The inhibitor-of-apoptosis protein Bir1p protects against apoptosis in *S. cerevisiae* and is a substrate for the yeast homologue of Omi/HtrA2

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Summary

Inhibitor-of-apoptosis proteins (IAPs) play a crucial role in the regulation of metazoan apoptosis. IAPs are typically characterized by the presence of one to three baculovirus IAP repeat (BIR) domains that are essential for their anti-apoptotic activity. Bir1p is the sole BIR-protein in yeast and has been shown to participate in chromosome segregation events. Here, we show that Bir1p is a substrate for Nma111p, which is the homologue of the human pro-apoptotic serine protease Omi/HtrA2 and which is known to mediate apoptosis in yeast. Bir1p is a cytoplasmic and nuclear protein, and yeast cells lacking *bir1* are more sensitive to apoptosis induced by oxidative stress. Consistently, overexpression of Bir1p reduces apoptosis-like cell death, whereas this protective effect can be antagonized in vivo by simultaneous overexpression of Nma111p. Moreover, chronologically aged cells that constitutively overexpress Bir1p show a delayed onset of cell death. Therefore, Bir1p, like its closest metazoan homologues deterin and survivin, has dual functions: it participates in chromosome segregation events and cytokinesis and exhibits anti-apoptotic activity.

Key words: Bir1p, IAP, Nma111p, Omi/HtrA2, Yeast apoptosis

Introduction

Apoptosis is a physiological form of programmed cell death vital to the development and maintenance of multicellular organisms. Apoptosis can be triggered by distinct extracellular and intracellular stimuli, and it can involve the activation of a unique class of cysteine proteases known as caspases (Earnshaw et al., 1999; Garrido and Kroemer, 2004; Kroemer and Martin, 2005; Riedl and Shi, 2004). Activation of caspases leads to irreversible proteolysis of their target proteins, and caspase activation therefore needs to be tightly regulated. Key regulators of caspases and hence apoptosis activation are a set of proteins called inhibitor-of-apoptosis proteins (IAPs).

IAPs have originally been identified in baculoviruses as factors that protect infected host cells from virus-induced cell death (Crook et al., 1993). Homologues have been identified in yeast, worms, flies and mammalian cells (Liston et al., 2003; Vaux and Silke, 2005; Verhagen et al., 2001). IAPs are characterized by the presence of one to three copies of baculovirus IAP repeat (BIR) domains, which are zinc-binding domains of typically 70 to 80 residues, essential for the anti-apoptotic activity of the IAPs. Additionally, IAPs contain a second zinc-binding motif known as RING domain, which exhibit E3-ubiquitin-ligase activity (Liston et al., 2003; Vaux and Silke, 2005; Verhagen et al., 2001). However, not all BIR-domain-containing proteins (BIRPs) appear to have anti-apoptotic properties, namely BIR-proteins in yeast, *C. elegans*, plants and protozoans (reviewed in Liston et al., 2003; Vaux and Silke, 2005; Verhagen et al., 2001). These BIRPs lack a RING domain and their BIR-domains are structurally different from the BIR-domains of IAPs. They are therefore referred as to type-II BIR domains, whereas IAPs harbour type-I BIR-domains. The type-II BIRPs, such as the *C. elegans* proteins Bir1 and Bir2 (Fraser et al., 1999) and the yeast Bir1p protein (Bouck and Bloom, 2005; Li et al., 2000; Uren et al., 1999; Yoon and Carbon, 1999), appear to have roles in chromosome segregation and cytokinesis rather than in apoptosis. However, the mammalian protein survivin, a type-II BIRP, has been shown to play a role in cell-cycle control as well as having anti-apoptotic properties (Ambrosini et al., 1997; Lens et al., 2003; Skoufias et al., 2000; Temme et al., 2003). Moreover, the *Drosophila* proteins Deterin and Bruce (also known as Apollon in mammals) and the murine protein TIAP, all type-II BIRPs, are able to inhibit apoptosis (Bartke et al., 2004; Hao et al., 2004; Jones et al., 2000; Vernooy et al., 2002).

Based on its genome, *S. cerevisiae* harbours only one BIRP, termed Bir1p, which belongs to the type-II family of BIRPs. Bir1p participates in cell division (Li et al., 2000; Uren et al., 1999; Yoon and Carbon, 1999), but a putative role for Bir1p in yeast apoptosis has not been studied, because yeast was supposed to have no apoptosis-like death programme. However, *S. cerevisiae* has recently become a useful model to study apoptosis (reviewed in Madeo et al., 2004) and homologues to key regulators of mammalian apoptosis have been identified, such as the yeast caspase-like protein Yca1p (Madeo et al., 2002), Cdc48 (Madeo et al., 1997) and Nma111p, the yeast homologue of Omi/HtrA2 (Fahrenkrog et

al., 2004), the mitochondrial fission factor Fis1p (Fannjiang et al., 2004) and the apoptosis-inducing factor Aif1p (Wissing et al., 2004). DNA fragmentation and compaction are important steps during apoptosis and in this context an apoptotic nuclease, Tat-D, has been identified in yeast (Qiu et al., 2005); DNA fragmentation during yeast apoptosis seems to depend, similar to metazoan apoptosis, on phosphorylation of histone H2B (Ahn et al., 2005a; Ahn et al., 2005b).

Nma111p belongs to the family of HtrA-like serine proteases, and we have recently shown that Nma111p, like its mammalian homologue Omi/HtrA2, has pro-apoptotic activity (Fahrenkrog et al., 2004). Omi/HtrA2 can promote apoptosis by binding and degrading cellular IAPs (Hegde et al., 2002; Martins et al., 2002; Sekine et al., 2005; Srinivasula et al., 2003; Yang et al., 2003), whereas a substrate for the yeast Omi/HtrA2 homologue Nma111p has not yet been identified.

Here, we identify Bir1p as a substrate for Nma111p because Nma111p overexpression leads to proteolytic degradation of Bir1p in vivo. Within yeast cells, Bir1p localizes to the cytoplasm and the nucleus and *BIR1* deletion mutants show typical apoptotic hallmarks and decreased survival rates after induction of apoptosis by hydrogen peroxide (H_2O_2). By contrast, yeast cells overexpressing Bir1p do not exhibit apoptotic markers and show better survival rates than wild-type cells after treatment with H_2O_2 and long-term cultivation. Moreover, simultaneous overexpression of Bir1p and Nma111p antagonizes the cell-death-protective activity of Bir1p. Therefore, Bir1p, like its closest homologues mammalian survivin, murine TIAP and *Drosophila* Deterin and Bruce, has anti-apoptotic properties.

Results

Bir1p is a substrate for Nma111p

Mammalian IAPs have been identified as targets for the pro-apoptotic serine protease Omi/HtrA2 (Bartke et al., 2004; Hegde et al., 2002; Jin et al., 2003; Martins et al., 2002; Sekine et al., 2005; Srinivasula et al., 2003; Suzuki et al., 2001; Suzuki et al., 2004; Verhagen et al., 2002; Yang et al., 2003). To analyse whether Bir1p, the unique BIRP in yeast, is a substrate for Nma111p, the yeast homologue of Omi/HtrA2 (Fahrenkrog et al., 2004), we inserted a C-terminal GFP-tag to the *BIR1* ORF in the yeast wild-type strain BYa. The resulting Bir1p-GFP strain was transformed with a plasmid pYES-ProtA-Nma111p (Fahrenkrog et al., 2004) to overexpress Nma111p. The effect of overexpressing Nma111p on Bir1-GFP expression was analysed by western blotting. As shown in Fig. 1A, Nma111p overexpression leads to the appearance of a degradation band of Bir1p-GFP that is lacking in the wild-type background (Fig. 1A, BYa). Overexpression of a serine protease mutant of Nma111p, Nma111p-S235C (Fahrenkrog et al., 2004), does not lead to degradation of Bir1p-GFP. Therefore, Bir1p is a substrate for Nma111p *in vivo*, and cleavage of Bir1p by Nma111p depends on the serine-protease activity of Nma111p.

Bir1p physically interacts with Nma111p but not with Yca1p

Next, we analysed whether Bir1p and Nma111p are interacting directly. Among the HtrA-family of serine proteases, Nma111p is unique, as it harbours an internal duplication of the HtrA-like sequence (Clausen et al., 2002; Pallen and Wren, 1997; Ponting, 1997). The N-terminal repeat of Nma111p retains the catalytic triade residues of HtrA-like serine proteases, whereas the C-terminal repeat contains an incomplete serine protease site that is supposed to be non-functional (Pallen and Wren, 1997; Ponting, 1997). To analyse whether Nma111p can interact with Bir1p and, if so, which repeat of Nma111p is used, we incubated recombinant GST-Bir1p fusion protein with two in vitro transcribed and translated ^{35}S-labelled fragments of Nma111p, Nma111p-N and Nma111p-C. As shown in Fig. 1B, the N-terminal repeat of Nma111p interacts with GST-Bir1p but not with GST alone, whereas the C-terminal repeat of Nma111p could be detected mainly in the unbound fraction. Therefore, Bir1p physically interacts with the N-terminal HtrA-like repeat of Nma111p.

To test whether Bir1p is also interacting with the yeast caspase Yca1p (Madeo et al., 2002), we expressed ^{35}S-labelled Yca1p in vitro and tested its ability to bind to GST, GST-Bir1p and GST-Nma111p. Yca1p is neither interacting with Bir1p nor with Nma111p directly (Fig. 1C), indicating that Bir1p, contrary to mammalian IAPs, cannot directly interact with the yeast caspase Yca1p.

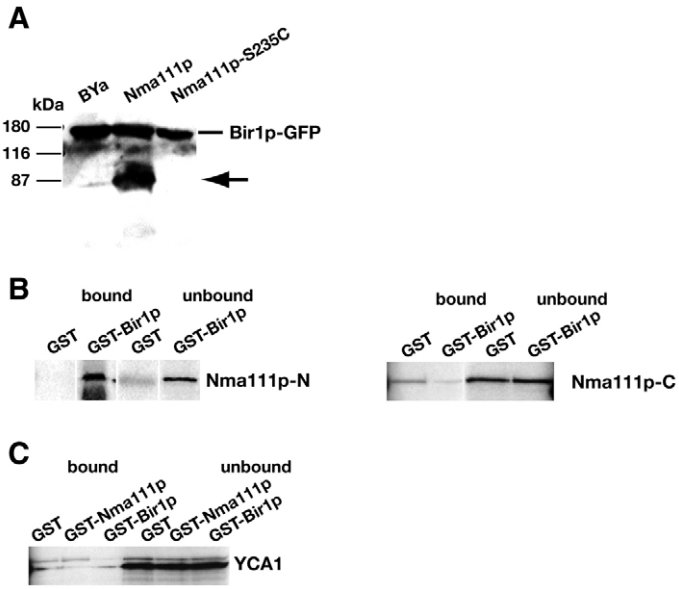


Fig. 1. (A) Western blot analysis of Bir1p-GFP expression. Bir1p-GFP cells transformed with either plasmid-borne N-terminally protein-A-tagged Nma111p or Nma111-S235C were grown in glucose medium and subsequently shifted to galactose medium for 24 hours. Bir1p-GFP cells grown in YPAD were used as control cells. Cell lysates were prepared, immunoblotted and probed with a monoclonal anti-GFP antibody. (B) In vitro binding-study of Nma111p and Bir1p. Recombinantly expressed GST-Bir1p and GST were incubated with in vitro-synthesized ^{35}S-labelled Nma111p-fragments. Unbound and bound fractions were analysed by SDS-PAGE and autoradiography. (C) In vitro binding study of Yca1p, Nma111p and Bir1p. Recombinantly expressed GST-Bir1p, GST-Nma111p and GST were incubated with in vitro-synthesized ^{35}S-labelled Yca1p. Unbound and bound fractions were analysed by SDS-PAGE and autoradiography.

Bir1p is a cytoplasmic and nuclear protein

We have recently shown that Nma111p is a nuclear protein

(Fahrenkrog et al., 2004) and were therefore aiming to analyse whether Nma111p and Bir1p localize to the same subcellular compartment(s). Earlier studies on the subcellular localization of Bir1p failed to localize the full-length protein (Uren et al., 1999; Yoon and Carbon, 1999), whereas an N-terminal fragment of Bir1p showed nuclear localization (Uren et al., 1999) and led to the assumption that Bir1p is a nuclear protein. To determine the subcellular localization of Bir1p more precisely, we used our Bir1p-GFP strain and analysed the localization of Bir1p-GFP by direct fluorescence microscopy. Since the expression levels of Bir1p in the yeast cells are typically relatively low, we were unable to detect Bir1p-GFP by direct fluorescence microscopy under steady-state conditions. Induction of Nma111p overexpression, however, leads to increased expression levels of Bir1p-GFP (data not shown), and we therefore analysed the localization of Bir1p-GFP in the Bir1p-GFP strain overexpressing Nma111p after induction by galactose (see above). As shown in Fig. 2 (left panel), Bir1p-GFP is evenly distributed within the yeast cells under recess of the vacuole indicating that Bir1p localizes to the cytoplasm and the nucleus of the yeast cells.

To confirm this subcellular localization of Bir1p under steady-state conditions, we transfected *BIR1*-disrupted cells (hereafter referred to as Δ*bir1* cells; see Materials and Methods) with a plasmid (pNOPPATA1W-Bir1p) to express a protein-A-tagged Bir1p fusion protein (ProtA-Bir1p; see Materials and Methods). Next, we determined the subcellular localization of ProtA-Bir1p by indirect immunofluorescence microscopy using a primary polyclonal anti-protein-A antibody and a secondary Alexa-Fluor-488-labelled anti-rabbit IgG antibody. The plasmid-borne ProtA-Bir1p fusion protein was detected in the cytoplasm and the nucleus of the yeast cells, exactly like the endogenous Bir1p-GFP (Fig. 2, middle panel). The same localization was observed for a Bir1p-TAP fusion protein by indirect immunofluorescence microscopy with the primary anti-protein-A antibody and the secondary Alexa-Fluor-488-labelled antibody (Fig. 2, right panel), when Bir1p was genomically fused to a tandem affinity purification-tag (TAP-tag) (Rigaut et al., 1999) at the C-terminus of the ORF (see Materials and Methods). Therefore, we conclude that Bir1p localizes to the cytoplasm and the nucleus of yeast cells.

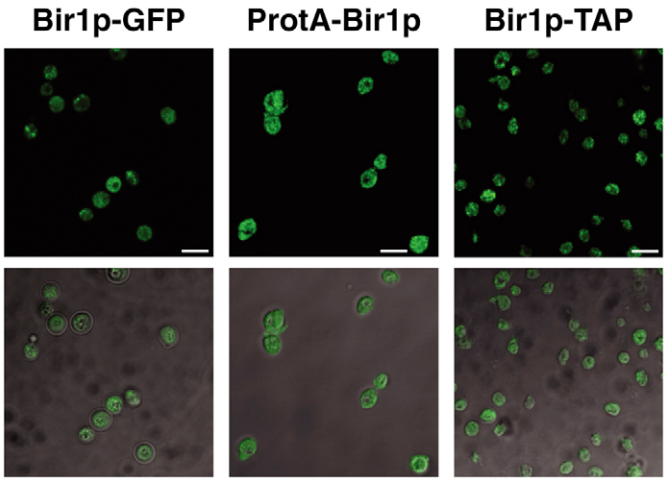


Fig. 2. Bir1p localizes to the cytoplasm and the nucleus of yeast cells. Confocal fluorescence micrographs (top), and coincident fluorescence and differential-interference contrast images (bottom) are shown. Haploid cells, whose endogenous Bir1p was C-terminally tagged with GFP and transformed with pYES-ProtA-Nma111p, were examined by direct fluorescence after induction of Nma111p overexpression (left panels). Δ*bir1* cells were transformed with pNOPPATA1W-Bir1p to express N-terminal protein-A-tagged Bir1p (ProtA-Bir1p) and examined by indirect immunofluorescence using a primary polyclonal anti-protein-A antibody and a secondary anti-rabbit IgG antibody labelled with Alexa Fluor 488 (middle panels). Haploid cells that were genomically tagged with a tandem-affinity purification tag at the C-terminus of endogenous Bir1p (Bir1p-TAP) and analysed by indirect immunofluorescence using a primary anti-protein A antibody and a secondary antibody labelled with Alexa Fluor 488 (right panels). Bars, 5 μm.

Δ*bir1* cells show apoptotic hallmarks

To explore whether Bir1p has anti-apoptotic activity, wild-type and Δ*bir1* cells were analysed for apoptotic hallmarks. *BIR1* is not an essential gene in most genetic backgrounds in *S. cerevisiae*, but Δ*bir1* cells grown in minimal medium show sporulation and chromosome segregation defects (Li et al., 2000; Uren et al., 1999; Yoon and Carbon, 1999). To avoid such defects, both wild-type and Δ*bir1* cells were grown in rich medium, in which Δ*bir1* cells show normal vegetative growth (Uren et al., 1999; Yoon and Carbon, 1999). Wild-type and Δ*bir1* cells were analysed for apoptotic hallmarks, such as chromatin condensation and fragmentation, DNA-strand breaks, and accumulation of reactive oxygen species (ROS). To test yeast cells for chromatin condensation, Δ*bir1* cells and wild-type cells were grown to mid-log phase, and apoptosis was induced by treating cells with 0.8 mM H_2O_2 for 4 hours. Next, cells were incubated with Sytox-Green nucleic acid stain to visualize the DNA. In wild-type cells, DNA is evenly distributed within the nucleus, whereas in Δ*bir1* cells DNA is strongly condensed within the nucleus (Fig. 3A, top panel).

Another phenotypic marker of apoptosis is DNA fragmentation, which can detected by the TUNEL assay (Gavrieli et al., 1992; Gorczyca et al., 1993). As shown in Fig. 3A (middle panel), after induction of apoptosis by H_2O_2, wild-type cells show a weak TUNEL-negative phenotype, whereas Δ*bir1* cells are strongly TUNEL-positive.

Accumulation of ROS is a key event in triggering apoptosis. Yeast cells can be tested for ROS production by incubation with Dihydrorhodamine 123 (DHR), which in the presence of ROS becomes oxidized to the fluorochrome Rhodamine 123 (Schulz et al., 1996). Only ~20% of wild-type cells show fluorescence after incubation with DHR, whereas ~50% of Δ*bir1* cells showed intense intracellular staining with DHR after treatment with H_2O_2 (Fig. 3A, bottom panel).

Cell survival of wild-type (BYa) and Δ*bir1* cells after induction of apoptosis with H_2O_2 was further tested in a plating assay (see Materials and Methods). Cells were treated with up to 1.2 mM H_2O_2, and Δ*bir1* cells showed a significant reduction in cell survival after treatment when compared with wild-type cells (Fig. 3B). Taken together, yeast Δ*bir1* cells undergo apoptosis more frequently when compared with wild-type cells, indicating that Bir1p has anti-apoptotic properties.

Overexpression of Bir1p protects yeast cells from cell death

To address further the question whether Bir1p inhibits yeast

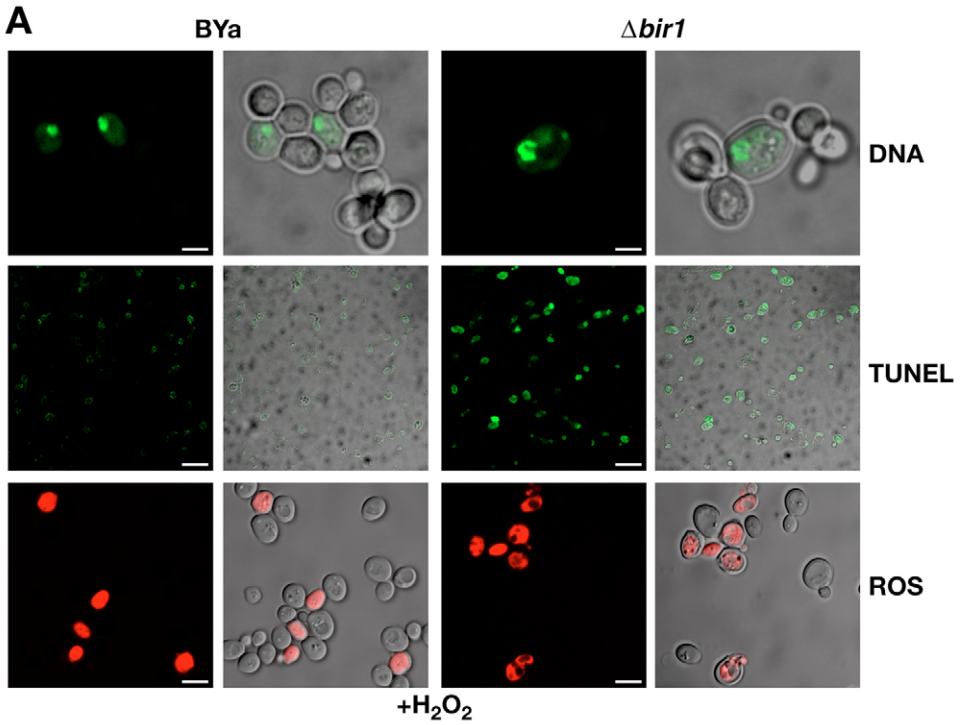


Fig. 3. Δ*bir1* cells undergo apoptosis. Confocal fluorescence (first and third column of panels), and coincident fluorescence and differential-interference-contrast (second and fourth column of panels) images are shown. (A) Wild-type (BYa) and Δ*bir1* cells were grown in YPAD medium, treated with 0.8 mM H_2O_2 for 4 hours and analysed for apoptotic hallmarks. DNA was visualized by Sytox-Green nucleic acid stain. DNA-strand breaks were detected by the TUNEL test and ROS by DHR staining. Bars, 2.5 μm (DNA); 5 μm (ROS); 10 μm (TUNEL). (B) Survival of wild-type (BYa) and Δ*bir1* cells after incubation with H_2O_2 for 4 hours at indicated concentrations. Error bars, mean + s.e.m.

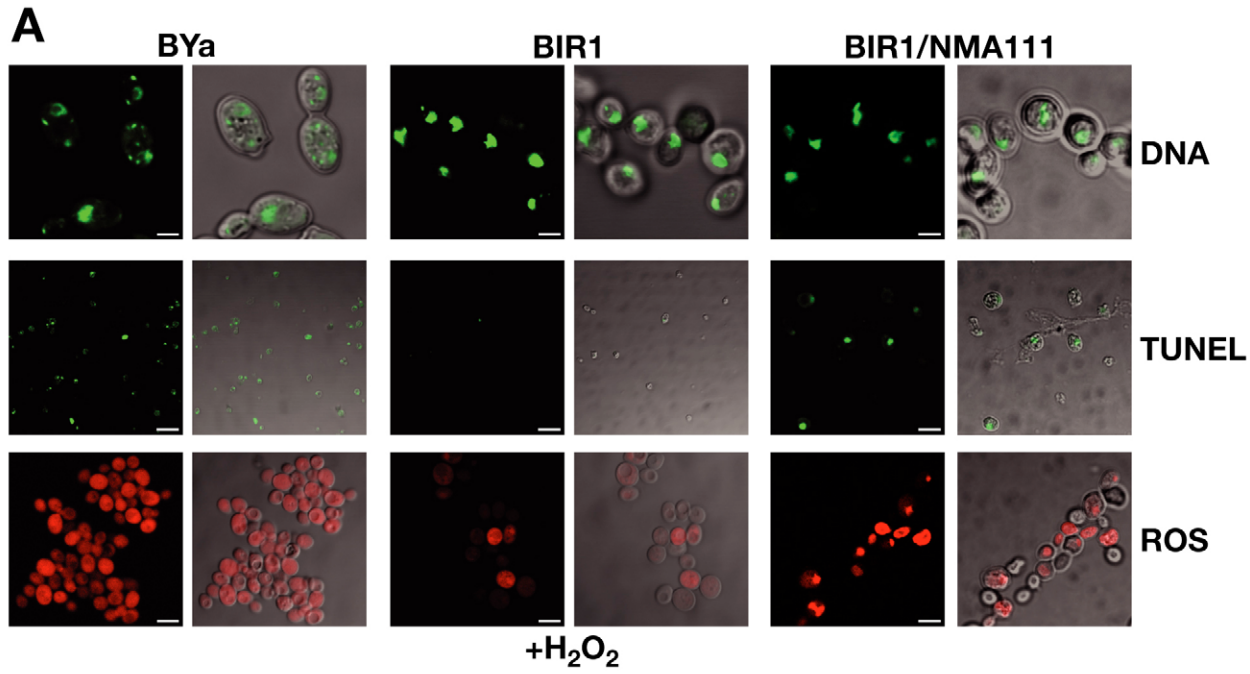
apoptosis directly, we measured cell death and apoptotic markers after overexpression of Bir1p. We transformed the yeast wild-type strain BYa with the plasmid pYES-ProtA-Bir1p to overexpress Bir1p under control of the *GAL1* promoter. A prominent expression of ProtA-Bir1p was observed after 26 hours of induction (data not shown). Overexpression of Bir1p prevents yeast from apoptosis-like cell death because cells treated with 0.8 mM H_2O_2 for 4 hours showed no DNA condensation, no significant ROS production and no DNA fragmentation (Fig. 4A, middle BIR1), whereas wild-type cells treated in the same way show all typical apoptotic hallmarks (Fig. 4A, left BYa). EM investigation of yeast cells overexpressing Bir1p (Fig. 5A,B) revealed no typical apoptotic hallmarks in the nucleus. By contrast, Δ*bir1* cells show invaginations of the nuclear envelope (Fig. 5C), chromatin condensation at the nuclear envelope (Fig. 5D) and vesicles on the outer face of the plasma membrane (Fig. 5E). Additionally, in some cells chromosome segregation defects were observed (Fig. 5F).

To test whether the observed degradation of Bir1p by Nma111p (Fig. 1) is correlated to apoptosis in yeast, we transformed the Bir1p-overexpressing strain with the plasmid pESC-Nma111p to allow simultaneous Nma111p-myc overexpression under control of the *GAL1* promoter. Significant overexpression of Nma111p-myc was observed by western blot 26 hours after induction (data not shown). When these cells that overexpress ProtA-Bir1p and Nma111p at the same time were treated with 0.8 mM H_2O_2 for 4 hours, and analysed for DNA condensation, fragmentation and ROS production, they showed the typical apoptotic hallmarks (Fig. 4A right, BIR1/NMA111), suggesting that degradation of Bir1p by Nma111p leads, in fact, to apoptosis-like cell death in yeast.

Survival of ProtA-Bir1p cells, in comparison to ProtA-Bir1p/Nma111p-myc and ProtA-Nma111p overproducers, was analysed in a plating assay (Fig. 4B). We have previously shown that overexpression of Nma111p induces increased cell death. Treatment with 0.4 mM H_2O_2 for 24 hours resulted in the cell death of ProtA-Nma111p-overexpressing cells and also of ProtA-Bir1p/Nma111p-myc overproducers (survival rates were ~6%, and ~28%, respectively), whereas ProtA-Bir1p cells treated in the same way showed significant better survival rates, i.e. ~50% (Fig. 4B). In the absence of H_2O_2, overexpression of ProtA-Bir1p for 24 hours also leads to an increase in survival (survival rate ~65% compared with ~50% of ProtA-Nma111p overexpressing cells as well as ProtA-Bir1p/Nma111p-myc overproducers). ProtA-Bir1p cells transformed with empty pESC vector instead of pESC-Nma111p showed similar survival rates with or without H_2O_2 treatment. Taken together, overexpression of Bir1p protects yeast cells from cell death, whereas proteolytical degradation of Bir1p by Nma111p leads to apoptosis-like death.

Constitutive overexpression of Bir1p causes late onset of cell death during chronological ageing

Chronological ageing, an ageing process in long-term



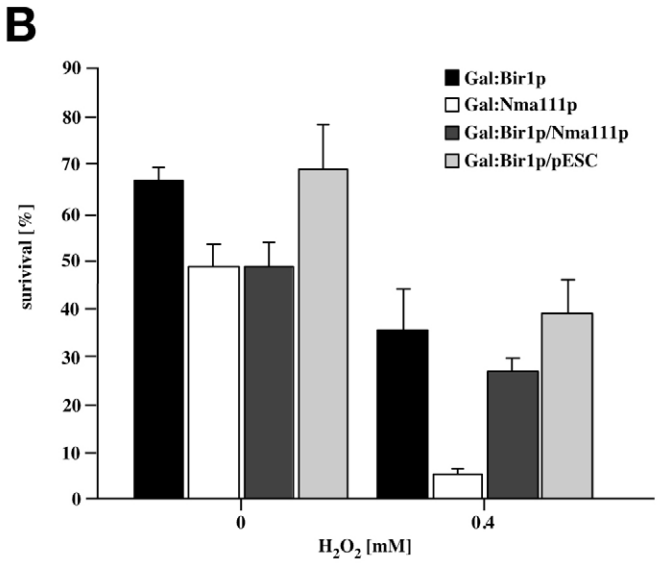


Fig. 4. Overexpression of Bir1p prevents apoptosis-like cell death. Confocal fluorescence (first, third and fifth column of panels), and coincident fluorescence and differential-interference-contrast (second, fourth and sixth column of panels) images are shown. (A) Wild-type cells (BYa), cells overexpressing Bir1p and cells simultaneously overexpressing Bir1p and Nma111p were treated with 0.8 mM H_2O_2 for 4 hours to induce apoptosis. DNA was visualized by Sytox-Green nucleic acid stain. DNA-strand breaks were detected by the TUNEL test and ROS by DHR staining. Bars, 2.5 μm (DNA), 5 μm (ROS); 10 μm (TUNEL). (B) Survival rates of yeast cells overproducing Bir1p compared with overproducers of Bir1p/Nma111p or Nma111p, without pre-treatment or after incubation with 0.4 mM H_2O_2 for 24 hours. Error bars, mean + s.e.m.

cultivated yeast cultures, leads to physiologically induced apoptosis in yeast (Herker et al., 2004; Laun et al., 2001). Therefore, we investigated whether Bir1p is involved in chronological ageing. We observed that disruption of *BIR1* does not significantly accelerate the onset of age-induced cell death, whereas, by contrast, constitutive overexpression of Bir1p under control of the NOP1 promoter (Hellmuth et al., 1998) or disruption of *NMA111* lead to delayed onset of cell death in chronologically aged cells after 5 days in culture (Fig. 6A). The survival rates were reproduced in nine independent experiments. When these yeast cells were tested for apoptotic markers after 5 days in culture, wild-type and Δ*bir1* cells showed typical hallmarks of apoptosis, such as a TUNEL-positive phenotype and the production of ROS (as detected by DHR-staining), whereas *Δnma111* cells, which do not express Nma111p, or Bir1p-overexpressers lack apoptotic markers (Fig. 6B,C).

Discussion

IAPs are important negative regulators of caspases that control their activation. Essential for the anti-apoptotic activity of IAPs are their BIR-domains. We show here that, the unique BIRP in *S. cerevisiae*, Bir1p, is a substrate for the pro-apoptotic serine protease Nma111p and Bir1p inhibits apoptosis in yeast. Also, we show for the first time that native Bir1p is localized in the nucleus as well as in the cytosol, contrary to the previous assumption that it localizes exclusively to the nucleus. Bir1p harbours a putative nuclear-localization signal (Uren et al., 1999) as well as a putative nuclear-export signal in its amino acid sequence, and might therefore shuttle between the cytoplasm and the nucleus, an ability that might be related to its anti-apoptotic function and/or its dual functions in yeast apoptosis, chromosome segregation and cytokinesis. The fact that Bir1p, like Nma111p and YCA1, localizes to the nucleus of yeast cells

further underlines the significant role of the nuclear localization of proteins in yeast apoptosis.

Bir1p is a substrate for Nma111p

Initiation of apoptosis by irreversible caspase activation is antagonized by IAPs, which, in turn, are negatively regulated by IAP-binding proteins, such as Omi/HtrA2 or Smac/DIABLO. We have recently shown that Nma111p, the yeast homologue of Omi/HtrA2, is able to mediate yeast apoptosis. Nevertheless, the molecular mechanism how Nma111p can activate yeast apoptosis has remained elusive. Here, we have identified Bir1p as a target for Nma111p and show that Bir1p acts as an inhibitor of apoptosis in yeast, suggesting that Nma111p exhibits its pro-apoptotic activity, at least in part, because of Bir1p degradation. Bir1p and Nma111p both localize to the nucleus of the yeast cell and interact directly, leading to the degradation of Bir1p by Nma111p in a serine-protease-dependent manner in vivo (Fig. 1). Similar scenarios have been described for mammalian IAPs, such as XIAP or Bruce/Apollon, which are targets for proteolytical degradation by Omi/HtrA2, the mammalian homologue of Nma111p (Bartke et al., 2004; Hegde et al., 2002; Jin et al., 2003; Martins et al., 2002; Sekine et al., 2005; Srinivasula et al., 2003; Suzuki et al., 2001; Suzuki et al., 2004; Verhagen et al., 2002; Yang et al., 2003). The interaction between IAPs and IAP-binding proteins is typically mediated by the BIR-domain of the IAPs and an IAP-binding motif (IBM) in the N-terminus of the IAP-binding protein (Verhagen et al., 2001), which is accessible for the BIR-domain after processing of the IAP-binding protein. Such an IBM, however, is missing at the N-terminus of Nma111p. Moreover, we never observed a catalytic processing of Nma111p as it has been described for other bacterial or mammalian HtrA-like proteins. Therefore, the Nma111p-binding and Bir1p-binding sites on both proteins remain to be identified. Preliminary results indicate that this involves the N-terminal regulatory domain of Nma111p as well as its second PDZ domain (D.W. and B.F., unpublished results).

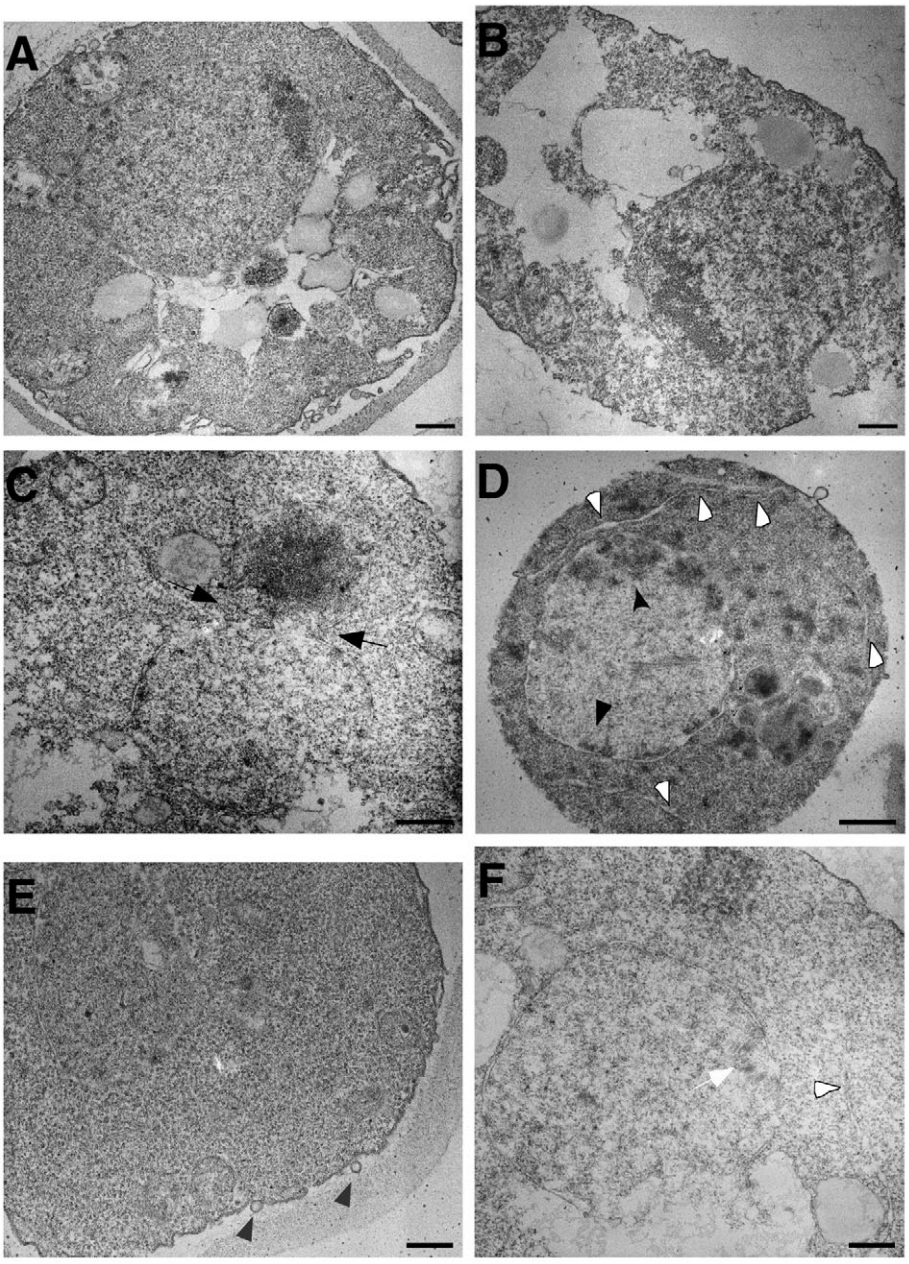


Fig. 5. Electron micrographs of Bir1p-overexpressing cells and Δ*bir1* cells. (A-B) Cells overexpressing Bir1p that had been treated with 0.8 mM H_2O_2 for 4 hours do not show any apoptotic hallmarks. (C-F) Δ*bir1* cells show strong invaginations of the nuclear envelope (black arrows in C), a very prominent endoplasmatic reticulum (white arrowheads in D and F), chromatin condensation at the nuclear envelope (black arrowheads in D), and tiny vesicles on the outer face of the plasma membrane (grey arrowheads in E). In few cells chromosome segregation defects were also detected (white arrows in F). Bars, 500 nm.

Bir1p is an inhibitor-of-apoptosis protein

Bir1p was originally identified as a protein involved in chromosome segregation and cytokinesis (Uren et al., 1999; Yoon and Carbon, 1999), whereas a putative role in apoptosis had not been studied and had been disfavoured. However, it has been shown that the role of Bir1p in chromosome segregation and cytokinesis can be attributed to its C-terminal, but not its BIR-domains, leaving the possibility open that Bir1p, owing to its BIR-domains, plays a role in yeast apoptosis. We show here that, Bir1p, indeed, acts as an inhibitor of apoptosis in yeast and, as such, Bir1p

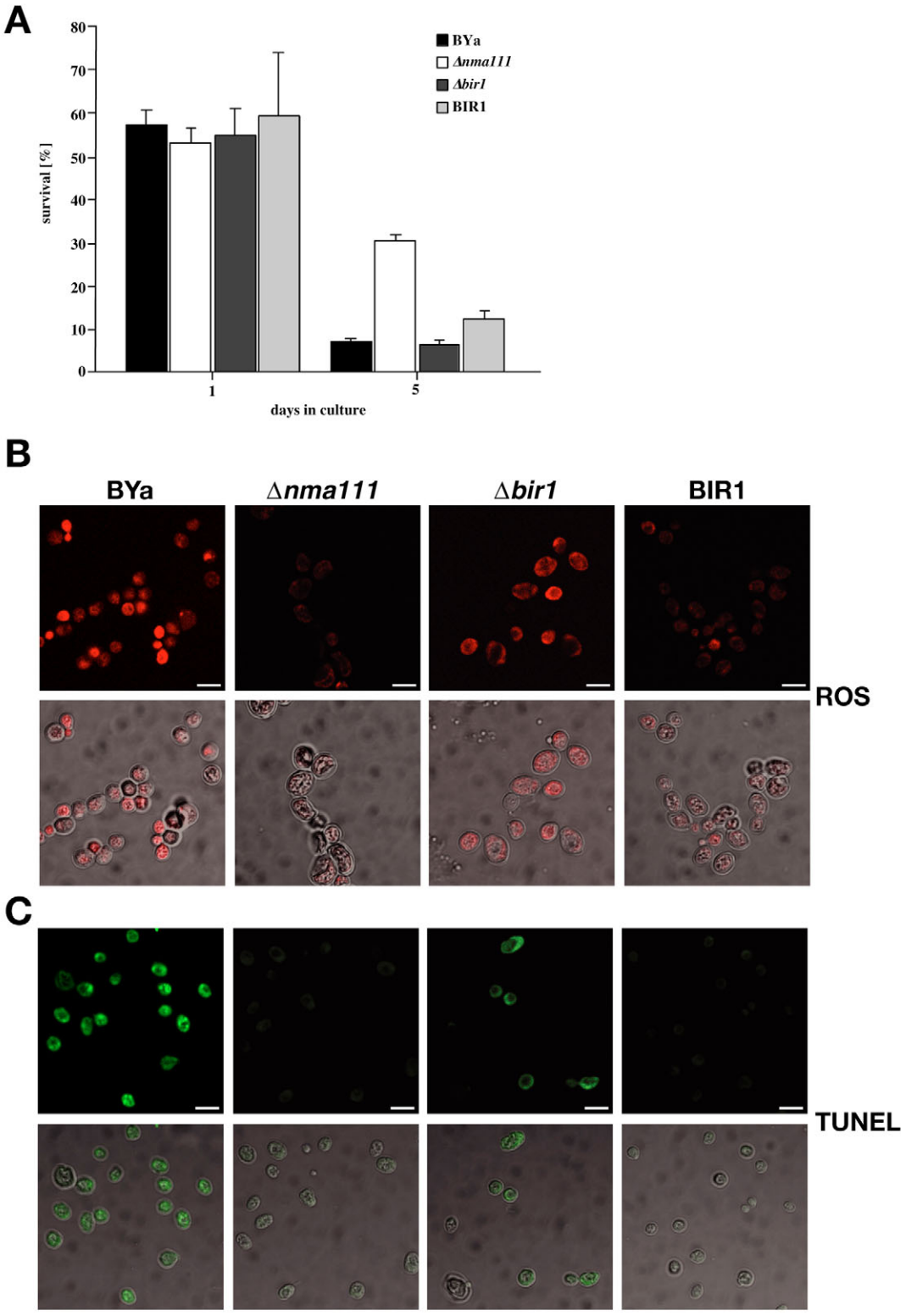


Fig. 6. Chronological ageing of wild-type, *Δbir1*-, *Δnma111*- and Bir1p-overexpressing cells. (A) Survival rates. Error bars, mean + s.e.m. (B) TUNEL assay and ROS detection of wild-type (BYa), *Δbir1*-, *Δnma111*- and Bir1p-overexpressing cells after 5 days of cultivation. Bars, 5 μm.

might behave similar to its closest mammalian homologue survivin and other survivin-like IAPs, such as BRUCE/Apollon, TIAP and deterin. Survivin, like Bir1p (Bouck and Bloom, 2005; Yoon and Carbon, 1999), plays a role in cytokinesis as part of the chromosome-passenger complex (Adams et al., 2001), whereas it is still controversially discussed how survivin inhibits apoptosis. Although it has been described that survivin directly interacts with caspase-3 and caspase-7 in vitro (Tamm et al., 1998), X-ray crystallography data of survivin's BIR domain failed to reveal any clues how survivin directly interacts with caspase-3 (Riedl et al., 2001). More recent data provide evidence that survivin probably acts

by stabilizing other cellular proteins, such as XIAP (Dohi et al., 2004) or the hepatitis B X-interacting protein (HBXIP) (Marusawa et al., 2003), rather than by directly interacting with caspases, thereby inhibiting them. Similarly, the survivin homologue dBRUCE does not block the activity of the *Drosophila* caspase Dronc (Vernooy et al., 2002). Rather – following induction of apoptosis – survivin associates with XIAP through the BIR repeats in both proteins, which causes increased stability of XIAP against ubiquitylation and proteosomal degradation, and synergistic inhibition of apoptosis due to inhibition of caspase-9 (Dohi et al., 2004).

A similar scenario is conceivable for Bir1p. It is unable to directly bind to the yeast caspase YCA1 (Fig. 1), indicating that inhibition of yeast apoptosis by Bir1p occurs indirectly rather than directly. Inhibition of apoptosis by type-II BIRPs might, therefore, more generally occur by binding and/or stabilizing other cellular proteins rather than by directly inhibiting caspase(s). Bridging factor(s) between Bir1p and Yca1p that prevent activation of Yca1p remain to be identified; however, the bridging protein(s) will not belong to the BIRP family because Bir1p, based on the yeast genome, is the sole BIRP in yeast. This might lead to the identification of a new class of apoptosis inhibitors, which, in turn, might ultimately also lead to the identification of new key players in mammalian apoptosis.

Taken together, our data indicate that Bir1p is a substrate for the pro-apoptotic serine protease Nma111p and an inhibitor of apoptosis-like cell death in yeast. As such, Bir1p is a bona fide homologue of *Drosophila* Deterin, and mammalian survivin and BRUCE/Apollon, which, similar to Bir1p, have anti-apoptotic properties and play role in chromosome segregation and cytokinesis. Compared with other type-II BIRPs, Bir1p is a rather large protein, suggesting that Bir1p plays a role in other cellular processes in addition to its role in yeast apoptosis and chromosome segregation and cytokinesis. Our findings further support that yeast and metazoan apoptosis have a common evolutionary origin, and that yeast is a useful species to identify new apoptosis regulators.

Materials and Methods

Strains and plasmids

Experiments were performed in BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), obtained from Euroscarf (European Saccharomyces Cerevisiae Archive For Functional Analysis). To generate the *Δbir1* (YJR089W) strain, vector pUG6 was used as template and the *kan*MX4 cassette was amplified by PCR with primers containing regions homologous to *BIR1*. The amplified cassette was transformed into BY4741 (BYa). Chromosomal C-terminally yEGFP-tagged *BIR1* was generated as described (Knop et al., 1999). Vector pYM12 was used as a template and the *kan*MX6-yEGFP-tag cassette was amplified by PCR with primers containing regions homologous to *BIR1*. The amplified cassette was transfected into BY4741. Similarly, *kan*MX6-GFP cassette was integrated into the *BIR1* locus in a *Δnma111* strain (Fahrenkrog et al., 2002). Yeast strain Bir1p-GFP was transfected with pYES-ProtA-Nma111p and pYES-ProtA-Nma111p-S235C, respectively (Fahrenkrog et al., 2004).

Genomic DNA was isolated from RH2881 (Fahrenkrog et al., 2004). To construct pYES-ProtA-Bir1p, *BIR1* was amplified by PCR, digested with *Nco*I and *Sal*I and ligated into vector pNOPPATA1W. ProtA-Bir1p was subcloned from pNOPPATA1W-Bir1p, partially digested and inserted into *BamH*I-*Not*I-digested pYES-CT. Correct ligation of ProtA-Bir1 was confirmed by western blot. The resulting plasmid pYES-ProtA-Bir1p was transformed into BY4741. The construct was expressed under the control of an inducible *GAL1* promoter.

To generate GST-Bir1p, *BIR1* was amplified by PCR, cut with *Nco*I and *Kpn*I and ligated into vector pGEX-CS (kind gift of D. Sitterlin, Université de Versailles St. Quentin en Yveline, France). Nma111p was amplified by PCR, digested with *Nco*I and *BamH*I, and ligated into pGEX-CS to yield GST-Nma111p.

To generate the N-terminal domain of Nma111p, nucleotides 1-1351 were amplified by PCR, digested with *Nco*I and *BamH*I and ligated into pBSpALTER (kind gift of D. Sitterlin). The C-terminal domain of Nma111p (nucleotides 1351-2994) was amplified by PCR, digested with *Nco*I and *BamH*I and ligated into pBSpALTER. YCA1 was amplified by PCR, digested with *EcoR*I and *BamH*I and ligated into pBSpALTER.

To generate pESC-Nma111p, Nma111p was amplified by PCR, digested with *BamH*I and *Sal*I and ligated into pESC-His (Stratagene, La Jolla, CA).

Immunofluorescence microscopy

For direct immunofluorescence microscopy, 20 μl of Bir1p-GFP cells were placed on poly-prep slides (Sigma, St Louis, MO), mounted and directly viewed with a confocal laser-scanning microscope (Leica TCS NT/SP1, Leica, Vienna, Austria). Images were recorded using the microscope-system software and processed with Adobe Photoshop (Adobe Systems, Mountain View, CA). Indirect immunofluorescence microscopy was performed as described (Fahrenkrog et al., 2004).

Test for apoptotic markers

DNA staining, Dihydrorhodamine 123 staining and TUNEL assay were performed as described (Fahrenkrog et al., 2004). Survival platings of *Δbir1* and BYa strains in the presence or absence of H_2O_2 were performed as described previously (Madeo et al., 2002). Overexpression of Bir1p in the presence or absence of H_2O_2 was performed as described (Fahrenkrog et al., 2004).

Survival plating

For survival plating, fresh overnight cultures of BYa and Δ*bir1* cells were diluted to 2×10^5 cells/ml and grown for a further 4 hours at 30°C. Cultures were exposed to 0.4 mM, 0.8 mM or 1.2 mM H_2O_2, for 4 hours. Aliquots of the cultures were taken, counted with a haemocytometer and diluted in distilled water to plate ~1000 cells per YPD plate. The numbers of surviving colonies were determined after a 2-day incubation at room temperature.

Cells expressing pYES-Bir1p or pYES-Bir1p/pESC-Nma111p were grown to OD_{600} ~0.1 in synthetic minimal medium containing 2% glucose, shifted to medium containing 2% galactose, exposed to 0.4 mM H_2O_2 for 20 hours, and proceeded for survival plating as described above.

Purification of recombinant Bir1p and Nma111p

GST, GST-Bir1p or GST-Nma111p were expressed in *E. coli* BL21 (DE3) cells. Protein expression was induced with 0.5 mM IPTG for 5 hours at 25°C. Cells were lysed by sonication in 2×PBS containing 1% Triton X-100 (to maintain Nma111p activity no protease inhibitors were added). Lysed cells were spun at 60,000 ***g*** for 1 hour, and the cleared lysates were stored at –80°C.

In vitro transcription and translation

Nma111p-N, Nma111p-C and YCA were obtained by in vitro transcription and translation with the TNT-coupled reticulocyte lysate system (Promega, Madison, WI) in the presence of revidue L-[^{35}S]methionine-cysteine (Amersham Bioscience, Uppsala, Sweden) following the instructions of the manufacturer.

In vitro binding assays

GST, GST-Bir1p or GST-Nma111p were bound to glutathione-sepharose beads (Amersham Bioscience, Uppsala, Sweden) for 1 hour at 4°C. Beads were washed twice with 2×PBS containing 10% glycerol and 0.5% Triton X-100. In vitro translated Nma111p-N, Nma111p-C and YCA1 were allowed to bind for 16 to 20 hours at 4°C. After binding, the beads were washed twice in 2×PBS containing 10% glycerol and 0.5% Triton X-100 followed by two washes in 2×PBS. Samples were eluted in 60 μl SDS sample buffer, analysed on a 10% SDS polyacrylamide gel and detected by autoradiography.

Chronological ageing

For ageing experiments, cultures were grown in SC medium consisting of 0.67% yeast nitrogen base (Difco, Sparks, MD) and 2% glucose supplemented with all amino acids. Cultures were inoculated from fresh overnight cultures (culture volume 10% of the flask volume), and aliquots were taken to perform survival plating and tests for apoptotic markers as described above.

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