

14-3-3 Protects against stress-induced apoptosis

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Expression of human Bax, a cardinal regulator of mitochondrial membrane permeabilization, causes death in yeast. We screened a human cDNA library for suppressors of Bax-mediated yeast death and identified human 14-3-3 β/α , a protein whose paralogs have numerous chaperone-like functions. Here, we show that, yeast cells expressing human 14-3-3 β/α are able to complement deletion of the endogenous yeast 14-3-3 and confer resistance to a variety of different stresses including cadmium and cycloheximide. The expression of 14-3-3 β/α also conferred resistance to death induced by the target of rapamycin inhibitor rapamycin and by starvation for the amino acid leucine, conditions that induce autophagy. Cell death in response to these autophagic stimuli was also observed in the macroautophagic-deficient *atg1 Δ* and *atg7 Δ* mutants. Furthermore, 14-3-3 β/α retained its ability to protect against the autophagic stimuli in these autophagic-deficient mutants arguing against so called 'autophagic death'. In line, analysis of cell death markers including the accumulation of reactive oxygen species, membrane integrity and cell surface exposure of phosphatidylserine indicated that 14-3-3 β/α serves as a specific inhibitor of apoptosis. Finally, we demonstrate functional conservation of these phenotypes using the yeast homolog of 14-3-3: *Bmh1*. In sum, cell death in response to multiple stresses can be counteracted by 14-3-3 proteins.

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Programmed cell death (PCD) is a critically important process that has a multitude of purposes in metazoans. It is involved in sculpting the final body that results after development and it serves to remove cells that may be a threat to the organism be they damaged or be they infected with foreign invaders.^{1,2} PCD is also of clinical importance as it is disrupted in a number of diseases.^{3–5} Inhibitors of the process would be beneficial in numerous pathophysiological situations where cell death is increased such as after ischemic/reperfusion events that are commonly seen in the heart.⁴ Similarly, the ability to selectively increase PCD in tumors represents another major impetus for much of the research that focuses on understanding the mechanisms regulating PCD.³

Our understanding of PCD has increased greatly over the last few years with the identification and characterization of novel negative regulators of different forms of PCD, including necroptosis.^{2,5–7} The identification of enzymes like deoxyuridine triphosphate (dUTPase), that utilize dUTP as a substrate, as a prosurvival sequence has served to focus attention on the fact that reactive oxygen species (ROS), calcium and ceramide are not the only second messengers whose levels increase in response to stress in order to trigger proapoptotic pathways.^{8,9} The activation of autophagy, often in response to environmental stresses such as starvation for key nutrients, appears to be another common mechanism involved in promoting the survival of cells in a number of stressful circumstances.^{10–12} In addition to its cytoprotective

role, autophagy is also well known as type II PCD.¹³ There remains a great deal of controversy regarding how widespread autophagic cell death is with increasing consensus that autophagic cell death may be a form of apoptotic or type I PCD.^{14–16} Alternatively, autophagic PCD may occur at the end of prolonged or acute periods of autophagy.¹⁷ Yeast has proven useful for delineating the basic framework of autophagy and this combined with its emergence as genetic model for PCD suggest that yeast will prove to be a useful tool to understand autophagic death.^{7,18–21} Here we report that a previously identified Bax suppressor 14-3-3 β/α is a functionally novel antiapoptotic sequence.²² We use 14-3-3 β/α along with yeast mutants defective in macroautophagy as tools to explore cross-talk between autophagic cell death and antiapoptosis.

Results

Identification of human 14-3-3 β/α as a Bax suppressor.

We report the characterization of a Bax suppressor identified in a previous screen, a 0.999 Kb cDNA called Bh113.²² To confirm the results of the screen, wild-type yeast were re-transformed with the vector expressing Bax with empty vector, with Bh113 or the previously characterized dUTPase.⁸ The three transformants were serially diluted and spotted on selective nutrient agar with glucose or galactose. As expected, cells harboring the Bax-containing plasmid

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Abbreviations: DHE, dihydroethidium; dUTP, deoxyuridine triphosphate; MEF, mouse embryonic fibroblast; PCD, programmed cell death; PI, propidium iodide; ROS, reactive oxygen species; TOR, target of rapamycin

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failed to grow on the galactose inductive media. In contrast, the cells harboring the Bax along with Bh113- or dUTPase-expressing plasmids showed significant growth on galactose (Supplementary Figure S1).

Analysis of the Bh113 nucleotide sequence revealed that it is an exact match to a portion of the 3015 nucleotide cDNA sequence encoding human 14-3-3 β/α (GenBank accession no. NM_139323). The coding sequence of the protein within the 14-3-3 β/α NM_139323 cDNA is located between nucleotides 188 and 928. Our Bh113 sequence encompasses nucleotides 15-1014 of the 14-3-3 β/α sequence. Thus, our Bh133 sequence contains the entire coding sequence of the 246 amino acids of the 14-3-3 β/α protein. The 14-3-3 β/α protein is part of a family of proteins that have numerous chaperone-like functions, including the ability to prevent cell death.²³ As previously demonstrated for a number of other sequences, the identification of 14-3-3 β/α as a Bax suppressor in yeast suggests that it may be a powerful antiapoptotic protein.²⁴

Human 14-3-3 β/α prevents cadmium-mediated cell death in yeast. To investigate the potential of 14-3-3 β/α in yeast, we used the fact that cadmium can induce a PCD that is reversed by the expression of a previously characterized Bax suppressor.⁸ Cultures of yeast transformed with different plasmids were diluted and aliquots spotted on nutrient agar galactose-containing plates with or without cadmium. Yeast cells harboring the 14-3-3 β/α or the dUTPase cDNAs are more resistant to the inhibitory effects of cadmium as compared with control cells harboring the empty vector (Figure 1a). To ascertain if 14-3-3 β/α actually prevents cadmium-mediated death, we grew cells in liquid media in the presence of cadmium and viability was determined using a vital dye. Cadmium decreased viability to $39.4 \pm 2.3\%$ in controls, while 14-3-3 β/α -expressing cells were protected with $79.1 \pm 2.1\%$ viability (Figure 1b).

14-3-3 β/α is antiapoptotic. As a first step in characterizing the types of cell death that could be regulated by 14-3-3 β/α , we used cycloheximide a known inducer of apoptosis.²⁵ Yeast cultures were treated with cycloheximide and their viability was determined by examining the proportion of cells that formed colonies (Figure 1c). Cycloheximide was dose dependent with a 36 and 11% remaining viable with 1 and 3 mM, respectively, in control cells. The viability was significantly increased to a respective 56 and 47% in cells expressing 14-3-3 β/α (Figure 1c). The accumulation of ROS is a typical feature of stressed cells and is also a typical hallmark of apoptosis.²⁶ To monitor ROS we treated cells with dihydroethidium (DHE), a non-fluorescent compound that enters cells and becomes cleaved by ROS to generate fluorescent ethidium. We could thus demonstrate that cycloheximide-mediated increases in ROS levels are significantly reduced in cells expressing 14-3-3 β/α compared with cells harboring vector alone (Figure 1d). To further characterize 14-3-3 β/α , we examined cells that were simultaneously stained with the nuclear vital dye propidium iodide (PI) and fluorescently labeled AnnexinV. Thus, we can detect cells that are undergoing cell surface exposure of phosphatidylserine (apoptosis; AnnexinV⁺) and loss of membrane

integrity (necrotic cell death; PI⁺).²⁷ In combination, this allows the discrimination between cells that are in early apoptosis (AnnexinV⁺/PI⁻), late apoptosis/secondary necrosis (AnnexinV⁺/PI⁺) and necrosis (AnnexinV⁻/PI⁺). Our analysis indicated that cycloheximide increased apoptotic markers which were significantly decreased in cells expressing 14-3-3 β/α (Figure 1e). Taken together, these results indicate that 14-3-3 β/α is capable of preventing cell death that has the hallmarks of apoptosis.

14-3-3 β/α prevents rapamycin-mediated growth inhibition and cell death. To evaluate the possibility that 14-3-3 β/α could serve to inhibit autophagic or type II PCD, we used conditions in which autophagy is activated in yeast. We initially used rapamycin, a common inhibitor of target of rapamycin (TOR), that inhibits cellular growth and activates autophagy.²⁸ In yeast, the overexpression of 14-3-3 allows the cells to grow in the presence of rapamycin.²⁹ Although its function is not known, the rapamycin-resistant phenotype is interpreted as 14-3-3 having a role in regulating TOR.³⁰ Although rapamycin is routinely used to induce autophagy, it does have additional effects including inducing cell death. The type of cell death induced by rapamycin is widely seen as apoptotic-like, but the possibility that prolonged activation of autophagy by rapamycin may lead to an autophagic-like cell death that is still being intensively investigated.^{28,31} Thus, we sought to determine whether rapamycin-mediated death is a form of autophagic PCD and whether it is inhibited by overexpressing 14-3-3 β/α . We thus set out to determine the effects of expressing 14-3-3 β/α on the sensitivity of yeast cells to rapamycin. As a prelude we determined the minimum concentration of rapamycin needed to inhibit the growth of cells. Using the spot growth assay we demonstrate that 100 nM of rapamycin induced a noticeable inhibition of growth in cells harboring the empty vector but that cells having the 14-3-3 β/α -expressing plasmid grew normally (Supplementary Figure S2). Given that 14-3-3 β/α can prevent cell death, this suggests that rapamycin induces growth arrest and cell death in yeast as it does in mammalian cells, and that 100 nM is the threshold concentration that initiates death.

To directly evaluate the deadly impact of rapamycin treatment, we examined its effect on the viability of cells harboring empty vector or the plasmid expressing 14-3-3 β/α . Rapamycin was added to growing cultures and aliquots of cells were removed daily and viability determined by examination after staining with vital dye. Although rapamycin decreased the viability of both cultures, cells expressing 14-3-3 β/α maintained higher viability compared with control cells (Figure 1f). For example, viability decreased to $29.5 \pm 1.1\%$ after 6 days of rapamycin treatment in cells harboring the empty vector, while the viability was $88.1 \pm 0.9\%$ in 14-3-3 β/α -expressing cells (Figure 1f). In the absence of rapamycin, viability of cells harboring empty vector remained high with $85 \pm 1.5\%$ viability after 6 days. This reinforces the concept that rapamycin induces a death that is prevented by 14-3-3 β/α .

Autophagy is not required for 14-3-3 β/α to protect against rapamycin. To investigate the role of autophagy

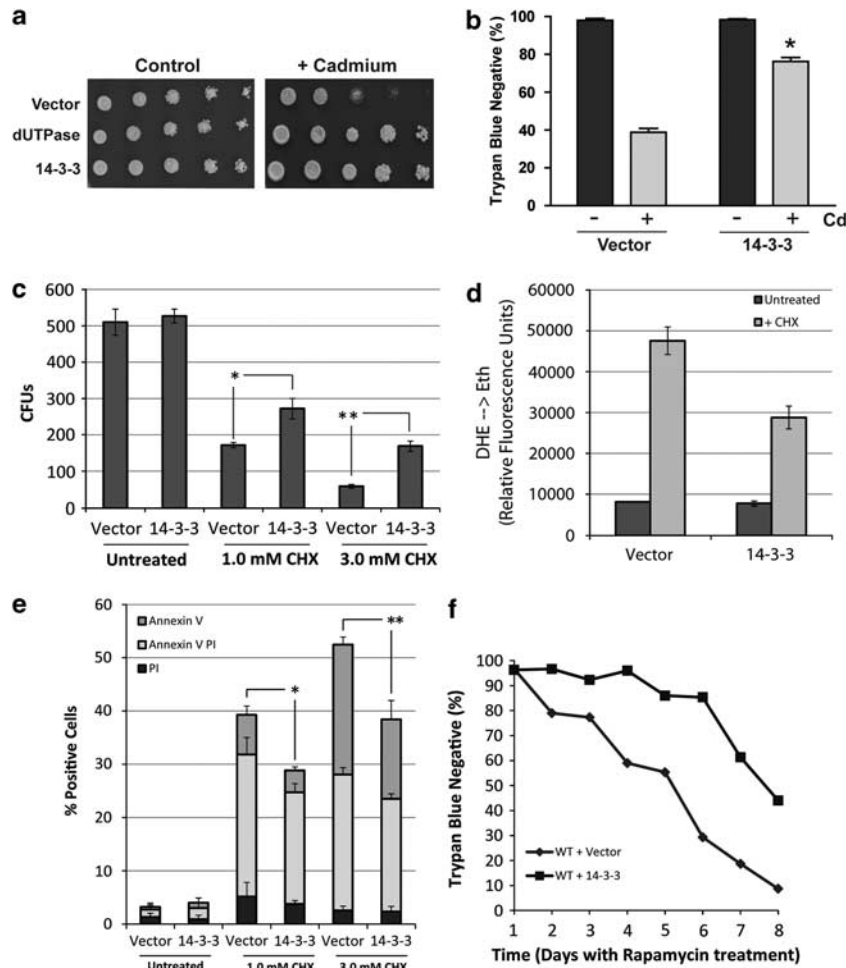


Figure 1 14-3-3 β protects against various triggers of cell death in yeast. (a) Freshly saturated cultures of yeast cells harboring control empty plasmid (Vector), as well as plasmids expressing the Bax suppressors dUTPase and 14-3-3 β/α (14-3-3) were serially diluted and aliquots were spotted onto YNB galactose-containing nutrient agar with no additions (Control) or with 1.5 mM cadmium sulfate (+ Cadmium). The plates were incubated at 30°C for 3 days. (b) Freshly saturated cultures of yeast cells harboring control empty plasmid (Vector) and the plasmid expressing and 14-3-3 β/α (14-3-3) were diluted and grown in galactose nutrient media for 4 h. Cells were then grown for 6 h without (black bars) or with 1.5 mM cadmium sulfate (gray bars). Viability was determined by microscopic examination of cells stained with the vital dye trypan blue. The data are shown as the percentage (%) of cells that does not stain with trypan blue and are thus viable (trypan blue negative). The data represent the mean of four independent experiments (\pm S.E.M.). *, indicates a significant difference between the viabilities of cadmium-treated Vector cells *versus* cadmium-treated 14-3-3-expressing cells (Student's *t*-test $P < 0.001$). (c–e) Human 14-3-3 β/α prevents cycloheximide-mediated apoptosis. Freshly saturated cultures of yeast cells with empty plasmid (Vector) and the 14-3-3 β/α -expressing plasmid (14-3-3) were diluted and grown in galactose nutrient media for 4 h prior to the addition of cycloheximide. (c) Viability of the cells was determined after a 24 h treatment with the indicated concentrations of cycloheximide using the clonogenicity assay. The data represent the mean of four independent experiments (\pm S.E.M.). * and ** indicates significant difference between the viabilities of control vector cells *versus* 14-3-3-expressing cells (* $P < 0.05$ and ** $P < 0.01$). (d) Fluorescence was monitored in DHE-treated cells using a fluorescent cell sorter. The results are reported as the mean (\pm S.D.) of two experiments that were performed in triplicate. (e) Cells were simultaneously challenged with fluorescently labeled annexin V and propidium iodide, and the proportion of cells labeled with either or with both were determined by cell sorter as described.²⁷ The data represent the mean of four independent experiments (\pm S.E.M.). * and ** indicates significant differences between the control vector cells *versus* 14-3-3-expressing cells (* $P < 0.05$ and ** $P < 0.01$). (f) Rapamycin-mediated cell death is inhibited by human 14-3-3 β/α . Freshly saturated cultures of yeast cells with empty plasmid (circles) and the 14-3-3 β/α -expressing plasmid (squares) were diluted and grown in galactose nutrient media for 4 h. Rapamycin was added (500 nM) and viability was monitored daily using the vital dye trypan blue over an 8-day period. The data are shown as the percentage (%) of cells that does not stain with trypan blue (trypan blue negative). The results are reported as the mean (\pm S.D.) of two experiments that were performed in triplicate

in rapamycin resistance, we used mutants lacking the genes encoding *Atg1p* and *Atg7p*, as they are able to grow normally but are unable to carry out macroautophagy, the form of autophagy that is induced by rapamycin and the form that offers the most striking protection against stress.¹⁸ In effect, these strains show an increased lethality in the face of numerous stresses, including starvation for leucine (see below, Figures 2c and d). We also used a *atg11Δ*, as it

grows normally, has normal macroautophagy and instead it is selectively defective in the minor autophagic pathway for mitophagy.¹⁸ The *atg1Δ* and wild-type strains with empty vector and the 14-3-3 β/α -expressing plasmid show similar growth in the absence of the drug, whereas all the strains expressing 14-3-3 β/α show enhanced growth in the presence of rapamycin when compared with control cells (Figure 2a). These results suggest that enhanced activation of autophagy

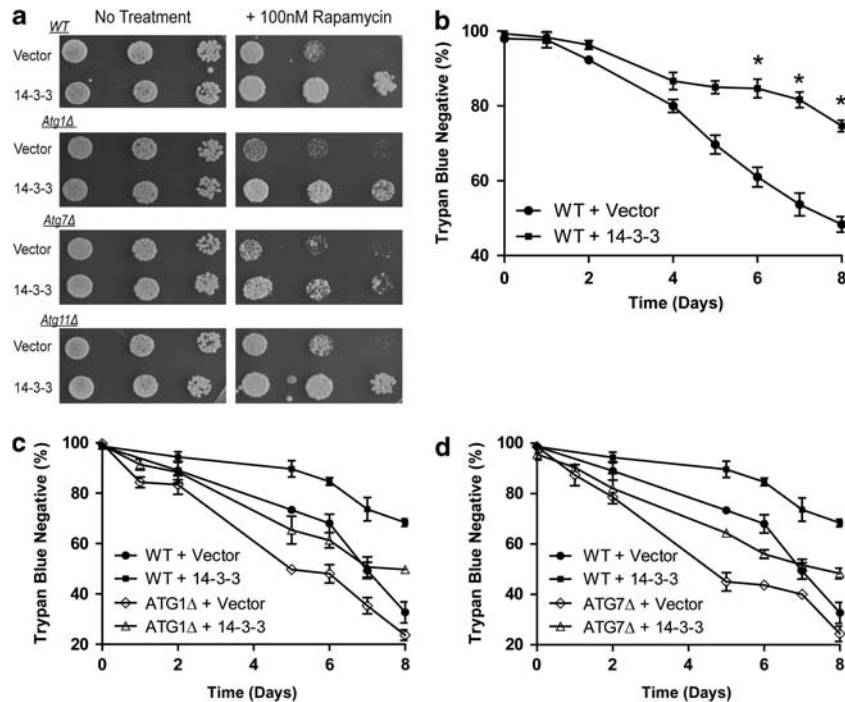


Figure 2 Cell death due to prolonged autophagy is rescued by 14-3-3. (a) 14-3-3-mediated rapamycin resistance is autophagy independent. Freshly saturated cultures of different yeast strains including wild type (WT) as well as the isogenic derivatives that are lacking the *Atg1*, *Atg7* and *Atg11* genes were individually transformed with control empty plasmid (Vector), and the plasmid expressing 14-3-3β/α (14-3-3) were serially diluted and aliquots were spotted onto YNB galactose-containing nutrient agar with no additions (no treatment) or with 100 nM rapamycin. The plates were incubated at 30°C for 2 days (no treatment) or for 4 days (+ 100 nM rapamycin). (b–d) 14-3-3 confers resistance to leucine starvation-mediated cell death in an autophagy-independent manner. Freshly saturated cultures of the leucine auxotrophic wild-type yeast cells harboring different plasmids were diluted and grown in complete minimal galactose-containing nutrient media for 4 h, washed with sterile water and resuspended at 2×10^6 cells/ml in complete minimal galactose-containing media without leucine. The cultures were incubated at 30°C and samples were taken at intervals to determine viability using the vital dye trypan blue. The data are shown as the percentage (%) of cells that does not stain with trypan blue (trypan blue negative). (b) Wild-type yeast cells with control empty plasmid (full circles) as well as the plasmid expressing 14-3-3β/α (full squares) underwent leucine starvation and viability was determined. The data represent the mean of four independent experiments (\pm S.E.M.). * indicates a significant difference between the viabilities of control vector cells versus 14-3-3-expressing cells (Student's *t*-test $P < 0.001$). (c and d) Wild type as well as mutants that are lacking the *atg1* (open diamonds) and *atg7* (open triangles) genes were individually transformed with the empty vector and the plasmid expressing 14-3-3β/α (14-3-3), and viability was determined following leucine deprivation. The data are shown as the percentage (%) of cells that does not stain with trypan blue (trypan blue negative). The experiments presented in c and d are the mean (\pm S.D.) of two experiments that were performed in triplicate. The data are presented in two separate panels for presentation purposes. The data for the WT cells (WT + Vector; WT + 14-3-3) are thus the same for c and d, but the data presented in b for these same strains were obtained from independent experiments

does not account for the protective effects of 14-3-3β/α and that the activation of autophagy is not required to mediate the inhibitory effects of rapamycin (Figure 2a).

Human 14-3-3β/α prevents leucine starvation-mediated cell death. We used leucine deprivation as an alternate method to activate autophagy and allow cell death to occur when the cells are 'exhausted' or depleted due to chronic autophagy.^{18,32,33} Thus, cultures of wild-type strain with empty vector or plasmid expressing 14-3-3β/α were subjected to leucine deprivation and viability was determined using vital dye. Viability of control cells decreased gradually over time and this was significantly blunted in cells expressing 14-3-3β/α (Figure 2b). In effect, by day 6, viability is reduced to $61 \pm 2.6\%$ in controls but is elevated to $84.7 \pm 2.5\%$ in cells expressing 14-3-3β/α (Figure 2b). The observed decrease in viability is specifically due to the absence of leucine, as the viability of cells harboring empty vector was largely unaffected when grown in the presence of media with leucine with $86 \pm 2.1\%$ remaining viable after 6 days. The autophagic-deficient *atg1Δ* and *atg7Δ* mutants

without and with the 14-3-3β/α-expressing plasmid were also subjected to leucine deprivation. As expected, loss of viability was more pronounced in the autophagic mutants when compared with wild-type cells (Figures 2c and d). At day 6, the viability of wild type was decreased to $68 \pm 3.6\%$, whereas the viability of the *atg1Δ* and *atg7Δ* mutants was 48 ± 3.6 and $43.7 \pm 1.2\%$, respectively (Figures 2c and d). This shows that viability during leucine starvation is enhanced by a functional autophagic pathway. The viability was enhanced in the wild-type and autophagic mutant strains by the expression of 14-3-3β/α (Figures 2c and d). This suggests that the observed cell death is not dependent on autophagy and that it is likely an apoptotic-type PCD.

Human 14-3-3β/α is physiologically relevant in yeast. Yeast cells have two distinct but almost identical 14-3-3 genes, *BMH1* and *BMH2*, both of which appear to function as multifaceted chaperonin-like proteins such as the mammalian 14-3-3 genes (Supplementary Figure S3a).^{23,34} Human 14-3-3β/α and yeast *Bmh1p* share 63% sequence identity, which is higher than the $\sim 30\%$ identity shared between most

other yeast–human orthologs (Supplementary Figure S3b).³⁵ Nevertheless, human 14-3-3 β/α is still a heterologous gene and it may not be physiologically relevant in yeast. To examine if 14-3-3 β/α is a functional 14-3-3 sequence, we examined if it could complement the rapamycin sensitivity of mutants lacking *bmh1* or *bmh2*.²⁹ Using the spot growth assay, we show that yeast cells lacking *Bmh1* or *Bmh2* show very little growth when compared with wild-type cells in the presence of rapamycin (Figure 3a). In contrast, *Bmh1 Δ , and to a lesser extent *Bmh2 Δ cells, show enhanced growth when overexpressing 14-3-3 β/α . These results suggest that 14-3-3 β/α is a functional ortholog of the yeast 14-3-3 genes.**

The yeast ortholog of human 14-3-3, BMH1, is anti-apoptotic. Antiapoptotic genes are characterized by the

increased and decreased resistance to apoptotic stresses, respectively, upon overexpression and knockout.⁵ To determine whether the yeast *BMH* genes are also antiapoptotic, we first examined the effects of apoptosis inducing levels of cycloheximide on yeast cells lacking *Bmh1 Δ and *Bmh2 Δ genes. Yeast cells lacking the *BMH1* gene show all the signs of being hypersensitive to the stress of cycloheximide (Figures 3b–d). When challenged with apoptotic-inducing stress they show an enhanced increase in their levels of ROS (Figure 3b), they show a decrease in their viability (Figure 3c) and they show increase in the percentage of cells that have overt hallmarks of apoptosis including staining with annexin V (Figure 3d). In contrast, *Bmh2 Δ cells are not hypersensitive to the apoptotic-inducing stress as we observed very little differences when compared with wild-type cells***

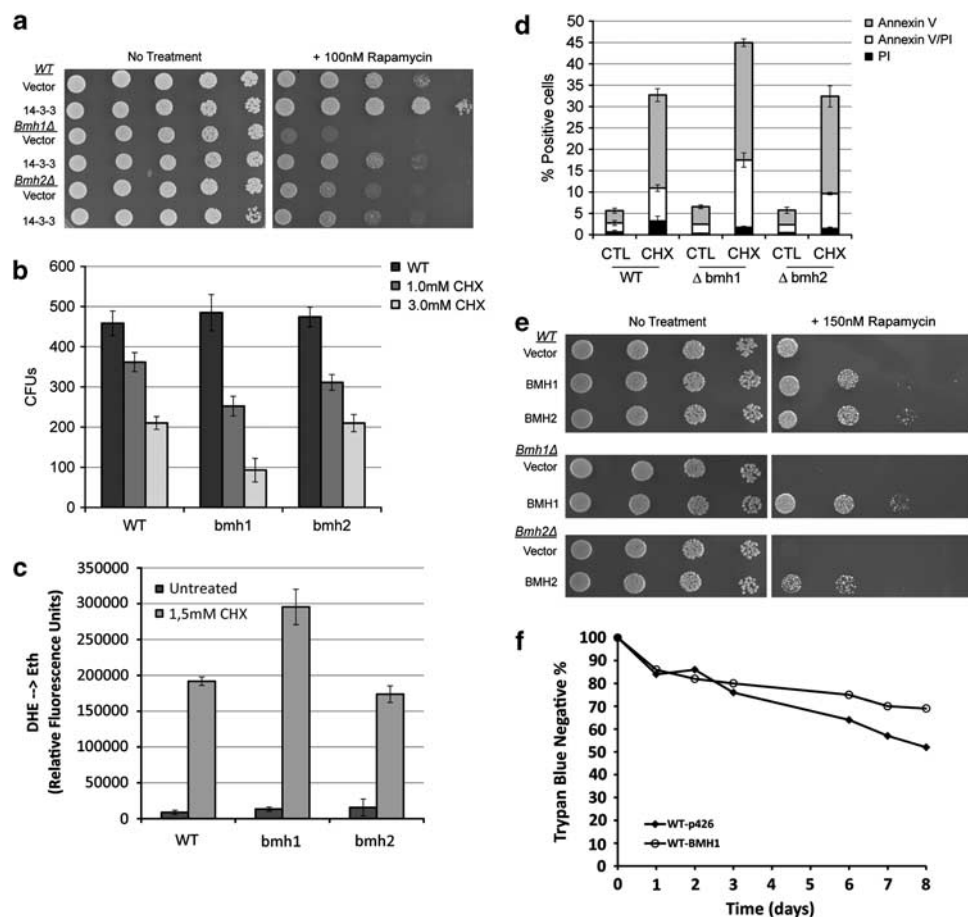


Figure 3 Yeast and human 14-3-3 proteins are functionally interchangeable. (a) Human 14-3-3 β/α can functionally replace the yeast *BMH1* and *BMH2* encoding the 14-3-3 genes. Freshly saturated cultures of different yeast strains including wild type (WT) as well as the isogenic derivatives that are lacking the yeast 14-3-3 *Bmh1* or *Bmh2* (*Bmh1 Δ or *Bmh2 Δ) genes transformed with different plasmids (Vector, 14-3-3) were serially diluted and aliquots were spotted onto galactose-containing nutrient agar without (no treatment) or with 100 nM rapamycin and the plates were incubated at 30°C. (b–d) Yeast mutants lacking the *BMH1* gene are hypersensitive to apoptotic-inducing stress. Yeast mutants lacking the *Bmh1 Δ and *Bmh2 Δ genes were treated with cycloheximide and different apoptotic parameters determined. The results are reported as the mean (\pm S.D.) of two experiments that were performed in triplicate. (b) Viability of the cells was determined after 5 h with cycloheximide using the clonogenic assay. (c) Fluorescence was monitored in DHE-treated cells using a fluorescent cell sorter. (d) Cells were simultaneously challenged with fluorescently labeled annexin V and propidium iodide, and the proportion of cells labeled with either or with both were determined by cell sorter as described.²⁷ (e) Rapamycin-mediated cell death is inhibited by yeast 14-3-3. Spot assays were carried out using freshly saturated cultures of wild-type cells (WT) and mutants lacking 14-3-3 (*Bmh1 Δ and *Bmh2 Δ) transformed with control plasmid (Vector) or plasmids overexpressing the yeast 14-3-3 genes (BMH1 and BMH2). The cells were spotted onto galactose-inducible selectable media without (no treatment) or with 150 nM rapamycin (+ Rapamycin). * $P < 0.05$ and ** $P < 0.01$. (f) Viability was determined, using the vital dye trypan blue, following leucine deprivation in wild-type cells transformed with the empty vector (open diamonds) and the plasmid expressing yeast *BMH1* (open triangles). The data are shown as the percentage (%) of cells that does not stain with trypan blue (trypan blue negative). The results are reported as the average of two experiments that were performed in triplicate******

(Figures 3b–d). These results are consistent with previous observations that the *BMH1* gene is likely the more overtly important of the two yeast 14-3-3 genes.³⁴

We then used yeast cells overexpressing both *BMH* genes to determine whether the sequences could also serve to protect against apoptotic-inducing stress. Using the spot assay, it is clear that wild-type yeast overexpressing either *BMH1* or *BMH2* show an increase in the resistance to the growth inhibitory effects of rapamycin (Figure 3e). Thus, both genes appear to have antiapoptotic functions when overexpressed. Yeast cells overexpressing *BMH1* also show increased viability compared with control cells in response to prolonged periods of autophagy induced by leucine starvation (Figure 3f). The viability of control cells was decreased to $52 \pm 2.3\%$ compared with $69 \pm 3.5\%$ after 8 days of leucine deprivation. Taken together, the results in Figure 3 indicate that at least one of the yeast 14-3-3 proteins, *Bmh1p*, is an antiapoptotic gene that has similar properties to human 14-3-3 β/α .

Discussion

The process by which cells protect themselves from inadvertently and prematurely inducing cell death in response to sublethal stresses involves a coordinated response to prevent the initiation of cell death.^{1,5} One common method is the use of antiapoptotic proteins that inhibit well-characterized proapoptotic pathways. Thus, Bcl-2 is a potent inhibitor of Bax such that the level of functional Bcl-2 serves as a threshold for Bax-mediated cell death (Figure 4). This is evident from the fact that decreases or increases in the levels of Bcl-2 changes

threshold for stress and their ability to prevent apoptotic cell death.³⁶ Here we show that human 14-3-3 β/α is capable of preventing cell death that has the hallmarks of apoptosis in yeast (Figures 1 and 2). Although the mechanisms are not yet known, 14-3-3 β/α is a chaperonin-type protein that likely alleviates cellular stress and subsequent activation of apoptotic pathways by assisting damaged proteins to maintain their physiologically relevant structure. Other chaperonin-type proteins that are antiapoptotic include a variety of heat-shock proteins, and some of these proteins may promote cell survival by binding to and inhibiting proapoptotic proteins.³⁷ The availability of yeast-based antiapoptosis assay system to study human 14-3-3 β/α will facilitate a genetic determination of the structural motifs and the cofactors required for its prosurvival function.

The processes by which autophagic-mediated cell death occurs has remained elusive and many of the experimental evidence used for its detection are in fact quite controversial.^{14–16} It is of interest that many studies have shown that negative regulators of autophagic death do not delay cell death at the end of autophagy, but instead they prevent the induction of autophagy. There are very few studies that actually show a causal relationship between cell death and autophagy.^{5,13} We thus examined the use of the 14-3-3 antiapoptotic sequence to determine whether it could prevent cell death under culture conditions where autophagy is known to be actually occurring. We thus could demonstrate that well-known conditions that serve to activate autophagy, the specific inhibition of TOR1 using rapamycin and starvation for the amino acid leucine both lead to cell death which could be inhibited by the expression of human 14-3-3 β/α (Figure 2).

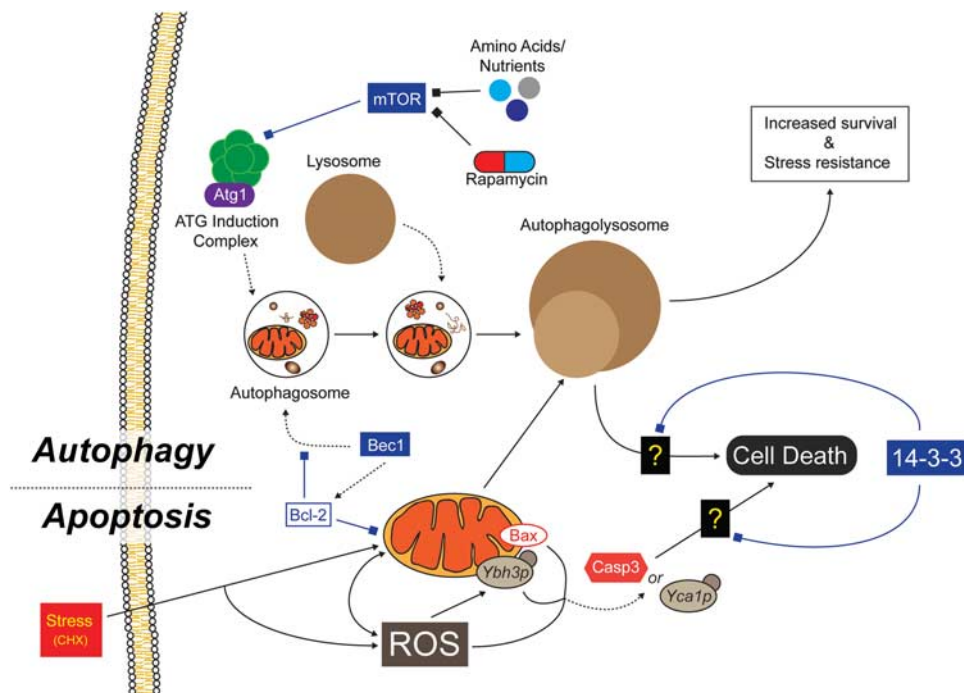


Figure 4 Schematic representation of proteins that regulate apoptosis and autophagy. The typical autophagic pathway including the role of mTOR and its inactivation by nutrients and rapamycin is shown on top. The role of the Atg1p in initiating autophagy is depicted. Proteins and effectors including ROS, Bcl-2 and Bec1 (Beclin1) that are involved in both apoptosis and autophagy are shown. This study shows that 14-3-3 can inhibit cell death due to stimuli that induce apoptosis and by stimuli that are commonly used to induce autophagy

Cells lacking the autophagic genes *atg1* or *atg7* were still able to undergo cell death with rapamycin or if starved for leucine and that this death could be still be prevented by the expression of human 14-3-3 β/α (Figure 2). This supports the notion that the cell death may not be due to autophagy. It does nevertheless remain possible that blocking autophagy may switch the cell from autophagic to apoptotic cell death and that 14-3-3 β/α is able to serve to inhibit both type I and II PCD (Figure 4). This reflects the fact that there is a great deal of cross-talk between apoptosis and autophagy with the most well-known cases involving the ability of activated autophagy to inhibit apoptosis.^{5,14} Other interesting examples involve apoptotic-resistant cells, such as mouse embryonic fibroblasts (MEFs) that are derived from embryos having a double knockout of the two genes encoding the proapoptotic Bcl-2 proteins Bax and Bak. These cells are reported to undergo autophagic cell death in response to apoptotic stimuli.³⁸ In spite of this we nevertheless favor an increasingly accepted scenario that autophagic cell death per se is a rare event, instead what is commonly observed is really a form of apoptosis that is associated with autophagy or possibly triggered by autophagy.^{14,16} The intense ongoing investigations into the processes of autophagy will lead to novel assays and markers that will eventually serve to resolve the confusion regarding the importance of autophagic death.^{2,14,16} The inability to find a true inducer of autophagic cell death in a screen that identified ~1400 chemical inducers of PCD serves as strong support.³⁹ An alternative approach would be to identify specific inhibitors of autophagic cell death, possibly by screening cDNA or chemical libraries in cells undergoing PCD after prolonged periods of autophagy. Identification of such specific prosurvival chemicals and proteins for cells undergoing necrosis provided a strong rationale for the support of genetically programmed forms of necrosis.^{19,27}

Materials and Methods

Yeast strains and plasmids. The *S. cerevisiae* BY4742 (*MATa* his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0) was used as the wild-type strain, and the mutant strains used were isogenic except for the deletion of an autophagic gene (*atg1A*, *atg7A* and *atg11A*) or of the yeast 14-3-3 encoding genes (*bmh1A* and *bmh2A*) (EUROSCARF). The 14-3-3 β/α and dUTPase encoding Bax suppressors, expressed under the control of the galactose-inducible *GAL1* promoter in pYES-DEST52, were isolated in our previous Bax suppressor screen of a human cardiac cDNA library.^{8,22} The galactose-inducible *BMH1* and *BMH2* overexpressing plasmids were obtained from Open Biosystems (Thermo Scientific, Lafayette, CO, USA).

Yeast transformations, growth, viability and cell death assays.

Yeast were grown in synthetic minimal media containing yeast nitrogen base (YNB), 2% glucose and supplemented with the required amino acids or bases. When expression of the *GAL1* promoter was required, the glucose was substituted with 2% galactose and 2% raffinose. Plasmids were introduced into yeast cells using lithium acetate and transformants were selected for by removing the appropriate nutrient. Two methodologies were used to determine viability. The clonogenicity assay consisted of removing aliquots of differentially treated cells serially diluted and triplicate samples of 200–500 cells were then plated on YEPD media, grown at 30°C and the number of colonies that formed after 2 days were counted.^{22,27} Viability was also determined by microscopical examination of cells treated with the vital dye trypan blue.²² Cells were placed in a final concentration of 0.1% trypan blue for 5 min and at least 300 cells were scored for each time point. Treatment of cells in liquid cultures consisted of generating freshly saturated overnight cultures of the different yeast transformants, which were then diluted in fresh, pre-warmed galactose-containing media, incubated for 4 h to induce *GAL1* gene expression, and subsequently treated with the indicated concentrations of

compound including cadmium, cycloheximide and rapamycin.²² For the spot growth assays, freshly saturated cultures grown in glucose media, serially diluted 5-fold and 5 μ l of the appropriate dilutions were spotted onto nutrient-containing galactose with and without the different chemical stresses. All spot assays were performed a minimum of three times with identical results. Flow cytometry using 30,000 cells per sample were used for cell death marker assays, including DHE staining (ROS production) and Annexin V/PI containing (apoptosis/necrosis marker) as previously described.²⁷

Conflict of Interest

The authors declare no conflict of interest.

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