Bre1p-mediated histone H2B ubiquitylation regulates apoptosis in *Saccharomyces cerevisiae*

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Summary

BRE1 encodes an E3 ubiquitin protein ligase that is required for the ubiquitylation of histone H2B at lysine 123 (K123). Ubiquitylation of this histone residue is involved in a variety of cellular processes including gene activation and gene silencing. Abolishing histone H2B ubiquitylation also confers X-ray sensitivity and abrogates checkpoint activation after DNA damage. Here we show that *Saccharomyces cerevisiae* Bre1p exhibits anti-apoptotic activity in yeast and that this is linked to histone H2B ubiquitylation. We found that enhanced levels of Bre1p protect from hydrogen-peroxide-induced cell death, whereas deletion of *BRE1* enhances cell death. Moreover, cells lacking Bre1p show reduced lifespan during chronological ageing, a physiological apoptotic condition in yeast. Importantly, the resistance against apoptosis is conferred by histone H2B ubiquitylation mediated by the E3 ligase activity of Bre1p. Furthermore, we found that the death of *Δbre1* cells depends on the yeast caspase Yca1p, because *Δbre1* cells exhibit increased caspase activity when compared with wild-type cells, and deletion of *YCA1* leads to reduced apoptosis sensitivity of cells lacking Bre1p.

Key words: Apoptosis, Histone H2B, *S. cerevisiae*, Ubiquitin, YCA1

Introduction

Apoptosis is a form of programmed cell death that has a central role in development and cellular homeostasis in higher eukaryotes. Knowledge of apoptotic regulation is particularly important for medical research, since apoptotic misregulation is implicated in many human diseases, such as Alzheimer's disease and Huntington's disease, immunodeficiency and cancer (Fadeel and Orrenius, 2005). Recent studies have established yeast as a model to study the mechanisms of apoptotic regulation. Defects in distinct cellular processes, such as actin dynamics (Gourlay et al., 2004), vesicular fusion (Madeo et al., 1997), DNA replication (Weinberger et al., 2005), histone chaperone activity (Yamaki et al., 2001) or histone deubiquitylation (Bettiga et al., 2004) can trigger apoptotic cell death in *Saccharomyces cerevisiae* and an apoptotic-like phenotype has also been demonstrated in yeast cells treated with various agents including hydrogen peroxide, acetic acid and pheromone (Ludovico et al., 2001; Madeo et al., 1999; Severin and Hyman, 2002). Notably, the yeast apoptotic machinery has functional orthologues of key mammalian apoptotic regulators, including the metacaspase Yca1p (Madeo et al., 2002), the apoptosis-inducing factor AIF (Wissing et al., 2004), the endonuclease EndoG (Buttner et al., 2007), the serine protease HtrA2/Omi (Fahrenkrog et al., 2004) and the inhibitor-of-apoptosis protein Bir1p (Walter et al., 2006). In addition, yeast apoptosis has been linked to cellular events such as mitochondrial fragmentation (Fannjiang et al., 2004), cytochrome c release (Ludovico et al., 2002), ageing (Herker et al., 2004; Laun et al., 2001) and phosphorylation of histone H2B (Ahn et al., 2005; Ahn et al., 2006).

Rapid protein modifications allow the cell to promptly adapt to environmental changes by different cellular responses, including apoptosis. The post-translational modification by covalent attachment of ubiquitin is one of the major biochemical mechanisms that regulate apoptosis (Lee and Peter, 2003). Ubiquitylation controls the level of proteins by targeting them for proteasomal degradation. Members of the inhibitor-of-apoptosis protein (IAP) family are targeted for degradation, but they also contain a RING domain with ubiquitylating activity, by which they are able to mark other proteins such as caspases for degradation (Wilson et al., 2002). Additionally, monoubiquitylation and nonclassical polyubiquitylation of components of the apoptotic pathway regulate apoptosis on a molecular level, beyond the degradation effects (Huang et al., 2000; Lee et al., 2002; Mimnaugh et al., 2001).

Histone proteins are well-known substrates for numerous covalent post-translational modifications and these modifications regulate a number of cellular processes including apoptosis (Ahn et al., 2005; Ahn et al., 2006; Cheung et al., 2003). Histone H2B is monoubiquitylated at Lys123 by the ubiquitin conjugase Rad6p and the E3 ligase Bre1p (Hwang et al., 2003; Robzyk et al., 2000; Wood et al., 2003b). *BRE1* disruption or lysine-to-arginine substitution at residue 123 of histone H2B (H2B-K123R), results in a complex phenotype that includes failures in gene activation (Henry et al., 2003; Kao et al., 2004; Wyce et al., 2007; Xiao et al., 2005) and lack of telomeric silencing (Briggs et al., 2001; Dover et al., 2002; Mutiu et al., 2007; Sun and Allis, 2002). Moreover, *Caenorhabditis elegans BRE1* was identified in a screen for anti-apoptotic proteins (Lettre et al., 2004). Only recently, histone H2B ubiquitylation has been implicated in DNA repair and checkpoint activation after DNA damage (Game et al., 2006; Giannattasio et al., 2005). As the DNA damage response machinery is closely linked to apoptosis in yeast and higher eukaryotes (Burhans et al., 2003) a relationship between histone H2B ubiquitylation and apoptosis might exist. This possibility is supported by the finding that loss of the ubiquitin-specific protease *UBP10*, which is involved in cleaving the ubiquitin moiety from

histone H2B, causes caspase activation and apoptosis in yeast (Bettiga et al., 2004).

We aimed to gain insight into the role of histone H2B ubiquitylation in apoptosis and found that *S. cerevisiae* Bre1p exhibits anti-apoptotic activity in yeast and that this is linked to histone H2B ubiquitylation and the metacaspase Yca1p.

Results

Bre1p protects against hydrogen-peroxide-induced cell death in budding yeast

The E3 ubiquitin ligase Bre1p is required for histone H2B ubiquitylation (Hwang et al., 2003; Robzyk et al., 2000; Wood et al., 2003b), which in turn is implicated in transcriptional regulation and DNA repair. Moreover, H2B ubiquitylation appears to have a role in apoptosis regulation because the loss of the ubiquitin-specific protease *UBP10*, which is involved in cleaving the ubiquitin moiety from histone H2B, causes caspase activation and apoptosis in yeast (Bettiga et al., 2004). Notably, the *C. elegans* homologue of the *S. cerevisiae* Bre1p was identified as a regulator of germ-cell apoptosis in worms (Lettre et al., 2004), further supporting the importance of H2B ubiquitylation in apoptosis regulation.

To explore a role for Bre1p-mediated histone H2B ubiquitylation in yeast apoptosis, wild-type cells, cells lacking *BRE1* (*Δbre1*) and cells constitutively overexpressing a protein-A-tagged Bre1p fusion protein (ProtA-Bre1p) under the control of the *NOP1* promoter were exposed to 0.6 mM hydrogen peroxide (H_2O_2) to induce apoptosis. After 8 hours of incubation, cell survival was determined by clonogenicity and cells were tested for apoptotic markers, such as DNA single-strand breaks and reactive oxygen species (ROS), which are causally linked to yeast apoptosis. To do so, cells were stained with dihydroethidium (DHE) to visualise accumulation of ROS and TUNEL labelling was used to detect single-stranded DNA breaks. As shown in Fig. 1A, yeast cells lacking *BRE1* exhibited an increased sensitivity to H_2O_2 (31±4% cell viability) compared with wild-type (wt) cells (53±6% cell viability). This increase in sensitivity to H_2O_2 of *Δbre1* cells was accompanied by enhanced ROS production (54±4% of *Δbre1* versus 25±2% of wild-type cells were DHE positive; Fig. 1B,C) and by an increase in apoptotic DNA fragmentation and TUNEL-positive cells compared with wild-type cells (25±3% versus 14±1% TUNEL-positive cells; Fig. 1B,D). By contrast, cells overexpressing ProtA-Bre1p showed resistance to H_2O_2 (78±7% cell viability; Fig. 1A) and a decrease in ROS accumulation (15±1% DHE-positive cells; Fig. 1B,C) and DNA fragmentation (5±1% TUNEL-positive cells; Fig. 1B,D). Taken together, our data indicate that Bre1p exhibits anti-apoptotic activity.

Disruption of *BRE1* causes an early onset of cell death during chronological ageing

Chronological ageing defines an ageing process of post-mitotic yeast cells that triggers apoptosis (Herker et al., 2004). Therefore, we next investigated whether or not Bre1p is involved in chronological ageing. To do so, we determined the chronological lifespan of cells lacking *BRE1* and found that these cells showed an early onset of age-induced cell death when compared with wild-type cells (Fig. 2A). After 2 days in culture, cells lacking *BRE1* showed survival rates of 23±4% compared with 75±5% in wild-type cells (Fig. 2A). When, after 2 days in culture, these yeast cells were tested for apoptotic markers, *Δbre1* cells showed typical hallmarks of apoptosis, such as the production of ROS as detected by DHE staining (77±3% of *Δbre1* versus 16±4% of wild-type cells were DHE positive; Fig. 2B,C) and an increase in apoptotic DNA fragmentation as detected by TUNEL labelling (26±4% of *Δbre1* versus 6±1% of wild-type cells were TUNEL positive; Fig.

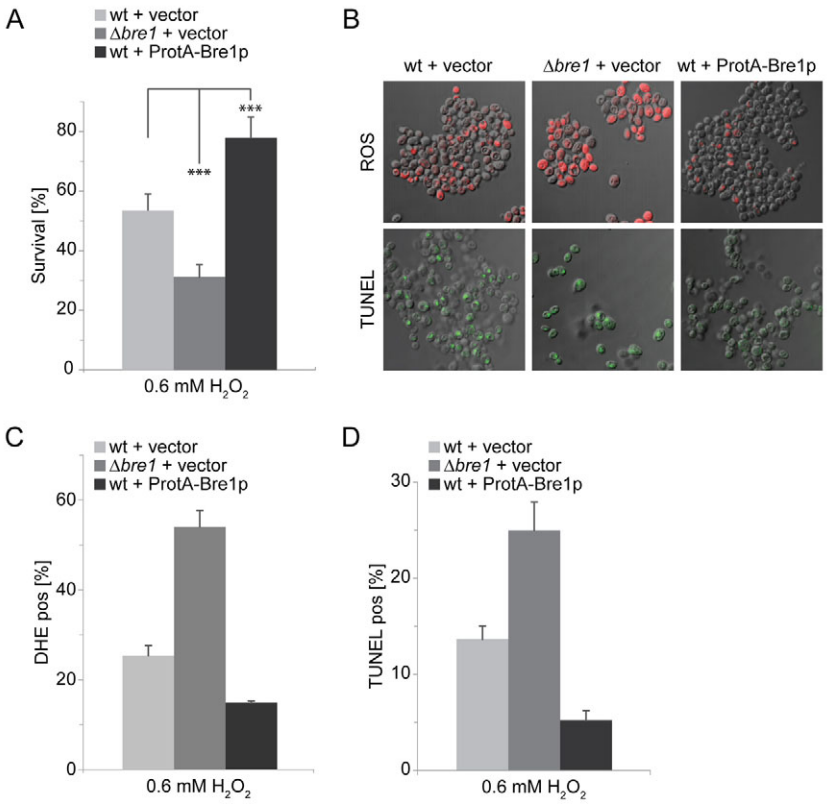

Fig. 1. *S. cerevisiae* Bre1p confers resistance to apoptosis induced by H_2O_2. (**A**) Wild-type (wt) and *Δbre1* yeast cells harbouring the empty vector control, and ProtA-Bre1p-overexpressing cells were treated with 0.6 mM H_2O_2 for 8 hours and survival was determined by clonogenicity. Data represent means ± s.d. (n=6; ***P<0.001). (**B**) ROS accumulation and DNA fragmentation in wild-type (wt) and *Δbre1* cells harbouring the vector control or overexpressing ProtA-Bre1p was determined by DHE staining and TUNEL staining, respectively. (**C**) DHE-positive and (**D**) TUNEL-positive cells were quantified by manually counting at least 500 cells. Data represent means ± s.d. (n=3).

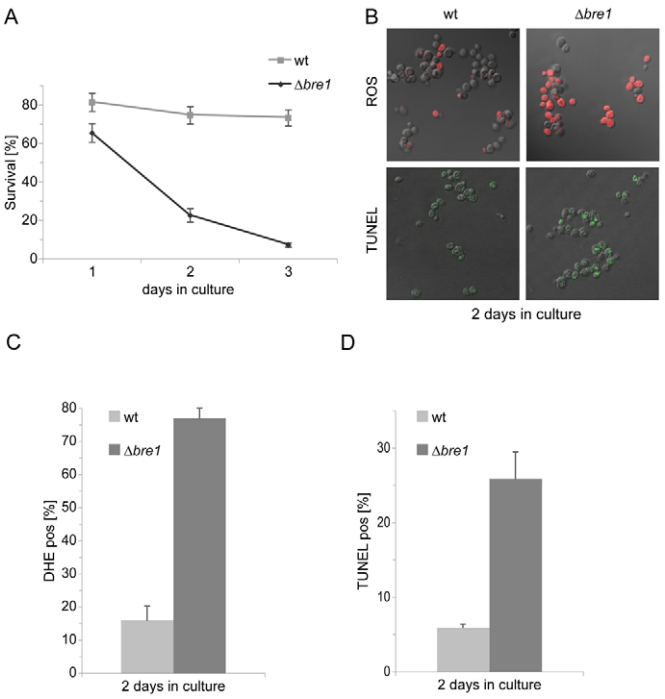

Fig. 2. Disruption of *BRE1* causes early onset of apoptosis during chronological ageing. (**A**) Survival of wild-type (wt) and *Δbre1* cells determined by clonogenicity during chronological ageing. Data represent means ± s.d. (*n*=9). (**B**) ROS accumulation and DNA fragmentation in wild-type and *Δbre1* cells after 2 days in culture determined by DHE staining and TUNEL staining, respectively. (**C**) DHE-positive cells were quantified after 2 days in culture using flow cytometry. In each experiment, 10,000 cells were evaluated. Data represent means ± s.d. (*n*=3). (**D**) TUNEL-positive cells were quantified by manual counting of at least 500 cells. Data represent means ± s.d. (*n*=3).

2B,D). These data further support the notion that Bre1p acts as inhibitor of apoptosis in yeast. Constitutive overexpression of ProtA-Bre1p, however, did not significantly influence chronological lifespan of yeast cells (data not shown).

The E3 ligase activity of Bre1p is required for its anti-apoptotic properties

A hallmark of Bre1p is a C-terminal C3HC4 (RING) zinc-finger domain (Hwang et al., 2003). RING domains are typically found in E3 ubiquitin ligases and frequently mediate the interaction with the E2 ubiquitin-activating enzyme (Deshaies and Joazeiro, 2009). The RING domains are therefore crucial for catalysing the transfer of ubiquitin from the E2 to the substrate. Accordingly, the RING domain of Bre1p confers E3 ubiquitin ligase activity, which is required for the ubiquitylation of histone H2B (Hwang et al., 2003; Wood et al., 2003a). To test whether the E3 ligase activity is required to grant resistance to age-induced apoptosis, we generated two different RING-domain mutants. First, two conserved cysteine residues (C648 and C651) within the RING domain were mutated to glycine and second, leucine L650 was mutated to glutamate (L650E). Although the mutation of the cysteines might affect the overall folding of the RING domain, L650 probably mediates the interaction of Bre1p with its E2 ligase, but does not affect the zinc coordination and stability of the protein. We next complemented *Δbre1* and *Δbre1* cells expressing H2B-GFP (*Δbre1 HTB1-GFP*), respectively, with plasmid-borne ProtA-Bre1p, ProtA-Bre1p(C648G, C651G) and ProtA-Bre1p(L650E) and tested the functionality of the ProtA-Bre1p, ProtA-Bre1p(C648G, C651G) and ProtA-Bre1p(L650E) as a measure of their ability to ubiquitylate histone H2B.

Monoubiquitylation of histone H2B can be detected in wild-type cells harbouring a functional GFP-tagged allele of the *HTB1* gene (encoding histone H2B) as a slower-migrating form upon SDS-PAGE and immunoblotting of whole-cell extracts with anti-GFP antibody. The ubiquitylated species was absent in *Δbre1* cells harbouring a GFP-tagged allele of *HTB1* (Fig. 3A), which is consistent with a previous study (Wood et al., 2003a). However, *Δbre1 HTB1-GFP* cells complemented with ProtA-Bre1p displayed no defect in H2B monoubiquitylation, whereas *Δbre1 HTB1-GFP* cells complemented with ProtA-Bre1p(C648G, C651G) and ProtA-Bre1p(L650E), respectively, lacked ubiquitylation of histone H2B similarly to *Δbre1 HTB1-GFP* cells (Fig. 3A). We conclude that the ProtA-Bre1p fusion protein is functional and that Bre1p requires the conserved cysteines C648, C651 and leucine 650 for its E3 ligase activity.

To explore the contribution of the E3 ligase activity of Bre1p to apoptosis resistance, we analysed the survival of *Δbre1* cells complemented with ProtA-Bre1p, ProtA-Bre1p(C648G, C651G) and ProtA-Bre1p(L650E) during chronological ageing. *BRE1*-null cells complemented with ProtA-Bre1p showed no significant difference in cell survival when compared with wild-type cells (89±5% cell viability versus 92±5% cell viability after 2 days in culture; Fig. 3A). Consistently, *Δbre1* cells complemented with ProtA-Bre1p and wild-type cells showed similar amounts of apoptotic markers with ~16% DHE-positive cells and 6-7% TUNEL-positive cells, respectively (Fig. 3D-F). By contrast, *Δbre1* cells complemented with ProtA-Bre1p(C648G, C651G) or ProtA-Bre1p(L650E) showed an early onset of cell death during chronological ageing similarly to *Δbre1* cells (21±2% and 23±1% cell viability versus 23±4% cell viability after 2 days in culture; Fig. 3B). 79±6% of *Δbre1* cells complemented with Bre1p(C648G, C651G) showed ROS accumulation and 25±3% were TUNEL positive, similarly to *Δbre1* cells (77±3% ROS-positive, 26±4% TUNEL-positive cells; Fig. 3D-F).

Consistent with the chronological ageing experiments, plasmid-borne ProtA-Bre1p, but not ProtA-Bre1p(C648G, C651G) or ProtA-Bre1p(L650E) rescued *Δbre1* cells from H_2O_2-induced cell death (Fig. 3C). H_2O_2-treated cells lacking *BRE1* that were complemented with ProtA-Bre1p showed a slightly better cell survival than wild-type cells (67±5% cell viability versus 54±6% cell viability; Fig. 3C) and fewer apoptotic markers (18±5% versus 25±2% DHE-positive cells as well as 8±3% versus 14±1% TUNEL-positive cells; Fig. 3D,G,H). However, *Δbre1* cells complemented with ProtA-Bre1p(C648G, C651G) or ProtA-Bre1p(L650E) showed H_2O_2 sensitivity that was similar to *Δbre1* cells (35±2% and 33±3% cell viability versus 31±4% cell viability; Fig. 3C). 48±3% of *Δbre1* cells complemented with Bre1p(C648G, C651G) showed ROS accumulation and 26±2% were TUNEL positive, similarly to *Δbre1* cells (54±4% ROS positive, 25±3% TUNEL positive; Fig. 3D,G,H). We conclude that the E3 ligase activity of Bre1p is required for its ability to confer resistance to apoptosis.

Bre1p confers apoptosis resistance by histone H2B ubiquitylation

Bre1p targets Lys123 in histone H2B for ubiquitylation. We therefore asked whether the ubiquitylation site of histone H2B is

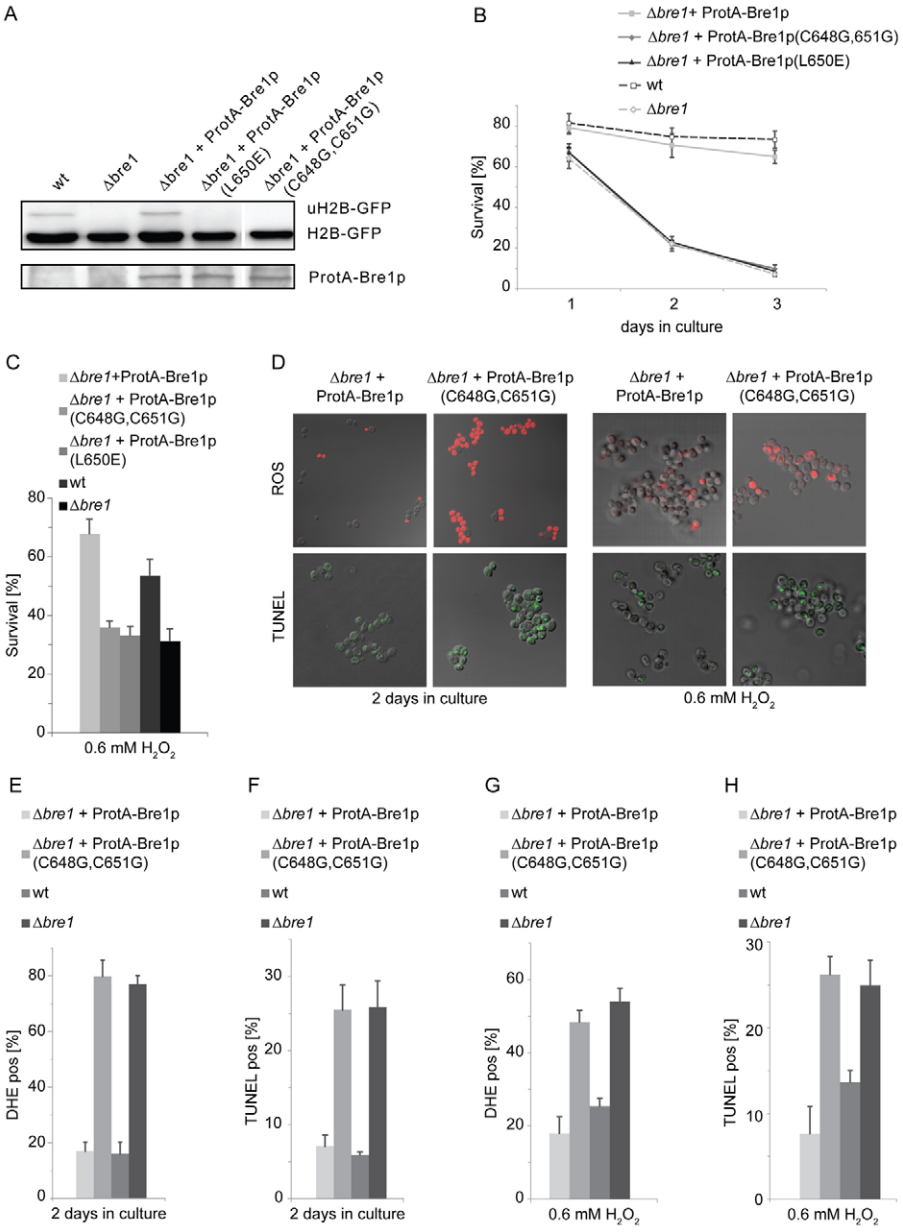

Fig. 3. The E3 ligase activity of Bre1p is required for apoptosis inhibition.
(**A**) Functionality of ProtA-Bre1p, ProtA-Bre1p(C648,651G) and ProtA-Bre1p(L650E) was tested as a measurement of the ability to ubiquitylate histone H2B. Wild-type (wt), *Δbre1* and *Δbre1* cells complemented with ProtA-Bre1p, ProtA-Bre1p(C648G, C651G) and ProtA-Bre1p(L650E) and harbouring a functional GFP-tagged allele of the HTB1 gene (encoding histone H2B) were grown in synthetic complete medium (SC) overnight. Whole-cell lysates were separated on a 12% acrylamide gel and the blot was probed with GFP (Dianova, clone: MA1-26343; Hamburg, Germany) and protein-A antibody. (**B**) Survival of wild-type, *Δbre1*, *Δbre1* cells complemented with ProtA-Bre1p, ProtA-Bre1p(C648G, C651G) and ProtA-Bre1p(L650E) was determined by clonogenicity during chronological ageing. Data represent means ± s.d. (n=9). (**C**) *Δbre1* cells complemented with ProtA-Bre1p, ProtA-Bre1p(C648G, C651G) and ProtA-Bre1p(L650E) were treated with 0.6 mM H_2O_2 for 8 hours and survival was determined by clonogenicity. Data represent means ± s.d. (n=9). (**D**) ROS accumulation and DNA fragmentation in *Δbre1* cells complemented with ProtA-Bre1p and ProtA-Bre1p(C648G, C651G) after 2 days in culture and after H_2O_2 treatment determined by DHE staining and TUNEL staining, respectively. (**E**) DHE-positive cells during chronological ageing were quantified after 2 days in culture using flow cytometry. In each experiment, 10,000 cells were evaluated. Data represent means ± s.d. (n=3). (**F**) TUNEL-positive cells during chronological ageing were quantified after 2 days in culture by manual counting at least 500 cells. Data represent means ± s.d. (n=3). (**G**) DHE-positive cells were quantified after H_2O_2 treatment by manually counting at least 500 cells. Data represent means ± s.d. (n=3). (**H**) TUNEL-positive cells were quantified after H_2O_2 treatment by manually counting at least 500 cells. Data represent means ± s.d. (n=3).

required for the anti-apoptotic property of Bre1p. To address this question, we analysed the chronological lifespan of the yeast strain *FLAG-htb1K123R*, which expresses a FLAG-tagged histone H2B variant containing a lysine-to-arginine substitution at Lys123 and therefore fails to be ubiquitylated (Sun and Allis, 2002). We found that these cells showed an early onset of cell death during chronological ageing, similarly to *Δbre1 FLAG-HTB1* cells that lack *BRE1* and express FLAG-tagged wild type histone H2B (36±7% cell viability versus 32±5% cell viability after 3 days in culture; Fig. 4A).

We next determined whether the lack of Bre1p and the lack of histone H2B ubiquitylation affect the same pathway leading to an early onset of cell death during chronological ageing. To do so, we disrupted *BRE1* in *FLAG-htb1K123R* cells and analysed the chronological lifespan of the resulting double mutant *Δbre1 FLAG-htb1K123R*. An additive phenotype for the double mutant is expected if the two mutations affect the chronological lifespan of yeast independently. However, the double mutant strain *Δbre1 FLAG-htb1K123R* showed no further decrease in survival during chronological ageing when compared with either single mutant (35±5% cell viability versus 36±7% and 32±5% cell viability, respectively, after 3 days in culture; Fig. 4A), indicating that both mutations affect the same pathway. Moreover, these data suggest that Bre1p confers resistance to age-induced apoptosis by mediating histone H2B ubiquitylation. Likewise, FLAG-*htb1-K123R* mutant cells showed H_2O_2 sensitivity that was similar to that in *Δbre1 FLAG-HTB1* cells (66±7% cell viability versus 69±7% cell viability; Fig. 4B), whereas the double mutant strain *Δbre1 FLAG-htb1K123R* exhibited no further decrease in survival when compared with either single mutant (70±6% cell viability versus 66±7% and 68±7% cell viability, respectively; Fig. 4B). Together, our data indicate that Bre1p

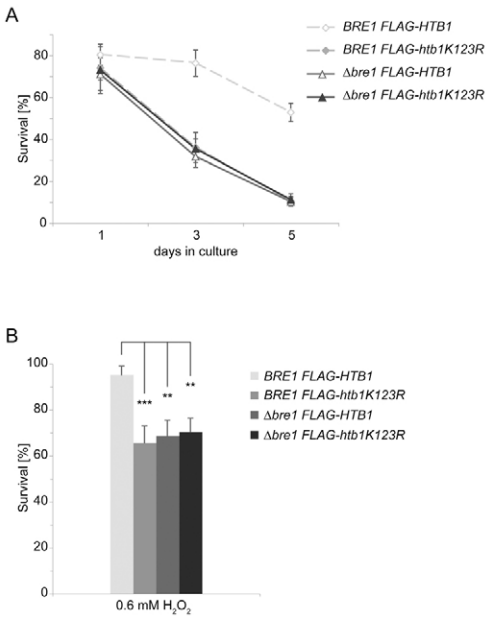

Fig. 4. Histone H2B ubiquitylation confers apoptosis resistance. (**A**) Survival determined by clonogenicity of wild-type cells (*BRE1 FLAG-HTB1*) and its derivates *BRE1 FLAG-htb1K123R*, *Δbre1 FLAG-HTB1* and *Δbre1 FLAG-htb1K123R* during chronological ageing. Data represent means ± s.d. (n=6). (**B**) The same strains were treated with 0.6 mM H_2O_2 and survival was determined by clonogenicity. Data represent means ± s.d. (n=6; ***P<0.001, **P<0.01).

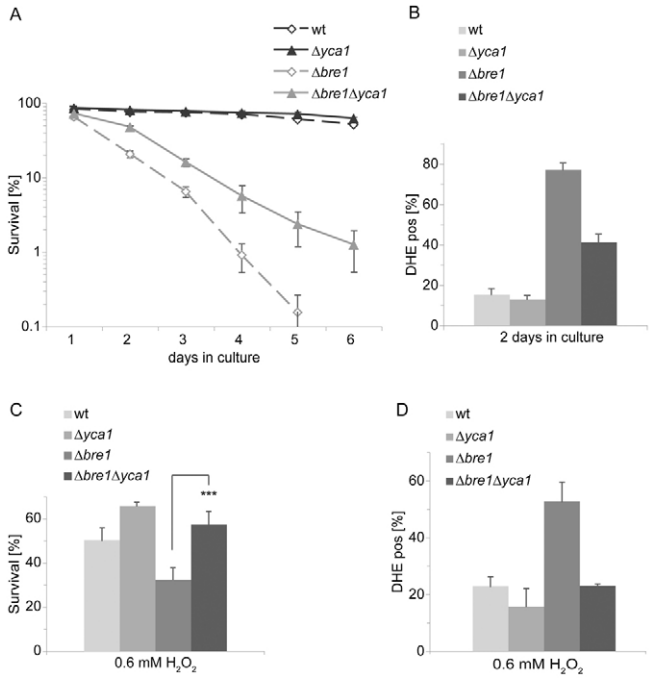

Fig. 5. Death of *Δbre1* cells depends on the yeast caspase-like protein Yca1p. (**A**) Survival determined by clonogenicity during chronological ageing of wild-type (wt), *Δyca1*, *Δbre1* and *Δbre1Δyca1* cells. Data represent means ± s.d. (n=6). (**B**) DHE-positive wild type (wt), *Δyca1*, *Δbre1* and *Δbre1Δyca1* cells were quantified after 2 days in culture using flow cytometry. In each experiment, 10,000 cells were evaluated. Data represent means ± s.d. (n=3). (**C**) Wild-type (wt), *Δyca1*, *Δbre1* and *Δbre1Δyca1* cells were exposed to 0.6 mM H_2O_2 for 8 hours and survival was determined by clonogenicity. Data represent means ± s.d. (n=6). (**D**) DHE-positive positive cells were quantified after H_2O_2 treatment by manually counting at least 500 cells. Data represent means ± s.d. (n=3).

diminishes apoptotic death by mediating histone H2B ubiquitylation.

Death of *Δbre1* cells depends on the yeast metacaspase Yca1p

Yeast cells lacking *BRE1* were sensitive to apoptotic stimuli and displayed morphological markers of apoptosis upon H_2O_2 treatment and during chronological ageing (Figs 1 and 2). Apoptosis in yeast can occur in a caspase-dependent or caspase-independent manner (Madeo et al., 2009). To investigate whether the yeast metacaspase Yca1p is involved in the death of *Δbre1* cells, we generated a *Δyca1Δbre1* double-mutant strain. The death rate during chronological ageing was decreased in *Δbre1Δyca1* cells compared with *Δbre1* cells (47±3% cell viability versus 21±2% cell viability after 2 days in culture; Fig. 5A), indicating that Yca1p is required for cell death of *Δbre1* cells. However, the death of *Δbre1* cells is not exclusively Yca1p-dependent during chronological ageing as the double mutant *Δbre1Δyca1* exhibited a higher death rate than wild type cells (47±3% cell viability versus 75±5% cell viability after 2 days in culture; Fig. 5A). In addition, *Δbre1Δyca1* cells displayed less ROS accumulation during chronological ageing when compared with *Δbre1* cells (41±4% versus 77±3% cells were DHE positive after 2 days in culture; Fig. 5B), but more than wild-type cells (41±4% versus 15±3% cells were DHE positive after 2 days in culture; Fig. 5B). Therefore, the death of *Δbre1* cells during chronological ageing is partially Yca1p dependent. Notably, unlike *Δbre1* cells, *Δbre1Δyca1* cells did not display apoptotic DNA fragmentation during chronological ageing as detected by TUNEL labelling (supplementary material Fig. S1), suggesting that *Δbre1Δyca1* cells die in a necrotic rather than apoptotic fashion. Annexin-V and propidium iodide (PI) costaining was used to further analyse apoptotic externalisation of phosphatidylserine and necrotic membrane permeabilisation. *Δbre1* cells, unlike *Δbre1Δyca1* cells, displayed externalisation of phosphatidylserine as detected by annexin-V staining (supplementary material Fig. S1), further supporting the notion that *Δbre1* cells die in an apoptotic manner, whereas the death of *Δbre1Δyca1* cells is of a necrotic nature.

Next, we tested the response of *Δbre1Δyca1* cells to H_2O_2 exposure. As shown in Fig. 5C, the death rate after H_2O_2 treatment was decreased in *Δbre1Δyca1* cells when compared with *Δbre1* cells (57±5% cell viability versus 32±5% cell viability; Fig. 5C). Unlike during chronological ageing, the death of *Δbre1* cells seems to be exclusively Yca1p dependent after H_2O_2 treatment, because the double-mutant *Δbre1Δyca1* cells show survival rates similar to wild-type cells (57±5% cell viability versus 50±6% cell viability; Fig. 5C). In addition, the population of *Δbre1Δyca1* cells with ROS accumulation after H_2O_2 treatment was smaller when compared with *Δbre1* cells but similar to wild-type cells (23±1% DHE-positive *Δbre1Δyca1* cells compared with 53±7% DHE-positive *Δbre1* and 23±3% DHE-positive wild type cells; Fig. 5D). Taken together, our data indicate that Yca1p is required to activate apoptosis in *Δbre1* cells in response to H_2O_2 treatment.

Apoptosis in yeast can also be Yca1p independent. The mitochondria-localised apoptosis-inducing factor Aif1p (Wissing

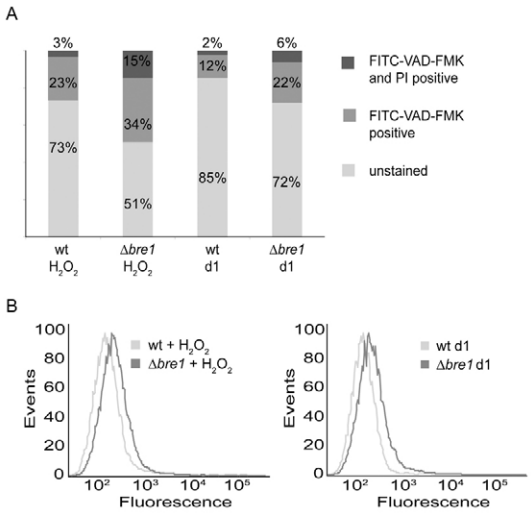

Fig. 6. Cells lacking *BRE1* show increased caspase activity.
(**A**) Chronological aged wild-type and *Δbre1* cells and cells treated with 0.6 mM H_2O_2 were labelled with FITC-VAD-fmk and propidium iodide (PI) and analysed by flow cytometry. One representative experiment from three independent experiments with similar results is shown. (**B**) Chronological aged wild-type and *Δbre1* cells and cells treated with 0.6 mM H_2O_2 were incubated with the caspase substrate $(L\text{-}Asp)_2$ rhodamine 110 (D_2R) and analysed by flow cytometry. One representative experiment from three independent experiments with similar results is shown.

et al., 2004), the endonuclease Nuc1p/EndoG (Buttner et al., 2007) and possibly the nuclear serine protease Nma111p (Fahrenkrog et al., 2004) can execute caspase-independent apoptosis. To test whether these pro-apoptotic factors are also involved in the Bre1p pathway, we generated distinct double-mutant strains, *Δbre1Δnuc1*, *Δbre1Δaif1* and *Δbre1Δnma111*. As shown in supplementary material Fig. S2, none of these strains displayed better survival during chronological ageing or upon H_2O_2 treatment when compared with *Δbre1* cells, indicating that these other pro-apoptotic factors do not contribute to the Bre1p pathway.

Cells lacking *BRE1* show increased caspase activity

Since disruption of *YCA1* desensitises *Δbre1* cells towards apoptotic stimuli, we asked whether or not the caspase activity of Yca1p is involved in cell death execution of *Δbre1* cells. To address this question, we tested whether *Δbre1* cells exhibited higher caspase activity after induction of apoptosis compared with wild-type cells. To monitor potential caspase activation, yeast cells were incubated with FITC-labelled VAD-fmk (FITC-VAD-fmk). FITC-VAD-fmk binds specifically to the active centre of metazoan caspases, which enables a flow-cytometric determination of cells with active caspases (Madeo et al., 2002). As FITC-VAD-fmk might have the limitation that it stains dead cells nonspecificly (Wysocki and Kron, 2004), we additionally used propidium iodide (PI) to distinguish between apoptotic (PI negative) and necrotic cells (PI positive). Wild-type and *BRE1*-disrupted cells were compared after stimulation with 0.6 mM H_2O_2 or after 1 day of chronological ageing. As shown in Fig. 6A, after treatment with 0.6 mM H_2O_2 34% of *Δbre1* cells showed caspase activity (FITC positive, PI negative) (corresponding FACS profiles are found in supplementary material Fig. S3), whereas only 23% of wild-type cells exhibited caspase activity. Consistently, we monitored caspase activity in about 21% of aged *Δbre1* cells, but only in 13% of wild-type cells (Fig. 6A). To confirm these findings, we used the caspase substrate $(L\text{-}Asp)_2$ rhodamine 110 (D_2R), which is designated for the detection of caspase activity in mammalian cells. D_2R is non-fluorescent, however, upon cleavage by a caspase, the released rhodamine 110 gives rise to a fluorescence signal, which enables a flow cytometric determination of caspase activity in cells. As shown in Fig. 6B, after 1 day of chronological ageing and after treatment with 0.6 mM H_2O_2, *Δbre1* cells exhibited higher caspase activity than wild-type cells. Taken together, our data show that *Δbre1* cells have higher caspase activity than wild-type cells, further supporting the notion that Bre1p acts in an Yca1p-dependent manner.

Discussion

BRE1 encodes an evolutionarily conserved E3 ubiquitin ligase that, in yeast, catalyses monoubiquitylation of histone H2B at lysine 123 (K123). Histone H2B K123 ubiquitylation is involved in a variety of cellular processes, such as gene activation, gene silencing and checkpoint activation after DNA damage (Briggs et al., 2001; Dover et al., 2002; Game et al., 2006; Giannattasio et al., 2005; Henry et al., 2003; Kao et al., 2004; Mutiu et al., 2007; Sun and Allis, 2002; Wyce et al., 2007; Xiao et al., 2005). Here, we uncover a new role for Bre1p and show that *S. cerevisiae* Bre1p protects yeast cells from H_2O_2-induced cell death, whereas deletion of *BRE1* enhances cell death and leads to decreased lifespan during chronological ageing. Also, we show that Bre1p activity in yeast apoptosis requires its E3 ubiquitin ligase activity, thereby linking yeast apoptosis to histone H2B monoubiquitylation. Furthermore, we show that Bre1p protects yeast cells from death in a Yca1p-dependent manner.

A role for Bre1p in programmed cell death

Enhanced levels of Bre1p protect yeast from H_2O_2-induced cell death and diminish the development of apoptotic hallmarks, such as ROS accumulation and DNA single-strand breaks (Fig. 1). By contrast, cells lacking Bre1p are more sensitive to H_2O_2 treatment (Fig. 1) and show a decreased lifespan during chronological ageing (Fig. 2A), which coincided with the appearance of apoptotic markers (Fig. 2B-D). A role for Bre1p in yeast cell death has not been assumed, but is consistent with the recent identification of its *C. elegans* homologue as a regulator of germ-cell apoptosis in worms (Lettre et al., 2004). Therefore, the anti-apoptotic function of Bre1p is probably evolutionary conserved and it will be interesting to see whether, for example, the human homologues of Bre1p, RNF20 and RNF40, are also implicated in apoptosis regulation.

The ability of Bre1p to reduce cell death is conferred by its E3 ubiquitin ligase activity and histone H2B ubiquitylation (Figs 3 and 4). Bre1p harbours a C-terminal zinc-binding motif known as the RING-finger domain (Hwang et al., 2003) that is frequently found in E3 ubiquitin ligases and is required for catalysing the transfer of ubiquitin from the E2 to the substrate (Deshaies and Joazeiro, 2009). RING domains appear to be crucial for apoptosis regulation, because members of the inhibitor-of-apoptosis protein (IAP) family comprise RING domains, which enable IAPs to mark other proteins such as caspases for proteasomal degradation (Wilson et al., 2002). Our data indicate that, in addition to polyubiquitylation, monoubiquitylation might have a role in apoptosis. We show that *Δbre1* cells complemented with the RING-finger mutants ProtA-Bre1p(C648G, C651G) and ProtA-

Bre1p(L650E) lack monoubiquitylated histone H2B (Fig. 3A) and exhibit increased apoptosis sensitivity similarly to *Δbre1* cells, whereas *Δbre1* cells complemented with a functional ProtA-Bre1p behave in a similar manner to wild-type cells (Fig. 3). These findings suggest that Bre1p requires its E3 ligase activity to confer H2B monoubiquitylation and apoptosis resistance and support the notion that RING domains have a major role in apoptosis regulation.

The importance of H2B monoubiquitylation for the anti-apoptotic activity of Bre1p is further supported by our observation that *htb1-K123R* mutant cells, which fail to ubiquitylate histone H2B, also exhibit increased apoptosis sensitivity, similarly to *Δbre1* cells (Fig. 4A,B). Interestingly, yeast cells that exhibit enhanced levels of ubiquitylated histone H2B owing to the lack of the ubiquitin-specific protease Ubp10p, which cleaves the ubiquitin moiety from histone H2B, are also prone to apoptosis (Bettiga et al., 2004; Emre et al., 2005; Gardner et al., 2005). These data indicate that high levels of histone H2B ubiquitylation can also predispose yeast to apoptotic stimuli in a similar manner to the lack of histone H2B ubiquitylation. However, this study did not address whether or not *Δubp10* cells show increased caspase activity due to high ubiquitylation levels of histone H2B. It is therefore possible that Ubp10p has other targets than ubiquitylated H2B and failure in deubiquitylation of these targets might cause sensitivity to apoptosis in these cells. To rule out this possibility, we analysed the apoptosis sensitivity of *Δbre1Δubp10*. We expected that this double mutant would exhibit increased apoptosis sensitivity when compared with *Δbre1* cells, if Ubp10p acted in a histone-H2B-independent manner. However, *Δbre1* and *Δbre1Δubp10* cells showed similar apoptosis sensitivity during chronological ageing (supplementary material Fig. S4A). After 2 days in culture, *BRE1*-null cells showed survival rates of 23±4% compared with 24±2% in *Δbre1Δubp10* cells (supplementary material Fig. S4A). In addition, *ΔbreΔubp10* and *Δbre1* cells showed similar survival rates after H_2O_2 treatment [31±5% cell viability versus 28±3% cell viability (supplementary material Fig. S4B)]. These data suggest that *UBP10* disruption causes apoptosis sensitivity because of failures in histone H2B deubiquitylation. Therefore, the lack of histone H2B ubiquitylation, as well as high levels of histone H2B ubiquitylation, appear to predispose yeast to apoptotic stimuli, indicating that H2B monoubiquitylation needs to be tightly regulated to ensure cell survival.

The yeast metacaspase Yca1p appears to be essential for approximately 40% of the investigated cell-death scenarios in yeast (Madeo et al., 2009) and we show here that apoptosis in *Δbre1* cells in fact depends on Yca1p, because *YCA1* disruption leads to reduced apoptosis sensitivity in cells lacking Bre1p (Fig. 5). Furthermore, the death of *Δbre1* cells depends neither on other pro-apoptotic factors, such as EndoG, Nma111p nor on Aif1p (supplementary material Fig. S2), indicating that Bre1p anti-apoptotic activity is, at least in part, caspase dependent. Consistently, we show that *Δbre1* cells exhibit increased caspase activity compared with wild-type cells (Fig. 6), suggesting that the caspase-like activity of Yca1p is implicated in the death of *Δbre1* cells.

Possible mechanisms

Our data suggest that lack of histone H2B monoubiquitylation leads to Yca1p-dependent apoptosis during chronological ageing and after H_2O_2 treatment. Histone H2B ubiquitylation has a role in the DNA-damage response, as well as in transcriptional control (Briggs et al., 2001; Dover et al., 2002; Game et al., 2006; Giannattasio et al., 2005; Henry et al., 2003; Kao et al., 2004; Mutiu et al., 2007; Sun and Allis, 2002; Wyce et al., 2007; Xiao et al., 2005). Therefore, cells lacking histone H2B ubiquitylation might undergo Yca1p-dependent apoptosis, either because of accumulated DNA damage, or because of alterations in transcription. Although H2B ubiquitylation has been implicated in transcriptional silencing (Briggs et al., 2001; Mutiu et al., 2007; Sun and Allis, 2002), other studies suggested a positive role for this modification in transcriptional initiation and elongation (Henry et al., 2003; Kao et al., 2004; Wyce et al., 2007; Xiao et al., 2005). Notably, the core apoptotic machinery, including caspases and other regulators of apoptosis, are regulated at the transcriptional level in higher eukaryotes (Zuckerman et al., 2009). Therefore, it is possible that this balance is disturbed in cells lacking H2B ubiquitylation and up- or down-regulated transcription of apoptotic regulators causes apoptosis of these cells. It would be interesting to see whether *YCA1* or other pro-apoptotic proteins are transcriptionally deregulated in *Δbre1* cells. Furthermore, comparison of global mRNA transcripts between wild-type and *Δbre1* cells could help to identify novel regulators of apoptosis.

Histone H2B ubiquitylation is required for Rad9p-mediated checkpoint activation after DNA damage and Rad51p-dependent DNA repair (Game et al., 2006; Giannattasio et al., 2005). Therefore, cells lacking histone H2B ubiquitylation might undergo Yca1p-dependent apoptosis because of accumulated DNA damage. However, neither *Δrad9* nor *Δrad51* cells exhibit apoptosis sensitivity similar to that observed in *Δbre1* cells (our unpublished results), indicating that apoptosis in *Δbre1* is not caused by defects in Rad9p or Rad51p pathways. Moreover, although *Δbre1* cells exhibit sensitivity towards DNA damage induced by methyl methanesulfonate, hydroxyurea and UV radiation, respectively, disruption of Yca1p does not lead to a rescue in survival of *Δbre1* cells under these conditions (data not shown). Therefore, we do not consider it likely that DNA damage causes Yca1p-dependent apoptosis in *Δbre1* cells.

In conclusion, we show that Bre1p confers resistance to apoptosis and Yca1p is required in the apoptosis pathway triggered by *BRE1* disruption. Bre1p is required for histone H2B ubiquitylation and its deletion, which influences transcriptional regulation and DNA repair, activates apoptosis. However, it remains to be seen whether transcription defects, failures in DNA repair, or both processes, activate the apoptotic program in cells lacking H2B ubiquitylation. Future studies in yeast will provide more details on the connection between transcription, DNA repair and apoptosis.

Materials and Methods

Plasmids, yeast strains and culture conditions

To construct the plasmid pBF326, which encodes ProtA-Bre1p, the coding region of *BRE1* was amplified from genomic DNA isolated from BY4742 cells, using the following primers: 5′-CAT GCC ATG GCA ATG ACG GCC GAG CCT GCT A-3′ and 5′-CGC GGA TCC TTA CAA GTG CAC TGT CAA TAA ATC-3′. The PCR product was digested with *Nco*I and *Bam*HI and cloned into pNOPATA1L (Hellmuth et al., 1998). Plasmids pBF346 and pBF499 coding for ProtA-Bre1p(C648G,C651G) and ProtA-Bre1p(L650E), respectively, were constructed by site-directed polymerase chain reaction (PCR) mutagenesis using pBF326 as template according to the manufacturer's instructions (Stratagene, QuikChange Site-Directed Mutagenesis Kit).

Yeast strains are listed in supplementary material Table S1. BY4742 (MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) and derivative strain *Δbre1* were obtained from Euroscarf. *Δubp10* strain was derived from BY4742 and constructed according to Longtine et al. (Longtine et al., 1998). Construction of the *Δbre1Δyca1*, *Δbre1ΔendoG*, *Δbre1Δnma111*, *Δbre1Δaif1* and *Δbre1Δubp10* double-mutant strains was performed according to published methods (Gueldener et al., 2002). Yeast strains YZS276 (*FLAG-HTB1*) and YZS277 (*FLAG-htb1K123R*) were a gift from C. David Allis (Rockefeller University, New York, NY) (Sun and Allis, 2002a).

Δbre1 FLAG-HTB1 and *Δbre1 FLAG-htb1K123R* were derived from YZS276 and YZS277, respectively and constructed as published (Longtine et al., 1998). *HTB1-GFP Δbre1* was derived from *Δbre1* strain and constructed as described (Longtine et al., 1998). Survival plating was conducted on YPAD (1% yeast extract, 2% peptone, and 2% glucose, 40 mg/ml Adenine) medium supplemented with 2% agar. For experiments testing oxygen stress and chronological lifespan, strains were grown in synthetic complete medium (SC) with 2% glucose (Fink, 1991). Transformation of yeast cells was performed by the lithium acetate procedure, as described (Gietz et al., 1992).

Survival plating and test for apoptotic markers

For experiments testing oxygen stress, cultures were inoculated at low cell density (2×10^5 cells/ml) in SCGlu, grown to late log phase (OD_{600}~2) and exposed to 0.6 mM hydrogen peroxide (H_2O_2) for 8 hours. For survival plating, yeast cultures were diluted in water, the cell concentration was determined using a Neubauer counting chamber and aliquots containing 500 cells were plated on YPAD plates. The number of colonies was determined after incubation for 2 days at 30°C. Percentage of cell survival was calculated for each strain by counting the number of colonies formed following H_2O_2 treatment relative to untreated cells. Apoptotic tests using DHE staining, annexin-V and PI staining and TUNEL staining were performed as described previously (Belanger et al., 2009; Buttner et al., 2007). In each sample, 10,000 cells were evaluated using flow cytometry (FACS-Aria, BD) and processed using BD FACSDiva software. Alternatively, around 500 DHE- and TUNEL-stained cells, respectively, were counted manually. For chronological ageing experiments, cultures were inoculated from fresh overnight cultures at low cell density (1×10^6 cells/ml) and aliquots were taken to perform survival plating and tests for apoptotic markers as described above.

In vivo staining of caspase activity by flow cytometry

5×10^6 cells were harvested, washed once in 1 ml PBS and incubated in PBS containing 10 μM FITC-VAD-fmk (CaspACE, Promega, Dübendorf) for 20 minutes at 30°C in the dark. Next, the cells were washed with PBS and resuspended in PBS containing 1 μg/ml propidium iodide (PI) and analysed by flow cytometry (FACS-Aria, BD). Cleavage of the caspase substrate (aspartyl)-Rhodamine 110 (D_2R) [CaspSCREEN (BioVision)] was measured by flow cytometry (FACS-Aria, BD) according to the manufacturer's instructions.

Immunoblotting

Protein lysates were prepared using a lysis buffer containing 100 mM NaCl, 50 mM Tris-HCl pH 7.5, 50 mM NaF, 5 mM EDTA and 0.1% IGEPAL. Lysate proteins were resolved on 12% acrylamide-Tris-HCl gels and transferred to an Immobilon PVDF membrane (Millipore, Billerica, MA). The filters were hybridised with anti-GFP antibody (Dianova, clone MA1-26343; Hamburg, Germany) and anti-protein-A antibody (Sigma), respectively and the peroxidase-conjugated secondary anti-mouse antibody (Sigma).

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