# The *S. cerevisiae* HtrA-like protein Nma111p is a nuclear serine protease that mediates yeast apoptosis

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## Summary

**The yeast *S. cerevisiae* can undergo programmed cell death that exhibits the typical cellular markers of apoptosis. The mammalian HtrA2 protein was recently reported to mediate apoptosis in a serine-protease-dependent manner owing to its ability to antagonise the inhibitor of apoptosis protein XIAP. Here, we report the identification and characterisation of the *S. cerevisiae* HtrA-like protein, which we termed Nma111p (for nuclear mediator of apoptosis), as a mediator of yeast apoptosis. Nma111p is a nuclear protein that, under cellular stress conditions (i.e. at elevated temperature or after induction of apoptosis by $H_2O_2$), tends to aggregate inside the nucleus without its expression level being upregulated, suggesting that aggregation of Nma111p is correlated to its death-mediating character. Nma111p belongs to the HtrA family of serine proteases and its pro-apoptotic activity depends on its serine-protease activity. Yeast cells that lack Nma111p survive better at 50°C than wild-type cells and the cells show no apoptotic hallmarks, such as chromatin condensation and fragmentation, or accumulation of reactive oxygen species, after the induction of apoptosis by $H_2O_2$. By contrast, overexpression of Nma111p enhances apoptotic-like cell death. Therefore, Nma111p, like its mammalian homologue HtrA2, mediates apoptosis.**

Key words: Apoptosis; HtrA; Nic96p; Nucleus; Yeast

## Introduction

Apoptosis is a form of programmed cell death thought to be crucial for the development and maintenance of multicellular organisms. Apoptosis involves characteristic morphological changes, such as plasma membrane blebbing, cell rounding and shrinkage, chromatin condensation, and nuclear fragmentation (Kerr et al., 1972). Recent findings suggest that a programmed form of cell death also exists in unicellular organisms as a defence mechanism (e.g., in response to viral infection, nutrient insufficiency and genome damage) to ensure that some cells survive to rescue the population (Fröhlich and Madeo, 2000; Madeo et al., 2002a; Skulachev, 2002).

In *Saccharomyces cerevisiae*, the first indication of an apoptotic-like death pathway came from the observation that a point mutation in the Cdc48 AAA-ATPase (*cdc48*$^{S565G}$) resulted in cells dying with the typical apoptotic phenotype: expression of phosphatidylserine on the outer leaflet of the plasma membrane, chromatin condensation and DNA fragmentation, and formation of cell fragments (Madeo et al., 1997). Yeast cells lacking STM1, a DNA binding protein involved in DNA repair, show increased resistance to oxygen stress (Ligr et al., 2001). Stress caused by hydrogen peroxide ($H_2O_2$) (Madeo et al., 1999), acetic acid (Ludovico et al., 2001; Ludovico et al., 2002) or overexpression of the mating-type pheromone (Severin and Hyman, 2002) can induce yeast apoptosis. More recently, a caspase-like protein termed YCA1 has been identified and shown to be involved in programmed cell death induced by $H_2O_2$, acetic acid and ageing (Madeo et al., 2002b). YCA1 belongs to the family of metacaspases, proteases that have a caspase-like fold (Uren et al., 2000). Its overexpression enhances apoptotic-like death of the cells, whereas its knockout reduces cell death (Madeo et al., 2002b). However, relatives of other classical regulators of metazoan apoptosis, such as the caspase-activating protein Apaf-1 or the anti-apoptotic protein Bcl-2 are missing in yeast (Jin and Reed, 2002; Leist and Jäättelä, 2001).

An inhibitor of apoptosis (IAP)-like protein termed Bir1p, has been identified in yeast (Uren et al., 1999; Yoon and Carbon, 1999; Li et al., 2000). Bir1p contains a conserved baculoviral inhibitor of apoptosis repeat (BIR) domain, a domain known to mediate the interaction of the IAP proteins with caspases. Although Bir1p seems to be involved in cell division (Uren et al., 1999; Yoon and Carbon, 1999; Li et al., 2000), its potential role in yeast apoptosis has not yet been studied.

IAP proteins are antagonised by several proteins, one of which is the human high temperature requirement (HTR)A2 protein (OMI) (Suzuki et al., 2001; Hegde et al., 2002, Martins et al., 2002; Verhagen et al., 2002). Human mitochondrial HtrA2/Omi was originally identified as a presenilin- and Mxi2-interacting protein (Gray et al., 2000; Faccio et al., 2000). Recently, it has been shown that HtrA2/Omi, like the *Drosophila* IAP-binding proteins Grim, HID and Reaper, has a pro-apoptotic character because of its antagonistic effect on the mammalian IAP homologue XIAP (reviewed in Salvesen and Duckett, 2002; Suzuki et al., 2001; Hegde et al., 2002; Martins et al., 2002; Verhagen et al., 2002).

A defining feature of the HtrA protein family is the combination of a catalytic serine-protease domain with at least one PDZ domain (Pallen and Wren, 1997; Harris and Lim, 2001; Clausen et al., 2002). PDZ domains are small globular domains that act as protein-protein recognition modules and

**Table 1. Yeast strains used in this study**

| Strain | Genotype | Reference/source |
|---|---|---|
| BMA41 | Mat**a**/α, *ade2-1/ade2-1, leu2-3, 112/leu2-3, 112, ura3-1/ura3-1, trp1Δ/trp1Δ, his3-11, 15/his3-11, 15, can1-100/can1-100* | Baudin-Baullieu et al., 1997 |
| BMA41/1**a** | Mat**a**, *ade2-1, leu2-3, 112, ura3-1, trp1Δ, his3-11, 15, can1-100* | Baudin-Baullieu et al., 1997 |
| BFY47 | Mat**a**, *ade2-1, leu2-3, 112, ura3-1, trp1Δ, his3-11, 15, can1-100, nma111::TRP1* | This study |
| BFY15 | Mat**a**, *ade2-1, leu2-3, 112, ura3-1, trp1Δ, his3-11, 15, can1-100, nma111::TRP1,* pNOPPATA1L-NMA111 | This study |
| BFY55 | Mat**a**, *ade2-1, leu2-3, 112, ura3-1, trp1Δ, his3-11, 15, can1-100,* nma111::NMA111-GFP-TRP1 | This study |
| RH2881 | Mat**a**, *his3, leu2, ura3, trp1, bar1* | H. Riezman, University of Geneva, Geneva, Switzerland |
| BFY119 | Mat**a**, *his3, leu2, ura3, trp1, bar1,* pYES2-CT-ProtA-NMA111 | This study |
| BFY120 | Mat**a**, *ade2-1, leu2-3, 112, ura3-1, trp1Δ, his3-11, 15, can1-100, nma111::TRP1,* pNOPPATA1L-NMA111-S235C | This study |
| BFY111 | Mat**a**, *his3Δ1, leu2Δ0, lys2ΔO, ura3Δ0, yca1::kanMX4* | Euroscarf, Frankfurt, Germany |
| PSY1266 | Mat**a**, ura3-52, leu2Δ1, trp1-63, CENTRP XPO1-GFP | Hood and Silver, 1998 |

are involved in many signalling cascades (Pallen and Wren, 1997; Harris and Lim, 2001; Clausen et al., 2002). Bacterial HtrA/DegP is a heat-inducible serine protease with two C-terminal PDZ domains that is essential for viability of bacteria after heat shock (Skorko-Glonek et al., 1995). Moreover, it has a dual function because it degrades misfolded proteins at higher temperatures but acts as molecular chaperone at low temperatures (Spiess et al., 1999). Here, we identify and characterise the yeast HtrA-like protein Nma111p. We identified Nma111p in a yeast two-hybrid screen by using the yeast nuclear pore complex (NPC) protein Nic96p as bait against a yeast genomic library (Fahrenkrog et al., 2000). Nma111p is a nuclear protein that interacts with the NPC and, like its mammalian homologue HtrA2/Omi, plays a crucial role in yeast apoptosis. The pro-apoptotic activity of Nma111p is dependent on its serine-protease activity. An *nma111* deletion yeast strain has an enhanced thermotolerance at elevated temperatures and shows increasing survival rates after induction of apoptosis by $H_2O_2$. By contrast, overexpression of Nma111p leads to a decrease in the survival rate of cells treated with $H_2O_2$.

## Materials and Methods

### Yeast strains and media

The yeast strains used in this study are listed in Table 1. All strains were grown at 30°C, unless otherwise stated. Media and genetic methods, including mating, sporulation and tetrad dissection, were as described elsewhere (Guthrie and Fink, 1991). Yeast cells were transformed by using the lithium-acetate method (Gietz et al., 1992).

### Plasmids

The following plasmids were used: pUN100-NOP1::ProtA-TEV (pNOPPATA1L; Hellmuth et al., 1998); pNOPPATA1L-Nma111, a PCR product of the YNL123W [open reading frame (ORF) extending from nucleotide +1 to +2994] inserted into *Nco*I-*Bam*HI digested pNOPPATA; pNOPPATA1L-Nma111-S235C, a PCR product of the YNL123W (ORF extending from nucleotide +1 to +2994 with nucleotide +235 mutated to change serine to glycine) inserted into pNOPPATA1L-Nma111 by site-directed mutagenesis; pFA6a-GFP-TRP1 (Longtine et al., 1998) (kindly provided by F. Stutz, University of Lausanne); Nma111-GFP, derived from pFA6a-GFP-TRP1 and inserted with 40 nucleotides of the region directly upstream of the YNL123W ORF (+2951 to +2991) and with 40 nucleotides of the 3′-untranslated region (UTR) (nucleotides +2995 to +3035) directly downstream of the YNL123W; pYES2-CT (Invitrogen, Groningen, The Netherlands); pYES2-ProtA-Nma111p, a PCR product of the YNL123W (ORF extending from nucleotide +1 to +2994) with an N-terminal protein-A tag subcloned from pNOPPATA1L-Nma111p and inserted into *Bam*HI-*Not*I digested pYES2-CT.

### Gene disruption

YNL123W deletion constructs were prepared by replacing nucleotides –10 to +3010 with the N-(5′-phosphoribosyl)anthranilate isomerase (TRP1) selectable marker gene generated by PCR. The YNL123W::TRP1 fusion was transformed into the diploid BMA41 strain (Baudin-Baillieu et al., 1997) and selected on SD-W (synthetic minimal medium lacking tryptophan) plates (Rothstein et al., 1991). TRP+ transformants were characterised for correct integration of YNL123W::TRP1 at the YNL123W locus by PCR analysis. Heterozygous TRP1+ transformants were sporulated in liquid sporulation medium and tetrads were dissected on YPD (complex medium containing 2% glucose).

### Genomic and recombinant tagging

For genomic tagging, a haploid strain (BMA41a) was transformed with the green-fluorescent protein (GFP)-TRP1 cassette flanked at the 5′ end with 40 bp of YNL123W from (nucleotide +2952 to +2991) and at the 3′ end with 40 bp of the YNL123W UTR (nucleotide +2996 to +3035). The resulting transformants were selected on SD-W plates. Correct integration of the GFP tag was confirmed by PCR and western blots.

For recombinant protein-A tagging, the YNL123W ORF was amplified from isolated yeast genomic DNA by PCR, thereby generating an *Nco*I and a *Bam*HI site at the 5′ and the 3′ end of the ORF, respectively. The PCR product was inserted into the *Nco*I-*Bam*HI digestion site of pNOPPATA1L. The resulting plasmid was transformed into the Δ*ynl123w* strain using the lithium acetate method (Gietz et al., 1992) and selected on SD-L (synthetic minimal medium lacking leucine) plates.

### Direct immunofluorescence microscopy of living cells

For direct immunofluorescence microscopy of living cells, the genomically tagged Nma111p-GFP strain was grown at 25°C in YPAD medium to mid-log phase. To induce heat shock or apoptosis, aliquots were shifted to 42°C and 50°C for 30 minutes, respectively, (heatshock) or were treated with 3 mM $H_2O_2$ for 3 hours (apoptosis). 1 ml aliquots were taken from each culture and cells were spun down at 1500 ***g*** for 1 minute. The supernatants were removed and the cell pellet resuspended in 200 μl YPAD (YPD containing 0.003% adenine sulfate). 20 μl of the cell suspension was placed on poly-L-lysine-coated slides (Polyprep slides; Sigma, St Louis, MO, USA), 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 NIH Image and Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

### Indirect immunofluorescence microscopy

Immunofluorescence microscopy of yeast cells was performed as described (Ayscough and Drubin, 1998), with the following modifications. Yeast cells were grown to mid-log phase and transformed into spheroplasts as described previously (Fahrenkrog et al., 1998). The spheroplasts were placed on poly-L-lysine-coated coverslips and fixed in –20°C methanol for 6 minutes and then –20°C acetone for 30 seconds. After fixation, the cells were washed three times with PBS at pH 7.4, containing 2% bovine serum albumin (BSA) and 0.1% Triton X-100. 15 μl of primary antibody (anti-protein-A, 15 mg $ml^{-1}$; Sigma, St Louis, MO, USA) diluted 1:1000 in PBS containing 2% BSA and 0.1% Triton X-100 were placed on each coverslip and incubated for 1 hour at room temperature (rt). After washing three times with PBS containing 2% BSA and 0.1% Triton X-100, 15 μl of a secondary Alexa488-labelled anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA) diluted 1:800 in PBS containing 2% BSA and 0.1% TritonX-100 were placed on each coverslip and incubated for 1 hour at room temperature (rt) in the dark. After incubation, the cells were washed five times with PBS. Coverslips were mounted upside down on slides using Mowiol (Hoechst, Frankfurt, Germany) and stored at 4°C in the dark until viewed. Micrographs were recorded with a confocal laser scanning microscope (Leica TCS NT/SP1, Leica, Vienna, Austria). Images were analysed using NIH Image and Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

### Site-directed mutagenesis

Mutations were introduced into pNOPPATA1L-Nma111 using the Quick-change site-directed mutagenesis system (Stratagene, Amsterdam, The Netherlands) following the manufacturer's instructions. Mutations were confirmed by DNA sequencing.

### Thermotolerance assay

Yeast cells were grown overnight at 25°C to an $OD_{600}$ of 0.1 to 0.5. Samples of 5 ml were taken from each culture, mixed with 5 ml of prewarmed medium (25°C, 49°C or 59°C) and incubated at 25°C, 37°C, 42°C or 50°C for 30 minutes, 60 minutes or 180 minutes. 1 ml aliquots of each incubated sample were taken, chilled on ice and serially diluted (1:1, 1:5, 1:25, 1:125, 1:625) with sterile water. 8 μl of each serial dilution were spotted on YPD plates and incubated for 2 days at 25°C.

### Chromatin staining

Cells were grown at 25°C to early-log phase in YPAD medium. To stain the DNA, 1 μmol $ml^{-1}$ Sytox-Green nucleic-acid stain (Molecular Probes, Leiden, The Netherlands) was added to the medium and cells were left for 1 hour at rt. Cells were next incubated for a further hour at 25°C or 50°C, fixed in 2% paraformaldehyde for 1 hour and washed with 1 ml YPAD. Cells were resuspended in 200 μl YPAD and an aliquot of 20 μl cells was placed on poly-L-lysine-coated slides, mounted and directly viewed with a confocal laser scanning microscope (Leica TCS NT/SP1). Images were recorded using the microscope-system software and processed with NIH Image and Adobe Photoshop.

To induce apoptosis, yeast cells were incubated with 3 mM $H_2O_2$ for 3 hours at rt followed by a wash with 1 ml YPAD. The cells were then resuspended in YPAD, incubated with 1 μmol $ml^{-1}$ Sytox-Green nucleic-acid stain for 1 hour at rt and processed for confocal laser scanning microscopy as described above.

### Dihydrorhodamine staining

To test for the presence of free intracellular radicals or strongly oxidising molecules [e.g. reactive oxygen species (ROS)], yeast cells were grown at 25°C to mid-log phase. Aliquots were taken and apoptosis was induced by incubating the cells in 0.8 mM $H_2O_2$ for 3 hours. Next, the cells were washed once in YPAD medium, incubated with 5 μg $ml^{-1}$ dihydrorhodamine (DHR; Sigma) for 2 hours at rt and processed for confocal laser scanning microscopy as described above.

### TUNEL assay

For the TdT-mediated dUTP nick end labelling (TUNEL) test, cells were grown to mid-log phase. Apoptosis was induced by adding 0.8 mM $H_2O_2$ for 4 hours. Cells were fixed in 3.7% formaldehyde for 15 minutes, digested with zymolyase and applied to poly-L-lysine-coated slides (Poly-prep slides, Sigma). The slides were rinsed with PBS, incubated in permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) on ice for 2 minutes, rinsed twice with PBS and incubated with 20 μl of TUNEL reaction mixture [18 μl TUNEL label containing fluorescein-dUTP, 2 μl TUNEL enzyme (In Situ Cell Death Detection Kit, Roche Diagnostics, Rotkreuz, Switzerland)] for 1 hour at 37°C in a humid chamber in the dark. Slides were rinsed three times in PBS, mounted with Mowiol and stored at 4°C in the dark until viewed. Micrographs were recorded with a confocal laser scanning microscope (Leica TCS NT/SP1). Images were analysed using NIH image and Adobe Photoshop.

### EM of apoptotic cells

Yeast cells were grown in YPAD medium to early-log phase. 3 mM $H_2O_2$ was added for 3 hours and the cells were prepared for electron microscopy as described (Fahrenkrog et al., 2000).

### Overexpression and survival plating

For overexpression, pYES2-ProtA-Nma111p cells were grown in SC (synthetic complete medium) medium containing 2% glucose to an $OD_{600}$ of ~1.5. Cells were harvested by centrifugation and resuspended in the same volume of SC medium containing 2% galactose. Expression was induced for 0 hour, 4 hours or 26 hours, and aliquots were taken before induction of expression and at each of the two time points. Protein expression levels were analysed by western blot using a polyclonal anti-protein-A antibody (Sigma) and a chemiluminescent detection system (Tropix Western Star, Applied Biosystems, Rotkreuz, Switzerland).

For survival plating, pYES2-ProtA-Nma111p expression was induced for 0 hour, 26 hours and 44 hours. After 26 hours, an aliquot was taken and cells were treated for 24 hours with 0.4 mM $H_2O_2$. Aliquots of the cultures were taken, and cells were counted in a Neubauer chamber and diluted in distilled water in order to plate ~1000 cells on YPAD plates. The number of surviving colonies were determined after a 2-day incubation at rt.

### Oxygen-stress tolerance

To test the oxygen-stress tolerance of Δ*nma111, ProtA-NMA111* and *ProtA-NMA111-S235C* cells, cultures were grown to mid-log phase and exposed to 0.4 mM, 0.8 mM or 1.2 mM $H_2O_2$ for 4 hours. Cells were harvested by centrifugation and plated to determine the survival rate as described above.

## Results

### Identification of Nma111p

We have previously used a yeast two-hybrid screen to identify proteins that interact with the yeast nucleoporin Nic96p

(Fahrenkrog et al., 2000). Among the positive clones that interacted with the bait Nic96p, we identified the ORF of YNL123W nine times by sequencing and database search. Interaction of Nic96p and the prey occurred at three overlapping fragments of the N-terminal domain of YNL123W. Database search revealed putative homologous ORFs in *Campylobacter jejuni*, *Escherichia coli*, *Caenorhabditis elegans*, *Schizosaccharomyces pombe*, humans and others (reviewed in Clausen et al., 2002; Gray et al., 2000; Pallen and Wren, 1997; Ponting, 1997). By itself, Nic96p did not activate the transcription of the reporter genes, indicating that the interaction between Nic96p and the protein encoded by the YNL123W ORF is specific. A database search revealed that, based on sequence similarities, YNL123W belongs to the HtrA family of serine proteases (Clausen et al., 2002; Gray et al., 2000; Pallen and Wren, 1997; Ponting, 1997: Skorko-Glonek et al., 1999). The ORF YNL123W encodes an ~111 kDa protein, hence we refer to it as Nma111p, for nuclear mediator of apoptosis.

## Immunolocalisation of Nma111p

To determine the subcellular localisation of Nma111p, the protein was tagged C-terminally by integrating a GFP moiety directly upstream of the stop codon of the genomic copy of YNL123W. The chimeric protein was expressed in yeast and visualised by direct immunofluorescence microscopy using a confocal laser scanning microscope. As shown in Fig. 1A, the Nma111p-GFP fusion protein localises to the nucleus of the yeast cells.

Because we have identified Nma111p using a two-hybrid screen with the nucleoporin Nic96p as bait, we next wanted to know whether we could detect a direct interaction of Nma111p with the NPC at the electron microscopy (EM) level. Therefore, we performed pre-embedding immunogold-EM with the yeast strain expressing Nma111p-GFP. Spheroplasted Nma111p-GFP cells were incubated with an anti-GFP antibody directly conjugated to 8 nm colloidal gold and processed for EM (see Materials and Methods). By doing so, we found that Nma111p-GFP does in fact interact with both the cytoplasmic and the nuclear NPC periphery (Fig. 1B). Quantification of the gold-particle distribution (Fig. 1C) with respect to the central plane revealed that 42% of the gold particles reside 30 to 50 nm from the central plane towards the cytoplasm, and 44% at distances of 35-100 nm from the central plane towards the nucleus. Their radial distances from the eightfold symmetry axis range from 0 to 40 nm (95% of the gold particles; Fig. 1C). Taken together, these findings indicate that Nma111p predominantly interacts with the NPC at the cytoplasmic fibrils and at the nuclear basket.

## Mutant *nma111* cells have a higher survival rate than wild-type cells

Database searches revealed that Nma111p is the only HtrA-like protein in *S. cerevisiae*. To explore possible function(s) of Nma111p within the yeast cell, we first performed a thermotolerance assay, because bacterial HtrA proteins are indispensable for bacterial survival at elevated temperatures (reviewed in Pallen and Wren, 1997).

First we generated a yeast strain with a disrupted YNL123W ORF. We used the diploid BMA41 strain and disrupted the YNL123W ORF by homologous recombination with the TRP1 selectable marker (Baudin-Baillieu et al., 1997). The heterozygous diploid YNL123W::TRP1 was sporulated and

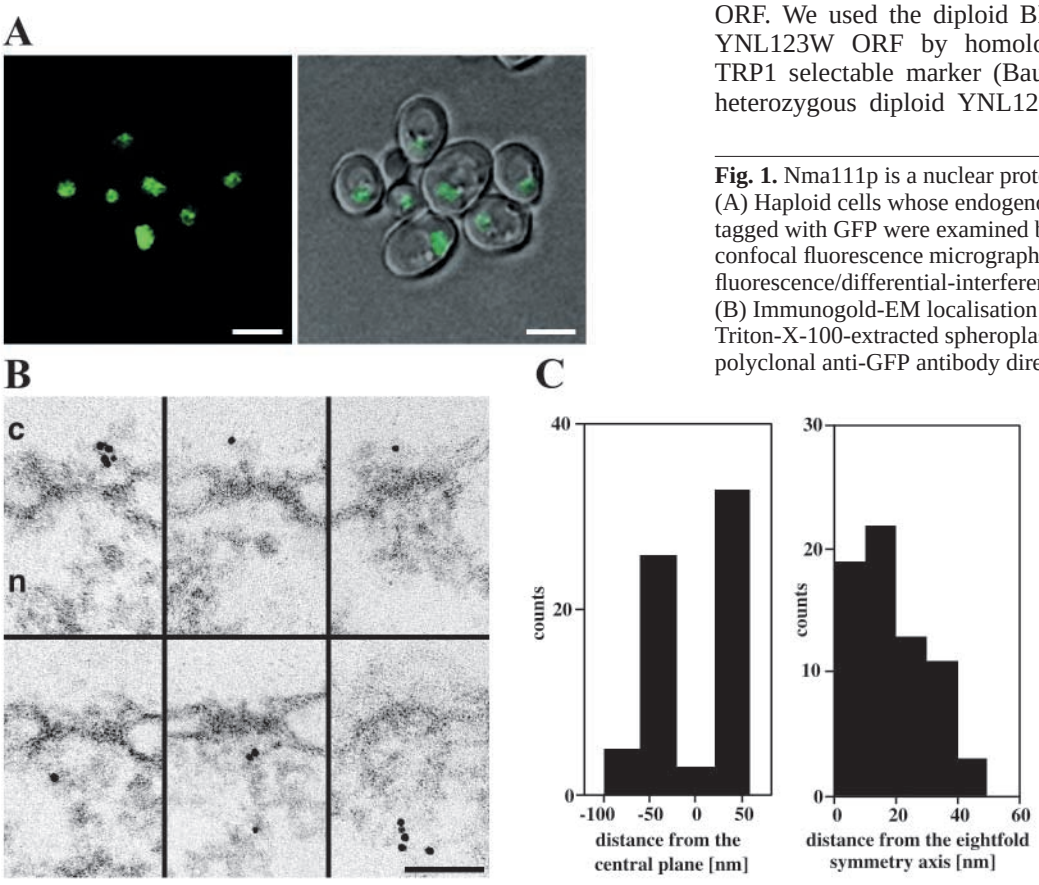


**Fig. 1.** Nma111p is a nuclear protein that interacts with the NPC. (A) Haploid cells whose endogenous Nma111p was C-terminally tagged with GFP were examined by direct immunofluorescence. A confocal fluorescence micrograph (left) and a coincident fluorescence/differential-interference-contrast image (right) is shown. (B) Immunogold-EM localisation of Nma111p-GFP at the NPC. Triton-X-100-extracted spheroplasts were preimmunolabelled with a polyclonal anti-GFP antibody directly conjugated to 8-nm colloidal gold. Selected examples of gold-labelled NPCs are shown in cross-sections along the NE. The antibody labelled the cytoplasmic face of the NPC (top) and the nuclear face of the NPC (bottom). c, cytoplasm; n, nucleus. (C) Quantitative analysis of the gold-particle distribution associated with the NPC relative to its two symmetry axes in Nma111p-GFP cells. The central plane of the NPC is within the NE, the eightfold symmetry axis is perpendicular to the plane of the NE. 68 gold particles were scored. Scale bars, 5 μm (A); 100 nm (B).

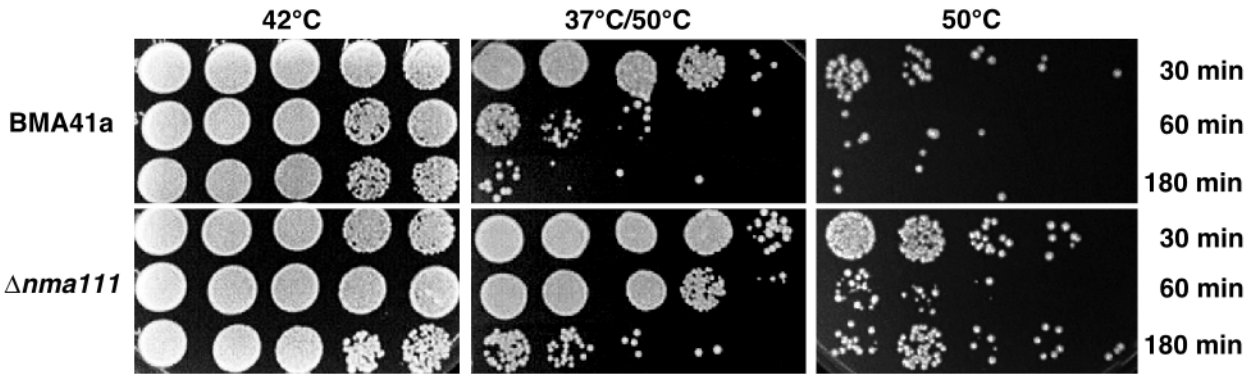


**Fig. 2.** Deletion of *nma111* increases the survival rate of yeast cells after shifting to elevated temperatures. The thermotolerance of wild-type and Δ*nma111* cells initially grown at 25°C was tested at 42°C and 50°C, respectively, for different durations. At 50°C, thermotolerance was also analysed by incubating the cells at 37°C for 30 minutes to induce the expression of heat-shock proteins before shifting the cells to 50°C. A serial dilution of cells was plated on YPAD plates and incubated for 2 days at 25°C. Plates show the number of surviving cells after heat treatment for 30 minutes, 60 minutes and 180 minutes.

tetrads were dissected. Four viable spores were recovered, indicating that Nma111p is not essential for vegetative growth. Segregants that carry YNL123W::TRP1 (Δ*nma111* cells) grow at similar rates to wild-type cells carrying YNL123W at temperatures between 16°C and 37°C, and exhibit no obvious structural defects at the EM level (data not shown).

To measure the thermotolerance, BMA41a wild-type cells and Δ*nma111* cells were grown in liquid medium at 25°C to early log phase. Cultures were then shifted to 37°C, 42°C or 50°C, or to 37°C for 30 minutes followed by a 50°C incubation, for 30, 60 and 180 minutes (Fig. 2). Colony forming ability was determined after plating and incubation at rt for two days. As in liquid cultures, Δ*nma111* cells grow indistinguishably from wild-type cells at 25°C and at 37°C (data not shown). After heatshock at 42°C, the growth behaviour of Δ*nma111* and wild-type cells also shows no significant difference (Fig. 2, left). Following incubation at 50°C, however, wild-type BMA41a cells die rapidly, whereas Δ*nma111* cells exhibit a significant increase in the survival rate (Fig. 2, right). Pre-incubation of yeast cells at 37°C for 30 minutes before incubation at 50°C to induce the expression of heat-shock proteins (Sanchez and Lindquist, 1990; Vainberg et al., 2000) increases the survival rate in both wild-type cells and mutant cells although the Δ*nma111* cells again survive better than the wild-type cells (Fig. 2, middle). These findings indicate that Nma111p promotes cell death at elevated temperatures independent of the expression level of heat-shock proteins.

### Δ*nma111* cells lack apoptotic hallmarks

Recently, it has been shown that HtrA2/Omi, one of the human homologues of yeast Nma111p, plays a critical role in apoptosis (Suzuki et al., 2001; Hedge et al., 2002; Martins et al., 2002; Verhagen et al., 2002). Hence, we next asked whether the cell-death-promoting effect of Nma111p is due to the induction of yeast apoptosis. To address this question, yeast wild-type, Δ*nma111* mutant and Δ*yca1* control cells, that lack the yeast caspase-like protein YCA1 (Madeo et al., 2002) were analysed for apoptotic hallmarks. Apoptotic-like death can be induced in *S. cerevisiae* by oxidative stress [i.e. by treating the cells with low doses of hydrogen peroxide ($H_2O_2$)] (Madeo et al., 1999). Phenotypic apoptotic markers include chromatin condensation and fragmentation, DNA breakage, and accumulation of ROS. To test yeast cells for chromatin condensation and fragmentation, wild-type cells, Δ*nma111* mutant cells and Δ*yca1* mutant control cells were grown to log phase, treated with 3 mM $H_2O_2$ for 3 hours to induce apoptosis and subsequently incubated with Sytox-Green nucleic-acid stain to visualise the DNA. The DNA staining pattern was analysed by confocal laser scanning microscopy. As shown in Fig. 3, top, the DNA of wild-type cells appears condensed and fragmented with a loss of mitochondrial DNA staining, whereas, in Δ*nma111* and Δ*yca1* cells, the DNA is distributed evenly within the nucleus and the mitochondria. The loss of mitochondria stain in wild-type cells is probably due to mitochondria damage caused by the high concentration of oxidants (Fleury et al., 2002).

Another hallmark of apoptosis is DNA single-strand breakage, which can be detected by the TUNEL assay (Gavrieli et al., 1992; Gorczyca et al., 1993). The TUNEL test detects free 3′ ends, which are generated by fragmentation of chromosomes, by attaching labelled nucleotides with terminal deoxynucleotidyl transferase. As shown in Fig. 3, middle, wild-type cells treated with 0.8 mM $H_2O_2$ for 4 hours showed a TUNEL-positive phenotype (intense nuclear fluorescence in all cells), whereas Δ*nma111* cells were TUNEL-negative, showing only a slight cytoplasmic fluorescence, just like the Δ*yca1* control cells.

A key event in triggering apoptosis is the accumulation of ROS, which might occur in yeast cells after, for example, exposure to $H_2O_2$ (Madeo et al., 1999). Wild-type, Δ*nma111* and Δ*yca1* cells treated with 0.8 mM $H_2O_2$ for 4 hours were tested for the production of ROS by incubation with dihydrorhodamine (DHR) 123. In the presence of ROS, DHR 123 is oxidised to the fluorescent chromophore rhodamine 123 (Schulz et al., 1996). As shown in Fig. 3, bottom, Δ*nma111* cells and Δ*yca1* control cells show no fluorescence, whereas ~50% of wild-type cells showed an intense intracellular staining by DHR.

EM investigation of wild-type cells treated with 3 mM $H_2O_2$ for 3 hours revealed some typical hallmarks of apoptosis (Madeo et al., 1997; Madeo et al., 1999). These included

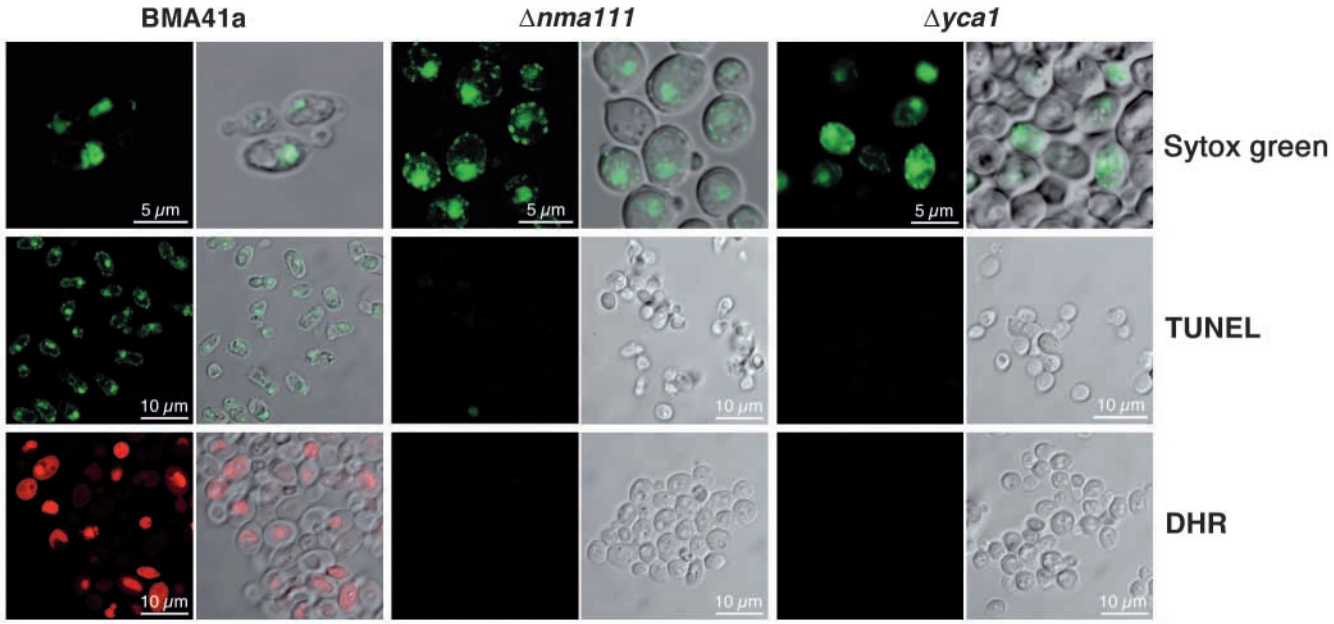


**Fig. 3.** *nma111* null cells do not undergo apoptosis. Wild-type (BMA41a), Δ*nma111* mutant and Δ*yca1* control cells were treated with 3 mM $H_2O_2$ for 3 hours to induce apoptosis. DNA was visualised by Sytox-Green nucleic-acid stain. Wild-type, Δ*nma111* mutant and Δ*yca1* control cells were analysed for DNA strand breaks by the TUNEL test and ROS by DHR staining after treatment with 0.8 mM $H_2O_2$ for 4 hours. Confocal fluorescence micrographs and coincident fluorescence/differential-interference-contrast images are shown.

chromatin condensation along the nuclear envelope (NE) (Fig. 4B,C, black arrows), NE blebbing (Fig. 4C, white arrow), vesicles decorating the outer face of the plasma membrane (Fig. 4D, black arrowheads) as well as a more prominent endoplasmic reticulum (Fig. 4A, white arrowheads). None of these apoptotic hallmarks are depicted in Δ*nma111* cells after treatment with $H_2O_2$ (Fig. 4E,F). The above described DNA staining, TUNEL assay, ROS accumulation and EM analysis therefore suggest that Nma111p promotes yeast apoptosis.

## Overexpression of Nma111p causes apoptotic-like cell death

To address further the question of whether Nma111p can induce yeast apoptosis by itself, we measured cell-death and apoptotic markers after overexpressing Nma111p. We transformed the yeast wild-type strain RH2881 with the pYES2-ProtA-Nma111p plasmid to overexpress Nma111p under control of the *GAL1* promoter. ProtA-Nma111p induction was analysed by immunoblotting using an anti-Protein-A antibody (Fig. 5A). A prominent band of the expected size (~130 kDa), which is absent in cells that were cultured in glucose medium, was observed after 26 hours of induction (Fig. 5A). Cell survival of ProtA-Nma111p overproducers was analysed by a plating assay (see Materials and Methods). Treatment with 0.4 mM $H_2O_2$ for 24 hours resulted in the death of yeast cells that were overexpressing ProtA-Nma111p (survival rate of less than 40%), whereas Δ*nma111* cells that were treated in the same way were largely unaffected (survival rate ~80%; Fig. 5B). In the absence of $H_2O_2$, overexpression of ProtA-Nma111p for 26 hours has less of an effect on cell death (survival rate ~75% compared with ~95% of Δ*nma111* cells; Fig. 5B). Consistent with the suggestion that overexpression of ProtA-Nma111p causes yeast apoptosis, DNA of such cells stained with Sytox-Green nucleic-acid stain appeared condensed (Fig. 5C top) and showed a positive TUNEL phenotype (Fig. 5C bottom). DNA condensation and breakage was already detectable in the absence of $H_2O_2$, but increased significantly in the presence of $H_2O_2$ (Fig. 5C). In brief, overexpression of ProtA-Nma111p triggers apoptosis in yeast cells.

## Nma111p aggregates in the nucleus both at higher temperature and under apoptotic conditions

To analyse whether the apoptosis-promoting effect of Nma111p correlates with a redistribution of Nma111p within the cell, we next determined the subcellular localisation of the protein after induction of cellular stress. For this purpose, Nma111p-GFP cells were grown to log phase at 25°C and subsequently shifted for 30 minutes to 42°C or 50°C. Nma111p-GFP is evenly distributed in the nucleus at 25°C (Fig. 6A) but the protein aggregates in the nucleus at 42°C (Fig. 6B) and at 50°C (Fig. 6C). In addition, at 50°C, some cells exhibited translocation of Nma111p-GFP into the cytoplasm, giving rise to a dotted staining pattern (Fig. 6D,E). These cytoplasmic dots do not localise with mitochondria (data not shown). Similarly, under apoptotic conditions, i.e. after treating the cells with 3 mM $H_2O_2$ for 3 hours, Nma111p-GFP aggregates inside the nucleus (Fig. 6F), but no translocation to the cytoplasm could be observed. Moreover, we found no alteration in the localisation of Xpo1p-GFP, a protein predominantly localised to the nucleus and at the NPC, under similar stress conditions (Fig. 6G-J). From these observations, we conclude that the localisation of Nma111p is distinctly altered inside the nucleus both at elevated temperatures and

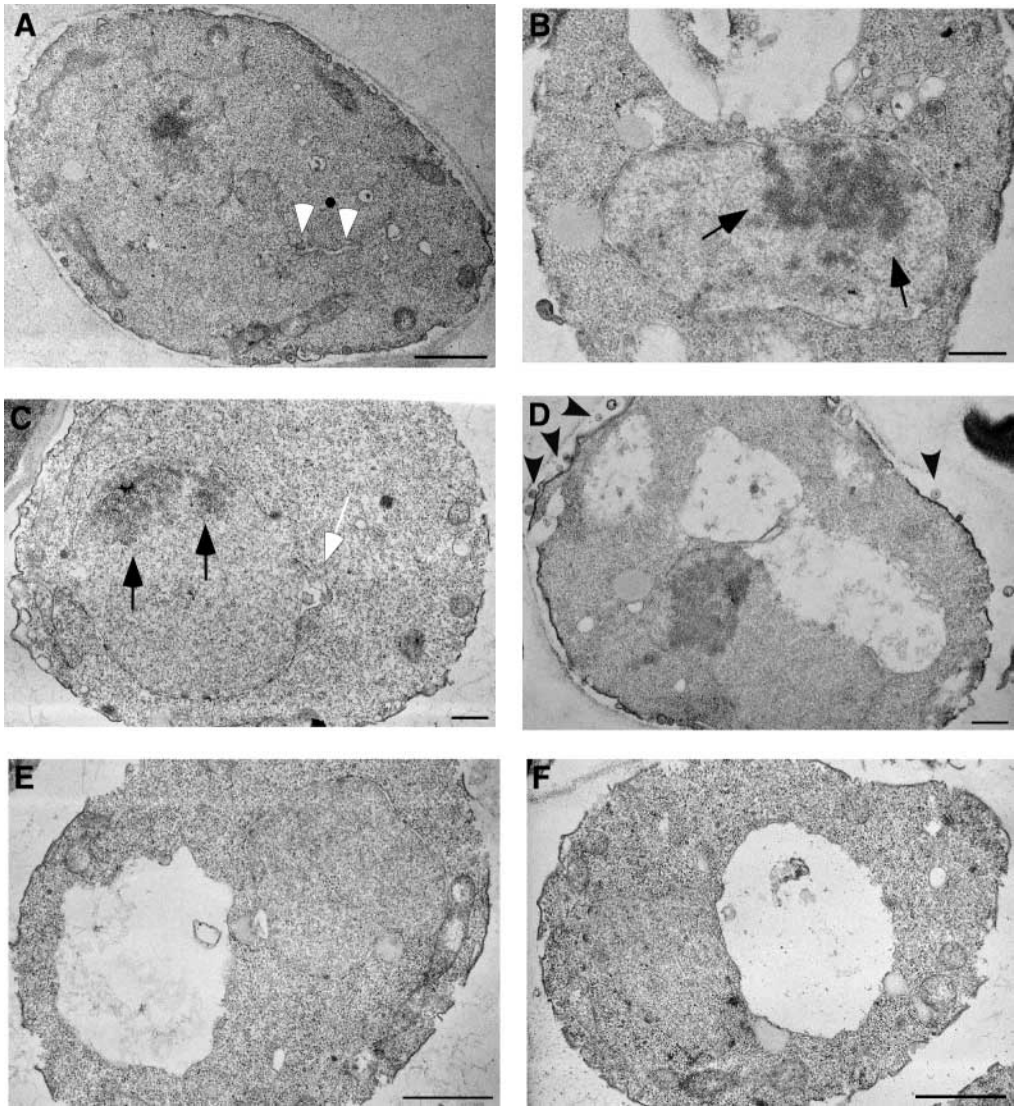


**Fig. 4.** Electron micrographs of wild-type and mutant *nma111* cells after induction of apoptosis with 3 mM $H_2O_2$. (A-D) Wild-type cells treated with $H_2O_2$ exhibit a more prominent endoplasmic reticulum (white arrowhead in A), chromatin condensation along the NE (black arrows in B,C), blebs from the NE (white arrow in C), and tiny vesicles on the outer face of the plasma membrane (black arrowheads in D). (E,F) Δ*nma111* cells do not exhibit any of these apoptotic hallmarks at the EM level. Scale bars, 500 nm.

under apoptotic conditions (i.e. by oxygen stress), and that this redistribution within the nucleus might be directly related to the apoptosis promoting effect of Nma111p.

### The serine-protease activity of Nma111p is required for its death-promoting activity

It was recently shown that human HtrA2 promotes cell death through its serine-protease activity (Suzuki et al., 2001; Hedge et al., 2002; Verhagen et al., 2002). Nma111p contains a sequence GGSGS (residues 233 to 237), which is similar to the consensus sequence GNSGG surrounding the active site serine of many trypsin-like serine proteases (Lipinska et al., 1990; Pallen and Wren, 1997; Clausen et al., 2002). To test whether serine235 is required for the death-promoting activity of Nma111p, we mutated this residue to cysteine by oligonucleotide site-directed mutagenesis (see Materials and Methods) in the plasmid pNOPPATA1L-NMA111. The resulting plasmid (pNOPPATA1L-NMA111-S235C) was transformed into Δ*nma111* cells. First, we determined the subcellular localisation of both the wild-type and the mutant ProtA-Nma111p fusion protein by indirect immunofluorescence microscopy. ProtA-Nma111p and ProtA-Nma111p-S235C are both nuclear proteins (Fig. 7A,B), similar to Nma111p-GFP (Fig. 6A), indicating that the S235C mutation has no effect on the correct localisation of the protein. Next, ProtA-Nma111p cells, ProtA-Nma111p-S235C cells and Δnma111 cells were incubated with 0.8 mM $H_2O_2$ for 4 hours and analysed for apoptotic hallmarks. As shown in Fig. 7C, ProtA-Nma111p cells showed DNA condensation within the nucleus as determined by Sytox-Green nucleic-acid stain, whereas, in ProtA-Nma111p-S235C and Δ*nma111* cells the DNA was evenly distributed between the nucleus and the mitochondria (Fig. 7D,E). In consistency with these results, ProtA-Nma111p cells treated with 0.8 mM $H_2O_2$ for 4 hours were TUNEL-positive (Fig. 7F), whereas ProtA-Nma111p-S235C and Δ*nma111* cells were TUNEL-negative (Fig. 7G,H).

Next, ProtA-Nma111p cells, ProtA-Nma111p-S235C cells and Δ*nma111* cells were tested for cell survival after $H_2O_2$ treatment. ProtA-Nma111p-S2355C and *nma111* mutants survive better than ProtA-Nma111p cells after treatment with up to 1.2 mM $H_2O_2$ (Fig. 7I).

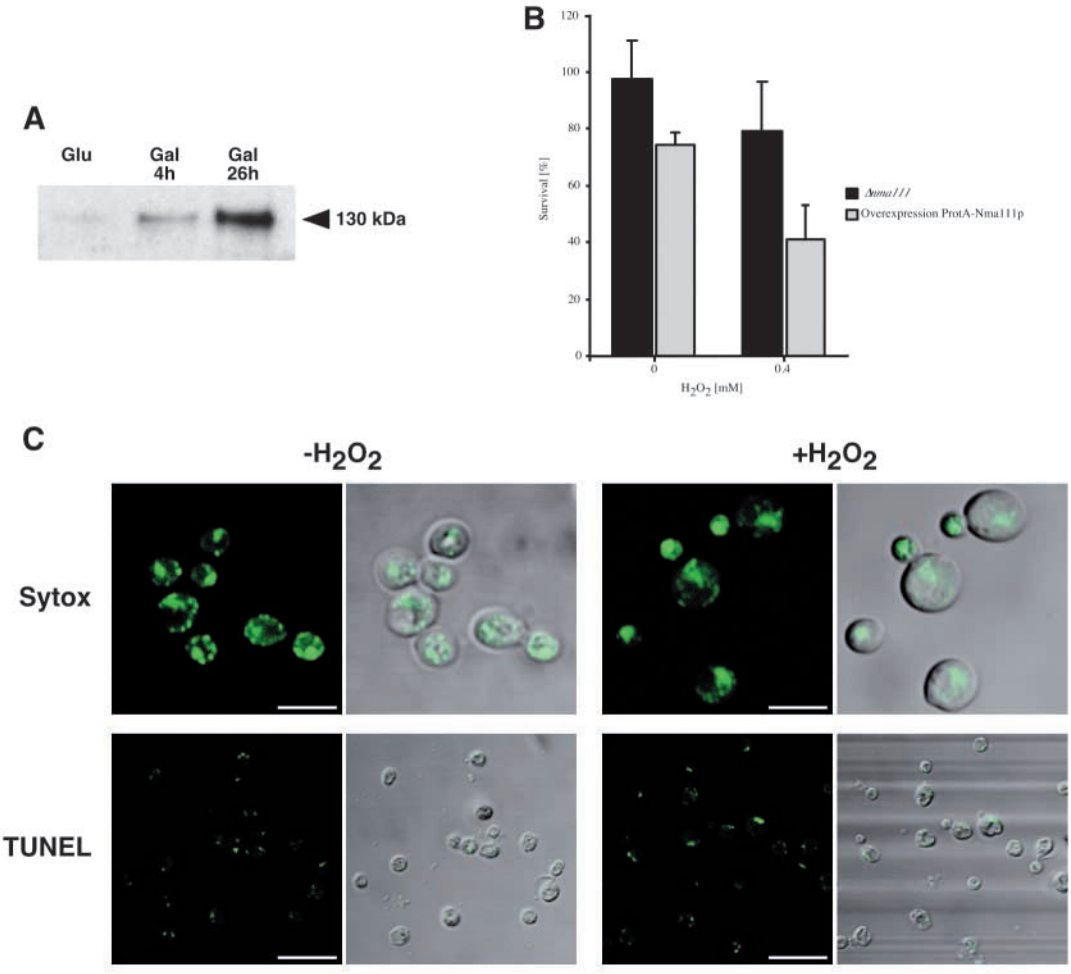


**Fig. 5.** Overexpression of ProtA-Nma111p leads to apoptotic-like cell death. (A) Extracts of cells carrying plasmid-borne N-terminally protein-A-tagged Nma111p that were grown in glucose medium for 4 hours or induced with galactose medium for 26 hours were immunoblotted and probed with an anti-protein-A antibody. (B) Survival rates of yeast cells overexpressing ProtA-Nma111 compared with Δ*nma111* cells without pretreatment or with incubation in 0.4 mM $H_2O_2$ for 24 hours. Data represent mean±s.d. (C) Sytox-Green DNA stain and TUNEL assay of ProtA-Nma111p overproducer. Scale bars, 5 μm (Sytox), 10 μm (TUNEL).

Taken together, the serine235 is required for the death-promoting activity of Nma111p.

## Discussion

In this study, we describe the characterisation of the *S. cerevisiae* HtrA-like protein Nma111p that was identified by its two-hybrid interaction with the yeast NPC protein Nic96p. Clearly, Nma111p acts as a mediator of yeast apoptosis. Cells lacking *nma111* show increased survival at elevated temperatures and after induction of apoptosis by oxygen stress, whereas overexpression of Nma111p leads to enhanced apoptotic-like cell death. We show further that the death-promoting activity of Nma111p depends on its serine-protease activity.

Based on database searches, the only yeast protein that belongs to the HtrA family of serine proteases is encoded by the ORF YNL123W. We named this protein Nma111p. HtrA-like serine proteases have been identified in bacteria and mammals (reviewed in Clausen et al., 2002; Pallen and Wren, 1997; Ponting, 1997), and a common feature of this protein family is the presence of PDZ domains at their C-terminus. PDZ domains mediate protein-protein interactions that are implicated in signal transduction events (reviewed in Ponting, 1997; Harris and Lim, 2001). Based on database searches, Nma111p is the only yeast protein that harbours PDZ domains and among the HtrA-family members it is unique because its PDZ-domain is duplicated (reviewed in Clausen et al., 2002; Salvesen and Duckett, 2002; Pallen and Wren, 1997; Ponting, 1997). The functional significance of this duplication is unknown and subject to further investigation. Preliminary results indicate that a C-terminally truncated version of Nma111p, in which the second PDZ-domain has been cleaved off, is no longer localised exclusively to the nucleus (B.F. and U.A., unpublished), which might indicate a connection between this PDZ domain and Nma111p's death-promoting function.

HtrA/DegP was first described in bacteria, where it is required for high temperature tolerance and acts both as a serine protease and a chaperone in a temperature-dependent manner (Spiess et al., 1999). The human homologue of Nma111p, HtrA2, was shown recently to induce apoptosis in a serine-protease-dependent manner and through its ability to antagonise the inhibitor of apoptosis protein XIAP (Suzuki et al., 2001; Hedge et al., 2002; Martins et al., 2002; Verhagen et al., 2002). In this context, Nma111p, like its mammalian homologue HtrA2, exerts a proapoptotic activity. Deletion of *nma111* causes an increase in the survival rate of cells treated with $H_2O_2$ and at elevated temperatures (Figs 1, 7), and this deletion prevents yeast cells from undergoing apoptosis (Figs 3, 4). By contrast, overexpression of Nma111p enhances apoptotic-like cell death (Fig. 5), and its death-promoting

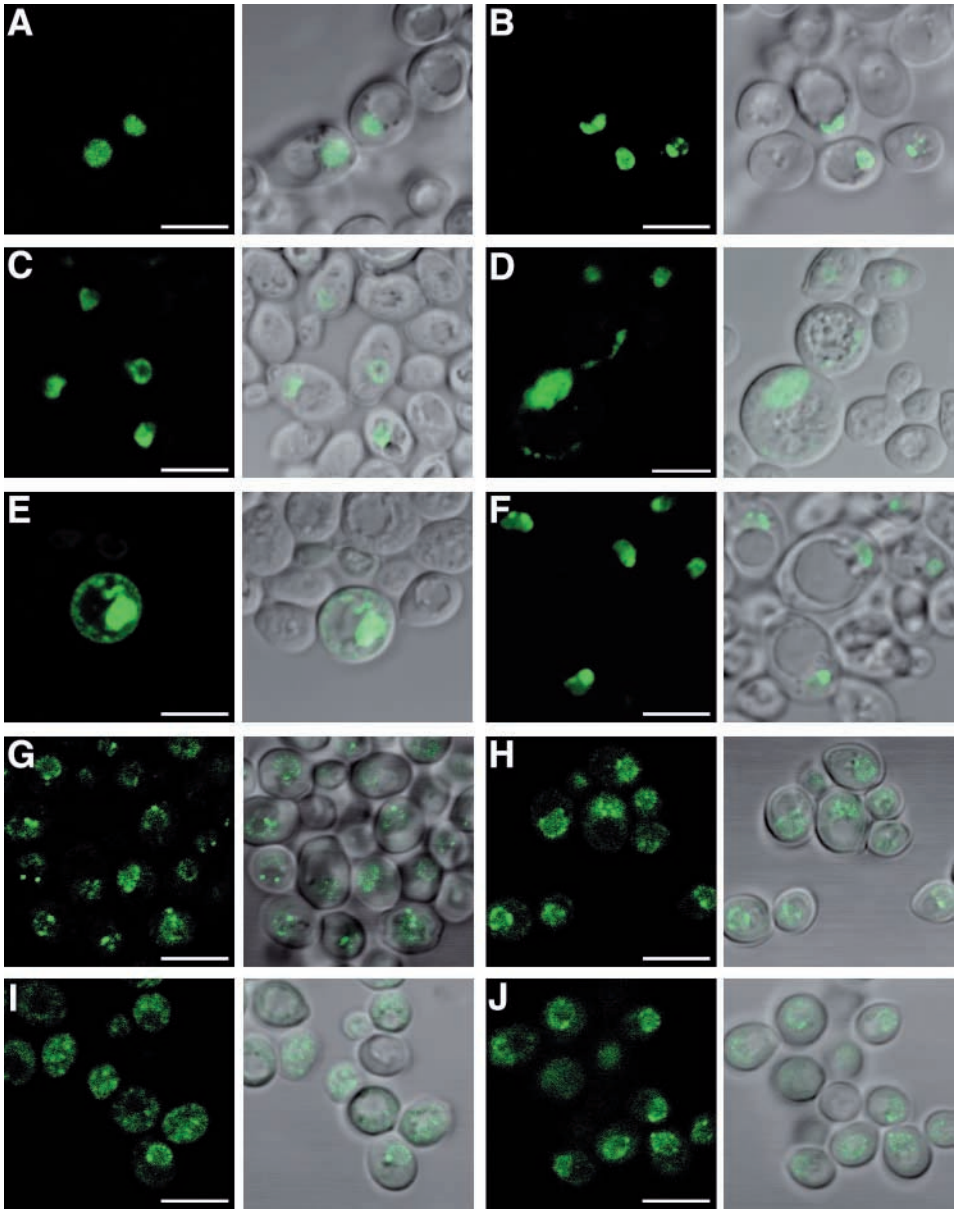


**Fig. 6.** Nma111p-GFP aggregates inside the nucleus at elevated temperatures and after induction of apoptosis. In yeast cells grown at 25°C, Nma111p-GFP is evenly distributed throughout the nucleus (A), whereas, in cells shifted to 42°C (B) or 50°C (C), the protein forms aggregates inside the nucleus. Moreover, in cells shifted to 50°C a partial translocation of Nma111p-GFP into the cytoplasm was observed (D and E). In apoptotic cells (F), after treating cells with 3 mM $H_2O_2$ for 3 hours, Nma111p-GFP also aggregates inside the nucleus. Nma111p-GFP was visualised by direct immunofluorescence of the GFP tag. Under similar conditions, the localisation of the control protein Xpo1-GFP remains unaffected: (G) 25°C; (H) 42°C; (I) 50°C; (J) 3 mM $H_2O_2$ for 3 hours. Confocal fluorescence micrographs (left) and coincident fluorescence/differential-interference-contrast images are shown (right). Scale bars, 5 μm.

activity depends on its serine-protease activity (Fig. 7). The *Drosophila* IAP antagonists Reaper, HID and Grim were initially thought to contain pro-apoptotic activities that repress the activity of *Drosophila* IAP proteins. More recent studies show that, on the contrary, they act by repressing the caspase-inhibitory function of the *Drosophila* IAP proteins (Martin, 2002). It therefore remains to be established if Nma111p promotes cell death because of a pro-apoptotic activity or if it also represses an antagonistic function, such as a yeast IAP protein.

IAP proteins are characterised by an ~70-amino-acids-long BIR (baculoviral IAP repeat) domain. They inhibit apoptosis by directly binding to caspases, thereby occluding their catalytic pockets, or by antagonising Reaper, HID and Grim, the *Drosophila* homologues of HtrA2 (reviewed in Deveraux and Reed, 1999; Reed and Bischoff, 2000; Salvesen and Duckett, 2002). Moreover, certain IAP proteins might participate in cellular functions other than apoptosis, in particular in cell division (Reed and Bischoff, 2000). In this context, Bir1p, the only IAP-like protein in *S. cerevisiae*, has been shown to exert a role in meiosis and in chromosome segregation rather than in yeast apoptosis (Uren et al., 1999; Yoon and Carbon, 1999; Li et al., 2000) (reviewed in Reed and Bischoff, 2000). However, the cell division defects observed in *bir1* mutant strains are attributed to the unique C-terminal domain of Bir1p and not to its N-terminal BIR domains (Yoon and Carbon, 1999; Reed and Bischoff, 2000), thereby leaving the possibility open that Bir1p has another, yet uncharacterised, function in yeast apoptosis. This is particularly interesting, because Bir1p, like Nma111p, is a nuclear protein (Uren et al., 1999), indicating that Nma111p and Bir1p might interact like their homologues in *Drosophila* and human, and that this interaction, in turn, might exert a role in the regulation of yeast apoptosis. IAP proteins, however, interact with their antagonists, for example HtrA2/Omi, through an N-terminal IAP-binding motif in the antagonist (Hedge et al., 2002; Verhagen et al., 2002). Such an IAP-binding motif is not obvious within the amino acid sequence of Nma111p.

Although *S. cerevisiae* lacks a caspase gene, a metacaspase termed YCA1 has been identified recently (Madeo et al., 2002b). Metacaspases are putative proteases that have a caspase-like fold and are present in plants, fungi and protozoa (Uren et al., 2000; Jin and Reed, 2002). YCA1 is implicated in cell death induced by $H_2O_2$, acetic acid and ageing (Madeo et al., 2002b). Overexpression of YCA1 in yeast enhances apoptotic-like death of yeast that is induced by $H_2O_2$ or acetic

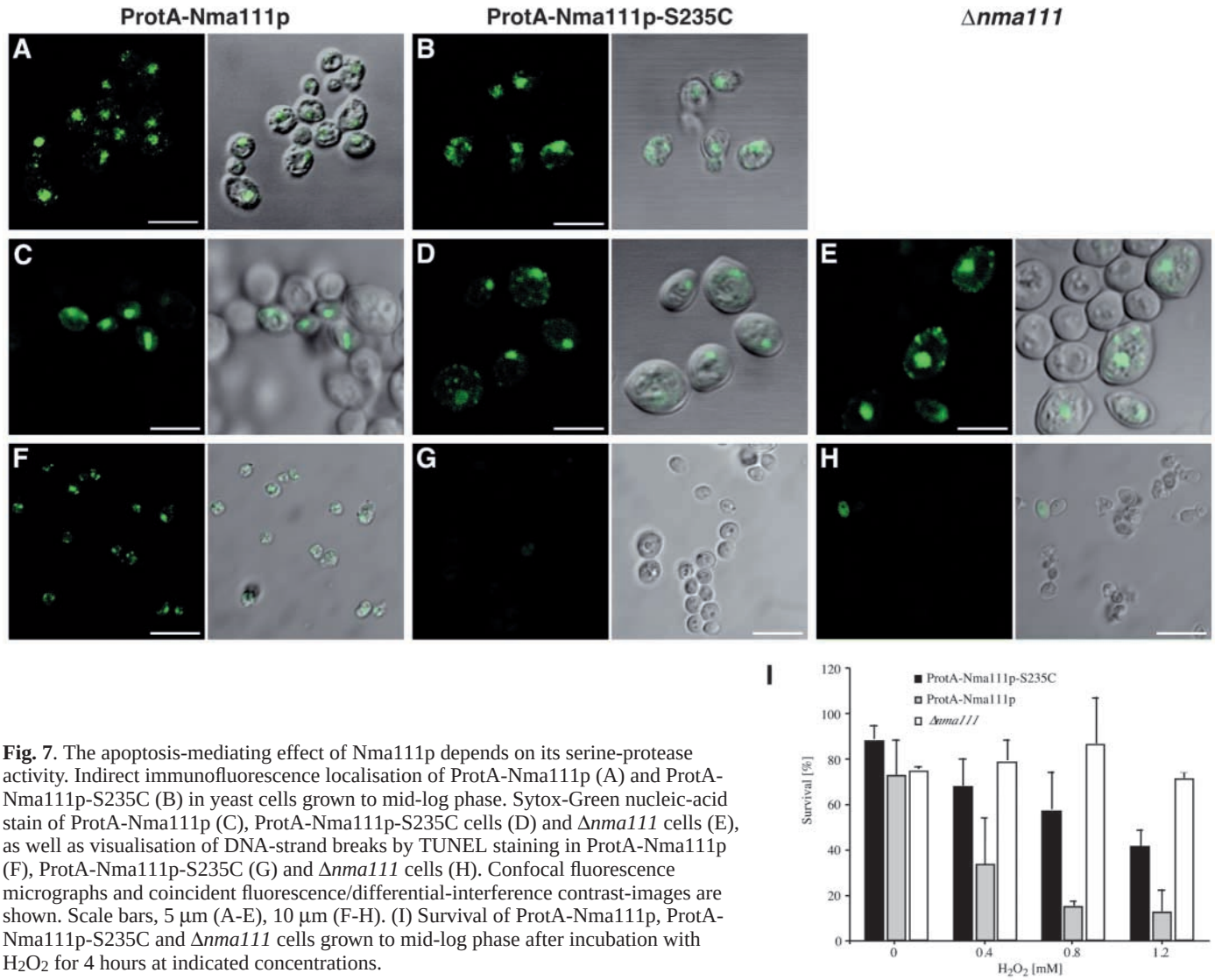


**Fig. 7**. The apoptosis-mediating effect of Nma111p depends on its serine-protease activity. Indirect immunofluorescence localisation of ProtA-Nma111p (A) and ProtA-Nma111p-S235C (B) in yeast cells grown to mid-log phase. Sytox-Green nucleic-acid stain of ProtA-Nma111p (C), ProtA-Nma111p-S235C cells (D) and Δ*nma111* cells (E), as well as visualisation of DNA-strand breaks by TUNEL staining in ProtA-Nma111p (F), ProtA-Nma111p-S235C (G) and Δ*nma111* cells (H). Confocal fluorescence micrographs and coincident fluorescence/differential-interference contrast-images are shown. Scale bars, 5 μm (A-E), 10 μm (F-H). (I) Survival of ProtA-Nma111p, ProtA-Nma111p-S235C and Δ*nma111* cells grown to mid-log phase after incubation with $H_2O_2$ for 4 hours at indicated concentrations.

acid, whereas *yca1* deletion reduces cell death. Interestingly, Δ*yca1* mutant cells, like Δ*nma111* cells, show higher thermotolerance, and an YCA1-GFP fusion protein is localised predominantly to the nucleus (B.F. and U.A., unpublished) (Szallies et al., 2002). Given that Nma111p and Bir1p are also nuclear proteins, YCA1, Bir1p and Nma111p might be regulated and interact in a similarly to their mammalian homologues. Furthermore, the nucleus appears to play a significant role in yeast apoptosis. This contrasts with mammalian cells, where caspases and other regulatory proteins of apoptosis are predominantly localised in mitochondria or the cytoplasm. For example, mammalian HtrA2 is predominantly localised to mitochondria and is released into the cytosol under apoptotic conditions. This, in turn, allows its interaction with XIAP, which subsequently allows the activation of the executioner caspases (Suzuki et al., 2001; Hedge et al., 2002; Martins et al., 2002; Verhagen et al., 2002).

Another pivotal step in mammalian apoptosis is the release of cytochrome *c* from mitochondria into the cytosol, resulting in the assembly of a caspase-9 activating complex (the apoptosome) in the cytosol and the subsequent activation of the executioner caspases (Leist and Jäättelä, 2001). Cytochrome *c* and mitochondria have also been shown to play a role in yeast apoptosis when induced by acetic acid (Ludovico et al., 2002), whereas other studies have reported that functional mitochondria are not required for yeast apoptosis (Gross et al., 2000; Kissova et al., 2000; Mazzoni et al., 2003). In the absence of RSM23 (YGL129c), a protein of the mitochondrial small ribosomal subunit, induction of yeast apoptosis by YCA1 is completely prevented again suggesting a role for mitochondria in the regulation of yeast apoptosis (Madeo et al., 2002b). Nevertheless, a functional or regulatory role of mitochondria in Nma111p-mediated yeast apoptosis remains to be established. However, based on the localisation of Nma111p to the nucleus and given that Nma111p probably does not leave the nucleus under apoptotic conditions but forms aggregates inside it (Fig. 6), a link between mitochondria and the Nma111p pathway appears rather unlikely.

The question of whether yeast does undergo apoptosis is still controversial. Clearly, yeast shows certain typical morphological and cytological hallmarks of apoptosis, thereby

supporting the idea it might undergo apoptosis. Our data further support this notion, demonstrating that the yeast HtrA-like protein Nma111p, like its mammalian and *Drosophila* homologues, can promote programmed cell death. Moreover, our data show that basic key player of apoptosis, such as Nma111p/HtrA2, already exist in yeast. However, the presence of a metacaspase and absence of a *bona fide* caspase, the lack of many known regulator proteins of apoptosis (such as the Bax/Bcl-2 family, Apaf-1 and p53) and the fact that the only yeast IAP protein (Bir1p) play a role in cell division rather than in yeast apoptosis, show that, despite all similarities, yeast apoptosis clearly differs from apoptosis in multicellular organisms. Nevertheless, yeast increasingly provides a useful system to elucidate the ancestral pathway(s) that might eventually have led to the manifestation of apoptosis in multicellular organisms. The discovery that Nma111p interacts with the NPC protein Nic96p further suggests that there is an interesting link between apoptosis, the NPC and nucleocytoplasmic transport. The functional significance of this link remains to be elucidated.

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