 500 ml. Filter sterilize through a 0.2 μm filter into a sterile bottle. Store at room temperature.
2. 10× galactose: 20 % (w/v) galactose. Dissolve 100 g of galactose in 300 ml of water, add water to 500 ml. Filter sterilize through a 0.2 μm filter into a sterile bottle. Store at room temperature.
3. 10× raffinose: 20 % (w/v) raffinose. Dissolve 100 g of raffinose in 300 ml of water, add water to 500 ml. Filter sterilize through a 0.2 μm filter into a sterile bottle. Store at room temperature.
4. YPglc/gal (complete liquid media): Dissolve 10 g peptone and 5 g yeast extract in 450 ml water. Autoclave for 15 min on wet cycle. Add 50 ml of 10× glucose or galactose (above) while still hot. Store at room temperature.
5. YPglc/gal agar (complete solid media): Dissolve 10 g peptone and 5 g yeast extract in 450 ml water. Add 10 g agar. Autoclave for 15 min on wet cycle. Store at room temperature. Melt in a microwave on low power, remove the required volume, and add 1/10th volume of 10× glucose or galactose before pouring into plates.
6. 10× dropout solution. Dissolve 3.5 g dropout supplement lacking leucine, tryptophan, histidine, and uracil (Sigma #Y2001) in 500 ml water. Filter sterilize through a 0.2 μm filter into a sterile bottle. Store at 4 °C.
7. 100× nutrient supplements: Dissolve 300 mg of leucine or 200 mg of tryptophan, histidine, or uracil in 100 ml of water. Filter sterilize through a 0.2 μm filter into a sterile bottle. Store at 4 °C.

8. Minimal liquid media. Dissolve 3.4 g of yeast nitrogen base without amino acids (BD#291940) in 400 ml of water. Add 50 ml of 10× dropout solution. Filter sterilize through a 0.2 μm filter into a sterile bottle. Store at 4 °C. Add 1/100th volume of 100× nutrient supplement(s) and 1/10th volume of 10× glucose, galactose, or raffinose as required.
9. Minimal agar media. Dissolve 3.4 g of yeast nitrogen base without amino acids (BD#291940) in 400 ml of water. Add 10 g of agar. Autoclave for 15 min on wet cycle. Store at room temperature. Melt in a microwave on low power and remove the required volume. Add 1/100th volume of 100× nutrient supplement(s), 1/10th volume of 10× dropout solution, and 1/10th volume of 10× glucose or galactose as required, before pouring into plates.

2.4 Other Reagents

1. Single stranded (carrier) DNA. Purchase fish sperm DNA (e.g., Roche #11467140001) and aliquot 5 ml per tube into multiple 10 ml tubes and freeze. Thaw one tube and denature DNA by heating at 98 °C for 15 min. Chill on ice for 10 min and aliquot 500 μl per tube. Store at −20 °C.
2. 10× LiAc: 1 M Lithium acetate. Dissolve 10.2 g Lithium acetate dihydrate in 90 ml of water. Add water to 100 ml. Autoclave 15 min on wet cycle.
3. 10× TE: 100 mM Tris–HCl pH 7.5, 10 mM EDTA. Dissolve the following in 90 ml of water: 1.2 g Tris, 0.37 g ETDA. Adjust the pH to 7.5 with HCl. Add water to 100 ml. Autoclave 15 min on wet cycle.
4. 1× LiAc/TE: 0.1 M lithium acetate, 10 mM Tris–HCl pH 7.5, 1 mM EDTA. Mix 50 ml 10× LiAc, 50 ml of 10× TE with 400 ml water. Autoclave 15 min on wet cycle.
5. TE: 10 mM Tris–HCl pH 7.5, 1 mM EDTA. Mix 50 ml 10× TE with 450 ml water. Autoclave 15 min on wet cycle.
6. 50 % PEG3350: Mix 250 g Poly(ethylene glycol)3350 in 500 ml of water until dissolved (this takes a few hours). Autoclave 15 min on wet cycle. Store at room temperature.
7. PEG/LiAc/TE: 40 % PEG3350, 0.1 M lithium acetate, 10 mM Tris–HCl pH 7.5, 1 mM EDTA. Into a sterile 50 ml tube, pipette 5 ml of 10× LiAc and 5 ml of 10× TE, and pour (sterilely) 40 ml of 50 % PEG. Mix (e.g., on rocker) for ~1 h.
8. Hybond-N+ nylon membrane: either purchase circular filters or cut to match the dimensions of the petri dishes
9. Xgal (5-Bromo-4-chloro-3-indolyl β-d-galactopyranoside): 20 mg/ml Xgal in dimethylformamide. Dissolve 100 mg of Xgal in 5 ml of dimethylformamide. Store at −20 °C protected from light.

10. 4 mg/ml ONPG (o-Nitrophenyl β-d-galactopyranoside). Dissolve 100 mg in 25 ml of Z buffer (below). Filter sterilize through a 0.2 μm filter into a sterile bottle. Store at −20 °C.
11. Z buffer: 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0. Dissolve 4.3 g of Na_2HPO_4, 4.8 g of NaH_2PO_4, 0.74 g of KCl, and 0.12 g of $MgSO_4$ in 900 ml of water. Adjust the pH to 7.0 and add water to 1 L. Autoclave 15 min on wet cycle. Store at room temperature. Just before performing assays, add 2.7 μl β-mercaptoethanol and *either* 15 μl of 20 mg/ml Xgal *or* 225 μl of 4 g/ml ONPG per ml of Z buffer.
12. 1 M Na_2CO_3.
13. Lysis buffer for plasmid DNA extraction: 2 % (v/v) Triton X-100, 1 % SDS (w/v), 100 mM NaCl, 10 mM Tris–HCl pH 8, 1 mM EDTA pH 8. Store at room temperature.
14. Phenol/chloroform/isoamyl alcohol, 25:24:1 (v:v:v). Store at 4 °C protected from light.
15. Glass beads (~500 μm diameter).
16. 3 M NaOAc pH 5.2. Store at room temperature.

2.5 Equipment

1. These methods require a plate-reading spectrophotometer. For Subheading 3.4, it must be able to be programmed to perform cycles of shaking for ~2 min, followed by reading absorbance at 600–620 nm, followed by incubation at 30 °C. This (or some equivalent arrangement) is necessary for obtaining absorbance measurements of resuspended yeast every 30 min for up to 48 h.
2. Shaking incubator that can be set at 30 °C, 230 rpm.
3. Plate incubator that can be set at 30 °C.
4. Microfuge that can be set to 14,000 × *g*, 4 °C.

3 Methods

3.1 Lithium Acetate Transformation

This is a common protocol for transforming *S. cerevisiae*, which is based on a method published by Ito et al. [22]. The quantities specified below are sufficient for ten transformations, but can be scaled-up if required.

1. Streak the yeast strain from a glycerol stock frozen at −80 °C onto a YPglc plate (if using a parental strain) or the appropriate selective agar plate containing glucose (if the strain already harbors a vector, such as a caspase or Bax/Bak expression plasmid).
2. After 2 or 3 days, when good-sized colonies have grown, inoculate a colony into 5 ml of liquid media containing glucose and grow, shaking at around 230 rpm, at 30 °C for ~18 h (*see* **Note 1**).

3. Expand the yeast into 50 ml of YPglc and grow, shaking for another 4–5 h.
4. Pellet the yeast by centrifuging for 5 min at 4000 × *g*.
5. Resuspend pellet in 25 ml 1×LiAc/TE, spin again (5 min, 4000 × *g*).
6. Resuspend pellet in 1 ml 1×LiAc/TE.
7. In sterile 1.5 ml tubes, prepare the DNA mixtures to be transformed. Pipette 10 µl of ssDNA (of a 10 mg/ml stock) into each tube, and add ~1 µg of each plasmid (*see* **Note 2**). To screen a cDNA library for inhibitors, transform a clone bearing the caspase/Bax/Bak plasmid with 8 µg of the library DNA spread across eight separate transformations. Also perform control transformations with empty vector and (if available) a plasmid encoding a known inhibitor (e.g., AcP35 for most caspases, Bcl-x_L for Bax/Bak).
8. Add 100 µl of yeast suspension to each tube.
9. Add 600 µl of pre-mixed PEG/LiAc/TE to each tube.
10. Invert ~5 times to mix.
11. Incubate at 30 °C for 30 min.
12. Add 70 µl of DMSO to each tube.
13. Invert ~5 times to mix.
14. Incubate at 42 °C for 15 min.
15. Pellet the yeast by centrifuging for 15 s at 14,000 × *g*. If transforming a single plasmid with a defined insert, resuspend the pellet in 70 µl of TE and spread the yeast onto a 10 cm selectable repressive plate (*see* **Note 3**). If transforming a cDNA library, merge the resuspended yeast transformed with the library, plate 1 % and 10 % on 10 cm repressing selective plates (to determine the transformation frequency) and the remainder onto three 15 cm inducing selective plates. Plate the control transformations onto 10 cm inducing selective plates.
16. Incubate the plates at 30 °C for 2–3 days (glucose plates) or 4–7 days (galactose plates).
17. Use Subheading 3.2 to extract library plasmid DNA from library screening plates to validate and identify candidate inhibitors, or use Subheading 3.3 to visualize the impact of the transgene(s) on yeast survival and growth.

3.2 Extracting Library Plasmid DNA from Yeast

1. Grow each of the transformants whose library plasmids are to be extracted for 12–18 h in 1 ml of selective liquid media containing glucose (or galactose, if isolating caspase/Bax/Bak inhibitors), shaking, at 30 °C.
2. Pellet the yeast by centrifuging for 15 s at 14,000 × *g*.

3. Resuspend in 1 ml of YPglc and grow, shaking, for 4–5 h (this rapid growth leads to weaker cell walls and better lysis).
4. Pellet the yeast by centrifuging for 15 s at 14,000 × *g*, discard supernatant.
5. Add a roughly equal volume of glass beads to the tubes and give them a quick spin to ensure all the beads are at the bottom. Samples can be frozen at this point if necessary.
6. Add 500 µl of lysis buffer to the tubes and thoroughly resuspend pellets.
7. Add 500 µl of phenol/chloroform/isoamyl alcohol to each tube.
8. Make sure the lids are securely closed and wear gloves. Vortex at top speed for 2 min.
9. Centrifuge for 3 min at 14,000 × *g*.
10. Take top layer to a fresh tube, add 50 µl of 3 M NaOAc and 500 µl of isopropanol. Mix by inversion a few times.
11. Precipitate DNA by spinning for 15 min, 14,000 × *g* at 4 °C
12. Gently discard supernatant.
13. Gently pipette 1 ml of ice-cold 70 % ethanol into each tube.
14. Centrifuge for 1 min at 14,000 × *g* then gently discard supernatant.
15. Spin again for 15 s, use a yellow tip to remove remaining liquid.
16. Place tubes in 42 °C heatblock for 2 min with lids open, to evaporate residual ethanol (don't leave longer than this as overdried DNA is difficult to dissolve).
17. Resuspend the pellet in 200 µl of TE, leave at 42 °C for 1–2 min (lids closed) to completely dissolve DNA.
18. Electroporate 2 µl into electrocompetent bacteria (*see* **Note 4**), plate on ampicillin-containing media.
19. Perform a standard "miniprep" procedure to extract DNA from the bacteria for transformation into yeast to confirm the inhibitor activity, and for sequencing.

3.3 Visualizing Caspase/Bax/Bak Activity and Inhibition in Yeast, by Semi-Quantitative Spotting onto Agar Plates

1. Grow each of the transformants to be tested for 12–18 h in 1 ml of selective repressive liquid media, shaking, at 30 °C. These transformants may, for example, bear a leucine-selectable plasmid encoding caspase/Bax/Bak plus a second histidine-selectable plasmid either lacking an insert or encoding a candidate inhibitor. In all assays, be sure to also include a transformant containing empty vectors bearing the appropriate selectable markers: these controls will indicate the amount of yeast growth expected on galactose-containing media if the caspase/Bax/Bak is completely inhibited.

2. Pellet the yeast by centrifuging for 15 s at 14,000 × *g*.
3. Resuspend in 500 µl TE and re-pellet.
4. Resuspend in 500 µl TE, mix 50 µl of each yeast suspension with 150 µl of water in separate wells of flat-bottomed clear 96-well plates, and measure absorbance at 620 nm (A_{620}) using a plate-reading spectrophotometer. (The yeast cell walls prevent lysis in water in the time required to read absorbance).
5. Use a sterile 96-well plate to prepare serial dilutions of each yeast clone to be analyzed: each clone in a separate column. In the top row, add the appropriate volume of each yeast suspension to achieve 250,000 colony forming units (CFU) in 200 µl of TE (*see* **Note 5**).
6. Pipette 160 µl of TE into rows 2–5 of the plate. Prepare serial fivefold dilutions of the yeast suspensions by taking 40 µl from the first row into the second, mixing, and then taking 40 µl from the second row into the third, and so on. Multichannel pipettors make this step a lot easier and quicker.
7. While the yeast are still in suspension, carefully pipette 5 µl from each well onto both a selective repressing plate (containing glucose) and also onto a selective inducing plate (containing galactose). This will result in 6250 CFU of each clone being "spotted" in the first row, and fivefold fewer being spotted in each subsequent row (*see* **Note 6**).
8. Leave the plates puddle-side facing up, with lids ajar, on the bench for ~15 min, to allow much of the liquid to soak into the plate and evaporate. Alternatively, if a laminar flow hood is available, leave the plates in it for ~5 min with the lids completely off.
9. Incubate the plates at 30 °C for 2 days (repressing glucose plate) or 3–4 days (inducing galactose plate). Figure 4 shows typical results from this kind of assay.

3.4 Visualizing Activity of BH3-Mimetics or Small Molecule Caspase Inhibitors in Yeast

This method uses optical density as a measure of yeast survival and propagation. **Steps 1–4** are the same as those for the spotting assay described above.

1. Grow each of the transformants to be tested (including the empty vector control) for 12–18 h in 1 ml of selective repressive liquid media. It is useful to assay at least three transformants bearing each plasmid combination, to determine the extent of clone-to-clone variability.
2. Pellet the yeast by centrifuging for 15 s at 14,000 × *g*.
3. Resuspend in 500 µl TE and re-pellet.
4. Resuspend in 500 µl TE, mix 50 µl of each yeast suspension with 150 µl of water in separate wells of flat-bottomed clear 96-well plates, and measure absorbance at 620 nm (A_{620}) using a plate-reading spectrophotometer.

5. Pipette 150 µl of minimal media containing 2 % raffinose (no glucose, no galactose) per well into as many wells of a sterile 96-well plate as the clones to be assayed.
6. Pipette 250,000 CFU of each yeast suspension (*see* **Note 5**) into the corresponding well of the 96-well plate.
7. Incubate, shaking at 30 °C for 5 h.
8. Towards the end of the incubation, prepare a second sterile clear, flat-bottomed, 96-well plate with the media +/− drugs to be tested. Into each well, pipette 150 µl of minimal media containing galactose plus either the BH3-mimetic drug (at one or more concentrations) or equivalent amount of solvent. It is important to measure the growth of each clone in media containing and lacking the drug. One convenient option is to arrange the plate so each clone occupies a column, and each row contains either just solvent or defined concentrations of the drug(s) in a constant concentration of solvent. Do not use the outer wells of the plate: instead pipette 150 µl of TE into the wells surrounding the samples to minimize evaporation. Equilibrate this plate at 30 °C.
9. Use a multichannel pipettor to resuspend the yeast growing in the first plate. Transfer 10 µl of each clone into the appropriate wells of the second plate containing media +/− drugs.
10. Place the plate into the plate-reading spectrophotometer programmed to maintain 30 °C and shake for 2 min then take readings at A_{620} every 0.5 h for up to 48 h.
11. Plot the absorbance of each sample relative to time, to visualize the effect of the transgenes and drugs on yeast growth.
12. Figure 7 shows examples of this kind of data.

3.5 Defining the Minimal Specificity of a Protease Using Yeast

This method uses Xgal staining to visualize reporter gene activity, as a readout of caspase-mediated fusion protein cleavage. It can be used as described below to identify proteins containing a caspase-sensitive cleavage site, and can easily be adapted to quickly test caspase-mediated cleavage of particular fusion proteins engineered to bear particular sequences between the CD4 and lexAB42 domains. Some indication of cleavage efficiency can be gleaned from the intensity of the blue color produced due to Xgal cleavage by β-galactosidase, and/or the speed with which that blue color is observed. For a more quantitative assessment of β-galactosidase activity, and hence caspase cleavage efficiency, the liquid ONPG assay outlined in Subheading 3.6 can be used.

1. Create or obtain (e.g., from us) a plasmid encoding a fusion protein containing the transmembrane portion of CD4 and the lexAB42 transcription factor, separated by a linker containing nucleotides encoding the sequence XXXD, under the con-

trol of the GAL1/10 promoter. We create these libraries using redundant oligonucleotides bearing NNS codons.

2. Create or obtain a second galactose-inducible plasmid encoding an active form of the caspase or other protease of interest.
3. Transform the EGY48 yeast strain with a caspase expression plasmid and pSH18-34 (a uracil-selectable plasmid encoding lexA-inducible β-galactosidase), using Subheading 3.1 (*see* **Note 7**).
4. Plate onto selectable repressing media and incubate at 30 °C for 2–3 days.
5. Transform the "XXXD" library into a transformant clone. Plate 90 % of the transformation onto one 15 cm selectable repressing plate and 10 % onto a second selective repressing plate, and incubate both for 2–3 days.
6. Prepare Xgal-drenched 3MM paper: cut two pieces of 3MM paper to fit inside a 15 cm petri dish. Pour 10 ml of Z buffer containing β-mercaptoethanol and Xgal into an empty 15 cm petri dish. Put the 3MM papers into the plate, avoiding bubbles. Remove the excess liquid by pipetting.
7. Carefully lay a nylon filter over a transformation plate bearing nicely separated colonies. Try to avoid dragging the filter, as this will smear the yeast.
8. Dip a 22 gauge needle in a colored dye (e.g., agarose gel loading dye, Coomassie blue stain) and punch asymmetrically located holes through filter into the agar. These colored holes will enable colonies on the Xgal-stained filter to be subsequently aligned with the corresponding colonies on the plate.
9. Use two pairs of forceps on opposite sides of the filter to carefully lift it off the plate without smearing.
10. Place the filter into a bath of liquid nitrogen to freeze, then remove and lie on a petri dish lid to thaw.
11. Transfer the filter onto the Xgal-soaked 3MM papers.
12. Wrap the petri dish with plastic wrap and incubate at 37 °C for 1–8 h. If/when colonies stain blue, align the filter with the transformation plate to identify the corresponding colonies.
13. Streak out positive colonies and repeat staining, to process a single clone. Extract library plasmid DNA (Subheading 3.2) for further analysis and to define the caspase-cleavable sequence.

3.6 Quantitative Assessment of β-Galactosidase Activity, as a Readout of Caspase Cleavage Efficiency

Although more quantitative than the Xgal-based method described above, this approach is less sensitive.

1. Grow each of the transformants to be tested for ~18 h in 1 ml of selective repressive liquid media. It is useful to assay at least three transformants bearing each plasmid combination, to determine the extent of clone-to-clone variability.

2. Pellet the yeast by centrifuging for 15 s at 14,000 × *g*.
3. Resuspend in 500 µl of TE and re-pellet.
4. Resuspend in 500 µl of TE, mix 50 µl of each yeast suspension with 150 µl of water in separate wells of flat-bottomed clear 96-well plates and measure A_{620} using a plate-reading spectrophotometer.
5. Based on the A_{620} readings, pellet all of the most dilute suspension and smaller volumes of the more concentrated suspensions, so roughly equal numbers of yeast are collected for each clone.
6. Resuspend each pellet in 1 ml of inducing selective medium.
7. Incubate, shaking at 30 °C for 10–24 h.
8. Pellet the yeast by centrifuging for 15 s at 14,000 × *g*.
9. Resuspend each sample in 400 µl Z buffer and measure and record A_{620}.
10. Pipette 100 µl into a fresh 1.5 ml tube and freeze at −80 °C from 15 min to overnight.
11. Prepare Z buffer containing β-mercaptoethanol and ONPG and equilibrate to 30 °C.
12. Thaw tubes to be analyzed in a 37 °C waterbath or heatblock (*see* **Note 8**).
13. Add 700 µl Z/β-ME/ONPG buffer to each tube. Note the time.
14. Incubate each tube at 30 °C until yellow color develops, up to 3 h.
15. For each tube, when distinct yellow color is visible, add 400 µl of 1 M Na_2CO_3 to stop the reaction and record the time that elapsed between resuspending in ONPG buffer (i.e., **step 12**) and adding Na_2CO_3.
16. Pellet the yeast by centrifuging for 5 min at 14,000 × *g*.
17. Measure A_{414} of each sample's supernatant.
18. Express β-galactosidase activity according to the formula:

$$\frac{2500 \times A_{414}\,(\text{step } 16)}{\text{time till yellow}\,(\text{seconds}) \times A_{620}\,(\text{step } 8)}$$

4 Notes

1. Good aeration is important, so use vented flasks or only fill closed containers (e.g., tubes) to 10 % or less of their capacity.
2. It is possible to simultaneously transform multiple plasmids with different selectable markers. The transformation efficiency does decrease, however: this method reproducibly yields

hundreds of colonies when two plasmids are co-transformed, and usually tens of colonies when three plasmids are transformed. Quadruple transformation are often unsuccessful, however. If three or four plasmids must be present, it would be advisable to perform sequential transformations.

3. If one or two plasmids are being transformed, it is often not necessary to plate the entire transformation mixture (unless it is crucial to maximize the numbers of transformants, as when screening libraries). To save time in this context, after the heat-shock step be careful not to mix the contents of the tube and simply plate 70 µl of the mixture from the bottom of the tube (most of the yeast will have settled there) directly onto the plate.
4. "Hit" clones from library screens would usually contain at least two plasmids, for example a leucine-selectable plasmid encoding a caspase and a histidine-selectable library-derived plasmid encoding a putative caspase inhibitor. We have used three techniques to specifically isolate the library plasmids: (A) DNA extracted from the yeast can be transformed into the KC8 strain of *E. coli* (Clontech), which harbors auxotrophic leuB, trpC, and hisB mutations that can be complemented by the *LEU2*, *TRP1*, and *HIS3* genes present on the yeast expression plasmids described above. KC8 cells transformed with DNA from yeast clones can be plated onto ampicillin-containing M9 minimal media lacking the appropriate amino acid, to select for bacterial transformants that acquired only the library plasmids (and not those encoding the caspase, for example). (B) DNA isolated from yeast can be transformed into any competent *E. coli* strain and plated onto complete media containing ampicillin, then colony-PCR can be performed using primers that anneal to sequences uniquely present in the library plasmid (e.g., within the *HIS3* gene). (C) We introduced the kanamycin-resistance cassette from pDORR221 (Invitrogen) into a restriction site located within the β-lactamase gene of pGALL-(*LEU2*) plasmid, thereby creating a plasmid that was leucine-selectable in yeast, and kanamycin-selectable (but not ampicillin-selectable) in bacteria. To screen for caspase inhibitors, caspase genes were subcloned into this vector, and cDNA libraries were constructed in the histidine/ampicillin-selectable pGALL-(*HIS3*) plasmid. This strategy enables simple isolation of library plasmids encoding potential caspase inhibitors, by transforming bacteria with yeast DNA then plating on media containing ampicillin.
5. Determine empirically the relationship between CFU and absorbance, use the plate-reading spectrophotometer you plan to use for these assays. Measure the A_{620} of a suspension of yeast bearing one or more plasmids, create around six serial 1:4

dilutions in TE and plate 70 μl of each onto selective repressing plates. Count the colonies on plates where this is possible, to determine the relationship between CFU and A_{620}.

6. To produce neat "spottings," position the tip just above the surface of the plate and slowly depress the pipettor to release the liquid: try not to gouge the agar. It can be helpful to place a template (we use a piece of paper marked with dots corresponding to the positions of the wells), under the agar plate. If this step is not done quickly, the yeast will settle to the bottom of the wells. They can be easily resuspended using a multichannel pipette if necessary. Spotting assays work best when the plates are relatively well dried, as the puddles soak in better and are less likely to dribble when the plates are inverted. To ensure the plates are suitably dry, after pouring the plates leave them in a laminar flow hood with the lids off for ~15–20 min before using.
7. The EGY48 strain contains a lexA-inducible LEU2 gene whose expression allows growth on media lacking leucine to be used as a readout of transcription factor activity (and hence caspase-mediated substrate cleavage). To avoid confounding selective pressure to retain the caspase plasmid with reporter gene activity, we avoid using leucine-selectable caspase expression plasmids in this system—instead we tend to express caspases from histidine and/or tryptophan-selectable plasmids.
8. Each sample may turn yellow at a different time, and the time taken to develop the yellow color is critical to accurately estimate reporter gene activity. To ensure the color development in each tube can be monitored properly, and each reaction stopped when they reach equivalent color intensity, it is advisable to process only a few samples at a time.

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