Histone modifications and the HtrA-like serine protease Nma111p regulate apoptosis in budding yeast

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von
David Walter
aus Mümliswil, Schweiz

Basel, 2010

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von

Prof. Dr. Ueli Aebi
Prof. Dr. Birthe Fahrenkrog
Prof. Dr. Joris Winderickx

Basel, den 30.03.2010

Dekan
Prof. Dr. Eberhard Parlow

Abstract

Apoptosis is a form of programmed cell death that plays a central role in development and cellular homeostasis in higher eukaryotes. Knowledge about apoptotic regulation is particularly important for medical research, since apoptotic misregulation is implicated in many human diseases, such as Alzheimer's and Huntington's disease, immunodeficiency and cancer. Recent studies have established yeast as model to study the mechanisms of apoptotic regulation. Changes in chromatin configuration are implicated in apoptotic regulation both in yeast and in higher eukaryotes. One mechanism that alters chromatin configuration is the covalent modification of histones, which associate with DNA to form the nucleosome, the fundamental unit of chromatin. In my thesis work, I have identified and characterized distinct interrelated histone modifications on histone H2B and histone H3 as regulators of apoptosis in yeast (Chapter 2 and 3). Histone H2B ubiquitination at lysine K123 by the E3 ligase *BRE1* is required in promoting methylation of histone H3 at lysine K4 and K79. These methylations are brought about by the conserved methyltransferases Set1p and Dot1p, respectively. We found that disruption of the E3 ligase *BRE1* or the methyltransferase *SET1*, which causes a lack of histone H2B K123 ubiquitination and histone H3 K4 methylation, respectively, causes metacaspase Yca1p-dependent apoptosis (Chapter 2 and 3). In contrast, we found that disruption of *DOT1*, which causes a lack of histone H3 K79 methylation confers apoptosis resistance (Chapter 3). Moreover, we found that Dot1p is required for Yca1p-dependent cell death of *Δset1* cells (Chapter 3).

How does disruption of *DOT1* confer apoptosis resistance? Yeast cells that fail to methylate histone H3 K79 due to *DOT1* disruption exhibit defects in the DNA damage response. Particularly, Dot1p mediated histone H3 K79 methylation is required for Rad9p-dependent checkpoint activation after DNA damage. In higher eukaryotes, the evolutionarily conserved DNA-damage response is a signaling cascade that senses DNA

damage and activates cellular responses including apoptosis. Strikingly, we found that Rad9p is required for cell death of *Δset1* similar to Dot1p (Chapter 5), suggesting that Dot1p mediates apoptosis through its function in the DNA-damage response. Thus, we suggest that apoptosis in budding yeast is linked to the DNA damage response similar to apoptosis in higher eukaryotes.

Together, these studies highlight the requirement of Dot1p-mediated histone H3 K79 methylation for an Yca1p-dependent cell death scenario and points to a novel role of the conserved histone H2B/H3 crosstalk in apoptosis regulation. Moreover, our results imply a requirement of the DNA damage response for apoptosis induction in budding yeast.

Another objective of this thesis was the characterization of the functional role of the HtrA1-like serine protease Nma111p in yeast apoptosis (Chapter 4). Nma111p functions as a nuclear serine protease that is necessary for apoptosis under cellular stress conditions. We have examined the role of nuclear protein import in the function of Nma111p in apoptosis. Nma111p contains two small clusters of basic residues toward its amino terminus, both of which are necessary for efficient translocation into the nucleus. Nma111p does not shuttle between the nucleus and cytoplasm during either normal growth conditions or under environmental stresses that induce apoptosis. The amino-terminal half of Nma111p is sufficient to provide the apoptosis-inducing activity of the protein, and both the NLS sequences and catalytic serine 235 are necessary for this function. Together, we provide compelling evidence that intranuclear Nma111p activity is necessary for apoptosis in yeast.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

A	alanine
AIF1	apoptosis inducing factor 1
Bcl-2	B-cell lymphoma 2
BH domains	BCL-2 homology domains
BIR	baculovirus IAP repeat
CLS	chronological life span
Da	Dalton
DHE	dihydroethidium
DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
DOT1	disruptor Of Telomeric silencing
D_2R	$(L\text{-}Asp)_2$ rhodamine 110
E	glutamic acid
Ef2p	elongation factor 2
ERCs	extrachromosomal rDNA circles
GFP	green fluorescent protein
HOX	homeobox
HtrA2	high temperature requirement protein A2
H_2O_2	hydrogen peroxide
IAP	inhibitor-of-apoptosis protein
K	lysine
kD	kilodalton
MOMP	mitochondrial outer membrane permeabilization
NLS	nuclear localization signal
NMA111	Nuclear mediator of apoptosis
NUC1/EndoG	Nuclease 1/Endonuclease G
PDZ	The name PDZ is derived from the first three proteins in which these domain was found: **P**SD-95 (a 95 kDa protein involved in signaling in

	the post-synaptic density), **D**lg (the *Drosophila* discs large protein), and **Z**O1 (the zonula occludens 1 protein involved in maintaining epithelial cell polarity).
PTP	permeability transition pore
RENT	regulator of nucleolar silencing
RING	really interesting new gene
RLS	replicative life span
RNA	ribonucleic acid
ROS	reactive oxygen species
SAGA complex	Spt-Ada-Gcn5-acetyltransferase complex
SET1	SET domain-containing protein 1
SDS-PAGE	sodium dodecyl sulfate-poly-acrylamide gel electrophoresis
SUMO	small ubiquitin-like modifier
S	serine
TOR	target of rapamycin
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end
VDAC	voltage-dependent anion channel
wt	wild type
YCA1	Yeast caspase 1
XIAP	X-linked inhibitor of apoptosis protein

1

General Introduction

1.1 Apoptosis

The term apoptosis comes from Greek αποπτοσισ, whose prefix "apo" (απο) can be taken as separation. The suffix ptosis (πτοσισ), translating as "falling off", has been generally known as the falling off of leaves from trees and refers to the morphological feature of the formation of apoptotic bodies (Figure 1.1). Apoptosis plays a complementary but opposite role to mitosis in the regulation of animal cell populations (Kerr et al., 1972). It is initially defined by its morphological and biochemical characteristics such as exposure of phosphatidylserine on the cell surface, cell shrinkage, apoptotic body formation, production of reactive oxygen species (ROS), chromatin condensation and nuclear fragmentation (Kerr et al., 1972). Apoptosis is essential in normal development and homeostasis and acts as a defense mechanism in response to cellular abnormalities in multicellular organisms (reviewed in (Fadeel and Orrenius, 2005)). Apoptosis occurs during normal embroyological development and during normal tissue turnover (Fadeel and Orrenius, 2005). Moreover, dysregulation of this cell death process has been postulated to play a role in the pathogenesis of a variety of human diseases (reviewed in (Fadeel and Orrenius, 2005)). Diminished apoptosis has been linked to both the development of tumors and autoimmune syndromes, whereas excessive apoptosis has been implicated in neurodegenerative diseases. These facets have made apoptotic pathways the objective of intense interest and as result, enhanced our understanding of the complex networks of apoptotic signal transduction pathways.

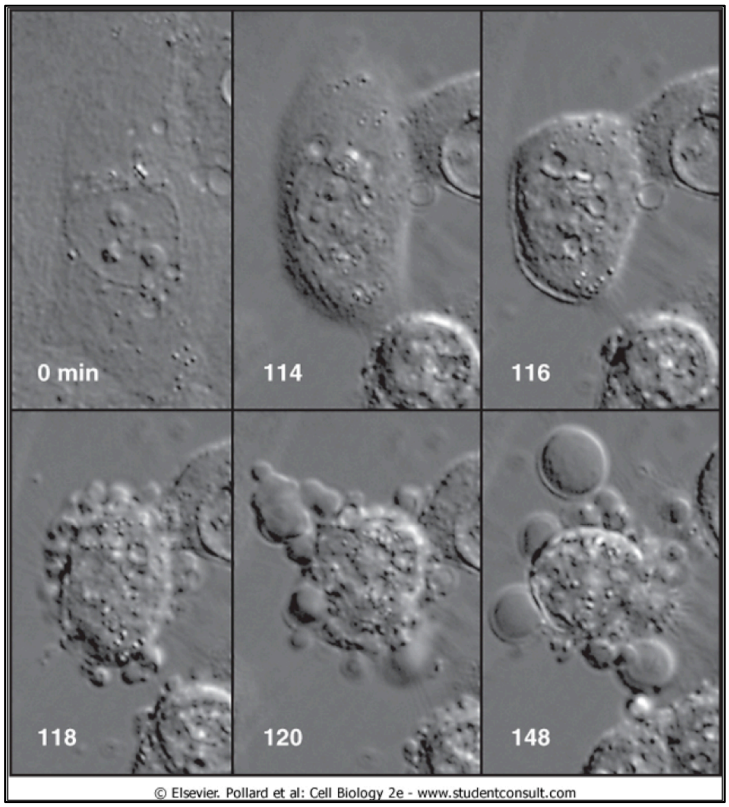

Figure 1.1: Apoptosis of a pig kidney cell following exposure to etoposide, a drug used in cancer chemotherapy.
The dramatic cytoplasmic blebbing results in the disassembly of the cell into membrane-enclosed vesicles called apoptotic bodies. Adapted from (Pollard and Earnshaw, Cell Biology 2E)

Apoptosis typically involves the activation of a unique class of cysteine proteases known as caspases (Riedl and Shi, 2004). These proteases bring about apoptosis by cleaving key cellular substrates after specific aspartate residues. Caspases are synthesized as inactive zymogens and two classes of caspases are involved in cell death, i.e. the initiator caspases and the effector caspases (Riedl and Shi, 2004).

Initiator caspases can be activated by two alternative pathways (Figure 1.2): one is mediated by death receptors on the cell surface — referred to as the extrinsic pathway; the other is mediated by mitochondria — referred to as the instrinsic pathway.

In the extrinsic pathway, the activation of cell surface receptors stimulates the assembly of the death-inducing signaling complex (DISC), within which procaspase-8 is activated (Peter and Krammer). In the intrinsic pathway, the translocation of proapoptotic Bcl-2 proteins, such as Bid to the mitochondria triggers the release of cytochrome c,

which stimulates the apoptotic protease activating factor 1 (Apaf-1)-dependent activation of procaspase-9 in the apoptosome (Figure 1.2) (Peter and Krammer). Activated initiator caspases in turn are capable of activating effector caspases, the ultimate executors of cell death.

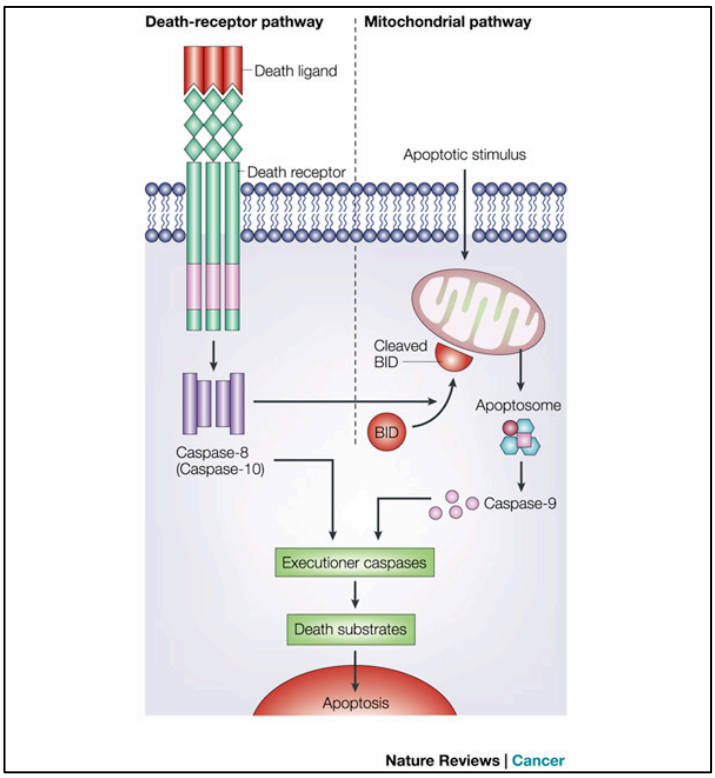

Figure 1.2: The two main apoptotic signaling pathways
Apoptosis can be initiated by two alternative pathways: either through death receptors on the cell surface (extrinsic pathway) or through mitochondria (intrinsic pathway). In both pathways, induction of apoptosis leads to activation of an initiator caspase: caspase-8 and possibly caspase-10 for the extrinsic pathway; and caspase-9, which is activated at the apoptosome, for the intrinsic pathway. The initiator caspases then activate executioner caspases. Active executioner caspases cleave the death substrates, which eventually results in apoptosis. Adapted from (Igney and Krammer, 2002)

1.1.1 Key regulator of apoptosis

Once activated, both initiator caspases and effector caspases can be modulated by a set of proteins, known as inhibitor-of-apoptosis proteins (IAPs). IAPs were initially identified in baculoviruses and found to prevent apoptosis of the host cell (Crook et al., 1993). IAP homologues have been identified in mammalian cells, worms, flies and yeast (Deveraux

and Reed, 1999) and they are characterized by the presence of one to three copies of the baculovirus IAP repeat (BIR) domains (Figure 1.3). BIR domains typically comprise 70 to 80 residues and hold a zinc ion that is coordinated by one conserved histidine and three cysteine residues. Via the BIR domain, IAPs are able to bind caspases, thereby preventing the interaction of caspases with their substrates (Riedl and Shi, 2004). Additionally, some IAPs contain a second zinc-binding motif known as RING domain (Figure 1.3), which exhibits E3-ubiquitin-ligase activity. By such a RING domain, IAPs recruit and direct E2-ubiquitin-conjugating enzymes to specific substrates, such as caspases (Hu and Yang, 2003; Suzuki et al., 2001b; Wilson et al., 2002), to catalyze the transfer of ubiquitin to the substrate and its subsequent degradation by the 26S proteasome. Furthermore, IAPs can trigger their self-degradation (Vaux and Silke, 2005), thereby leading to enhanced caspase activity.

IAPs are distinguished into type-I or type-II BIR-domain-containing proteins (BIRPs) depending on the structure of the BIR-domains and the presence or absence of a RING domain (Figure 1.3). Type-II BIRPs, such as the *C. elegans* proteins Bir1 and Bir2, yeast Bir1p or human survivin are known to play roles in chromosome segregation and cytokinesis (Fraser et al., 1999). Moreover, the type-II BIRPs Bir1p (Li et al., 2000; Uren et al., 1999; Walter et al., 2006; Yoon and Carbon, 1999), survivin (Ambrosini et al., 1997; Lens et al., 2003; Skoufias et al., 2000; Temme et al., 2003), *Drosophila* Deterin and Bruce and the murine TIAP are able to inhibit apoptosis, unlike *C.elegans* Bir1 and Bir2, by a yet unknown mechanism (Bartke et al., 2004; Hao et al., 2004; Jones et al., 2000; Vernooy et al., 2002).

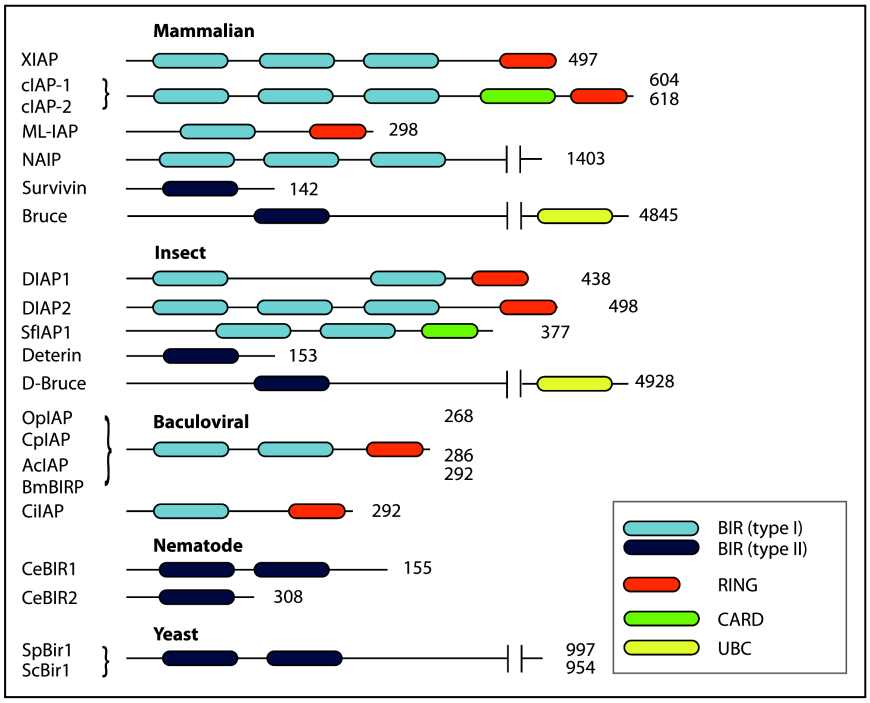

Figure 1.3: Schematic representation of BIR-containing proteins.
BIR proteins are characterized by the presence of multiple domains, such as BIR, RING, CARD, and UBC domains. Their approximate positions are represented with the total amino acid length shown to the right of each protein. RING domains confer E3 ubiquitin protein ligase activity; UBC refers to the ubiquitin-conjugating domain, which has E2 activity. The presence of both these domains in components of the apoptotic machinery suggests a link between apoptosis and protein degradation. Adapted from (Verhagen et al., 2001). Abbreviations: BIR: baculoviral IAP repeat; RING: RING (really interesting new gene) zinc-finger; CARD: caspase recruitment domain; UBC: Ubiquitin-conjugating enzymes

IAP-mediated inhibition of caspase activity, however, is not sufficient to regulate apoptosis. Hence, not only the activity but also the activation of caspases is tightly regulated. The latter involves the release of pro-apoptotic factors like cytochrome *c* from the mitochondrial inter-membrane space (Earnshaw, 1999), which facilitates the formation of the apoptosome. Major regulators of mitochondrial integrity and mitochondrion-initiated caspase activation are proteins of the Bcl-2 family (Cory and Adams, 2002; Danial and Korsmeyer, 2004). *BCL-2* (B-cell lymphoma 2) was initially identified as a gene whose product causes resistance to apoptosis in lymphocytes (McDonnell et al., 1989; Vaux et al., 1988). Subsequent studies, however, identified a number of both pro- as well as anti-apoptotic Bcl-2-related proteins. The Bcl-2-family is defined by homology shared within four conserved regions in Bcl-2, termed BCL-2

homology (BH) domains, and is grouped into three subfamilies depending on their apoptotic properties, i.e. the pro-apoptotic Bax- and BH3 only families as well as the pro-survival Bcl-2 family (Figure 1.4).

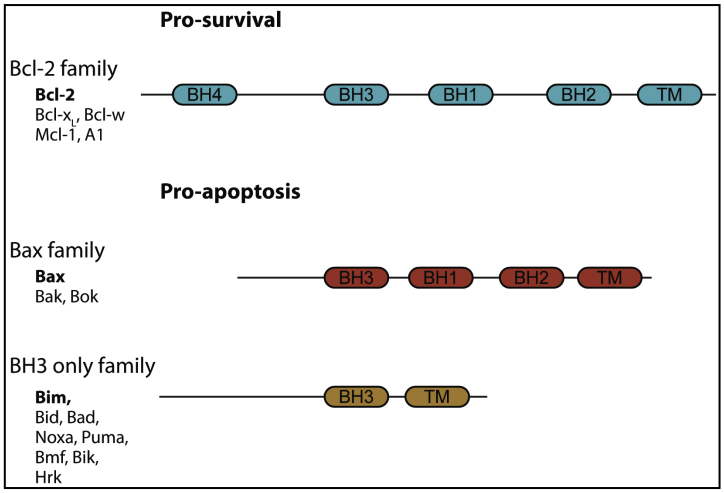

Figure 1.4: Three subfamilies of Bcl2-related proteins.
Bcl-2-related proteins are characterized by the presence of multiple copies of a Bcl-2 homology (BH) domain. Typically Bcl-2 family members additionally have a carboxy-terminal transmembrane domain (TM), with the exception of A1 and members of the BH3-only family (Bad, Bid, Noxa, Bmf and Puma).

1.2 Yeast Apoptosis

Apoptosis is a highly regulated cellular death program that is crucial for the development and maintenance of multicellular organisms. In the past few years, however, it became evident that apoptosis might occur not only in multicellular, but also in unicellular organisms, such as the yeast *S.cerevisiae* (reviewed in (Frohlich et al., 2007)). *S. cerevisiae* shows typical apoptotic hallmarks when treated with various agents including hydrogen peroxide and acetic acid (Ludovico et al., 2001; Madeo et al., 1999a; Narasimhan et al., 2001; Severin and Hyman, 2002). Other than the morphological characteristics, a growing list of homologues to apoptotic regulators in metazoans has been identified (Figure 1.5) (Buttner et al., 2007; Fahrenkrog et al., 2004; Madeo et al., 2002; Walter et al., 2006; Wissing et al., 2004). In addition, yeast programmed cell death has been linked to cellular events such as mitochondrial fragmentation (Fannjiang et al., 2004), cytochrome *c* release (Ludovico et al., 2002), ageing (Fabrizio et al., 2004; Herker et al., 2004) and phosphorylation of histone H2B (Ahn et al., 2005) (Figure 1.5). Taken together, these findings support the general view that a basic machinery of apoptosis is present and functional in yeast.

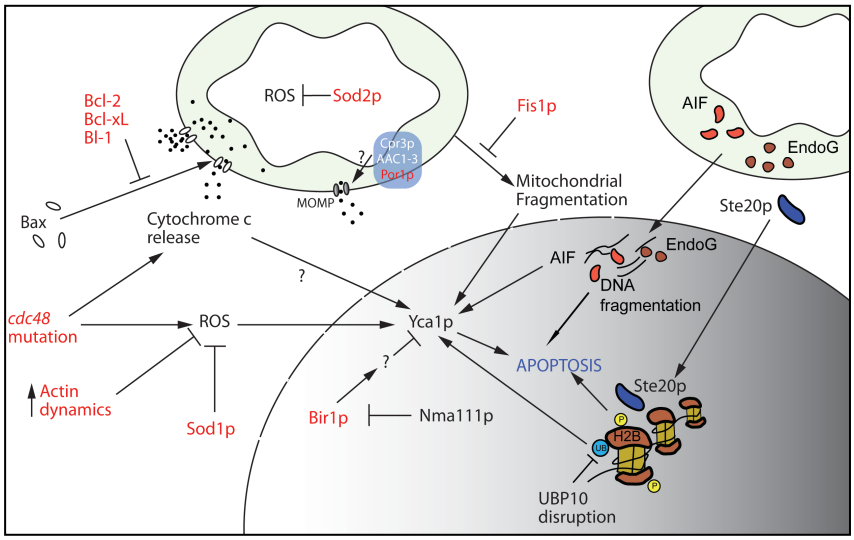

Figure 1.5: Regulation of the apoptotic machinery in yeast.
The key players regulating the basic molecular machinery of apoptosis such as the caspase-like protein Yca1p, the inhibitor-of-apoptosis protein Bir1p, the Omi orthologue Nma111p and the endonucleases EndoG/Nuc1p and Aifp are conserved from yeast to higher eukaryotes (see chapter **1.2.2**). Cellular processes such as mitochondrial fragmentation and cytochrome c release from the

mitochondria are also conserved. Moreover, key regulators of mammalian apoptosis such as Bcl-2-like proteins can interfere with the apoptotic machinery in yeast (see chapter **1.2.1**). Question marks indicate interrelations, which have been hypothesised but yet not clearly demonstrated. Components of the putative PTP are highlighted in blue color. Proteins and processes that protect against apoptosis are emphasized in red. PTP: permeability transition pore, MOMP: outer mitochondrial membrane permeabilization.

1.2.1 Heterologous expression of apoptosis regulators

Yeast as unicellular organisms was long supposed to lack an apoptosis-like death program. Therefore it had been used as "clean room" for investigating the interaction of mammalian proteins involved in apoptosis, such as proteins from the Bcl-2 family. Heterologous expression of Bax, a pro-apoptotic Bcl-2 family member, results in a lethal phenotype in yeast, which can be antagonized by co-expression of the anti-apoptotic members of the Bcl-2 family, including Bcl-2 and Bcl-x_L (Jurgensmeier et al., 1997; Poliakova et al., 2002; Sato et al., 1994) and such studies helped to identify the domains of Bcl-2 that are relevant for suppression of apoptosis (Hanada et al., 1995). Moreover, Xu and colleagues identified BI-1 (Bax inhibitor 1), an intracellular multi-membrane-spanning protein in *S.cerevisiae*, which is conserved in mammals, plants, and fungi, as Bax antagonist (Xu and Reed, 1998), indicating that yeast harbours an intrinsic response machinery to Bcl-2-like proteins. This is further supported by the finding that yeast cells expressing Bax show typical morphological changes that characterize apoptosis (Ligr et al., 1998), and that Bax induces the release of cytochrome *c* from mitochondria (Manon et al., 1997), a hallmark of Bax action in mammalian cells. In contrast, Kissova et al. suggested that yeast cells expressing Bax show characteristics of autophagy rather than apoptosis (Kissova et al., 2006). However, the expression of human cellular prion protein (PrP) can prevent Bax-mediated apoptosis, both in human and in yeast cells (Bounhar et al., 2006; Li and Harris, 2005) by inhibiting the first step of Bax activation, namely a conformational change of Bax (Bounhar et al., 2001; Roucou et al., 2005; Roucou et al., 2003), similarly to other known Bax inhibitors including Bcl-2. Whether yeast prion protein has a similar protective function remains to be investigated.

In addition, heterologous expression of anti-apoptotic members of the Bcl-2 family in yeast confers a cytoprotective effect in the absence of Bax (Trancikova et al., 2004) and causes increased long-term survival (Longo et al., 1997) and enhanced resistance to

H_2O_2 (Chen et al., 2003). Together these findings suggest that members of the Bcl-2-familiy can interfere with a highly conserved cell death program in yeast and higher eukaryotes.

1.2.2 Regulators of Yeast apoptosis

1.2.2.1 Reactive oxygen species and anti-oxidants regulate apoptosis

During aerobic growth and in response to environmental stresses such as temperature or diauxic shift, lack of nutrients, and UV damage, cells produce ROS. Because these molecules are toxic to the cell, an elaborate system with a variety of enzymes has evolved that is responsible for cleaning the cell of ROS, and strict regulation of this system is essential for normal growth (reviewed in (Temple et al., 2005)). In this context, ROS have also been determined to be the main mediator of apoptosis (Madeo et al., 1999a). Chronologically aged yeast cells die with typical hallmarks of apoptosis, in particular enhanced levels of ROS, whereas a reduction of intracellular ROS has been shown to extend the replicative as well as the chronological lifespan (Piper, 2006). The cytosolic and mitochondrial superoxide dismutases, Sod1p and Sod2p, respectively, are required for reduction of ROS in the cells and for long-term survival of yeast (Longo et al., 1996) and consistently overexpression of either the two proteins increases the lifespan (Fabrizio et al., 2004) underlining the protective role of the antioxidant system for longevity. Similarly, overexpression of the non-essential yeast catalase *CTT1*, which reduces intracellular H_2O_2 levels, has been shown to protect cells against apoptosis induction by acetic acid (Guaragnella et al., 2008).

An important factor with antioxidant and therefore anti-apoptotic activity is the tripeptide glutathione (Drakulic et al., 2005; Madeo et al., 1999a). The depletion of glutathione in yeast cells leads to massive DNA fragmentation and enhanced sensitivity towards H_2O_2 (Madeo et al., 1999a). In accordance with this observation, levels of cytoplasmic *O*-acetylhomoserine sulfhydrolase, a protein central for glutathione synthesis that is encoded by *MET17*, are enhanced in the $cdc48^{S565G}$ mutant (Braun et al., 2006), indicating that intracellular antioxidant levels are important for regulation of ROS and that low levels of ROS prevent the induction of programmed cell death.

Recently, a direct relation between actin dynamics ROS production and apoptosis has been demonstrated (reviewed in (Gourlay and Ayscough, 2005)). Actin-stabilizing drugs or mutations have been shown to lead to an increase of ROS and decreased cell viability, whereas destabilization of the actin cytoskeleton by deletion of *SCP1* encoding for an actin-bundling protein causes a decrease in ROS and an increase in lifespan (Gourlay et al., 2004). These observations indicate the importance of maintaining the dynamic stage of the actin cytoskeleton for the regulation of ROS levels and the prevention of programmed cell death.

The response to environmental stresses leads to an increased amount of ROS in yeast cells. Several proteins have been shown to protect against programmed cell death by regulating the stress response. *SVF1* is a gene that was identified in a yeast genetic screen in search for factors that function in a survival pathway analogous to that of human Bcl-x_L (Vander Heiden et al., 2002). Little evident similarity of *SVF1* to known mammalian genes is observed, but, however, it can partly be replaced by human anti-apoptotic Bcl-x_L (Brace et al., 2005; Vander Heiden et al., 2002). Moreover, Svf1p facilitates diauxic shift from glycolytic to oxidative metabolism in yeast, which leads to enhanced levels of several antioxidant enzymes, including Sod1p, Sod2p and glutathione synthase (Maris et al., 2001). Svf1p also protects cells against oxidative stress caused by lower growth temperature, which in turn leads to the induction of antioxidant genes such as *SOD1*, *GSH1* and *CTT1* in the cell (Brace et al., 2005; Zhang et al., 2003), whereas *svf1* deletion mutants are more sensitive towards oxidative stress caused by low growth temperature, exposure to chemical inducers or ROS precursors (Brace et al., 2005). Together these observations indicate that Svf1p's anti-apoptotic function is most critical during rapid changes in environmental conditions when protection against oxidative stress becomes necessary.

In conclusion, these results emphasize the importance of an elaborate protective system against ROS consisting of a variety of antioxidant enzymes such as superoxide dismutases, catalases and enzymes for glutathione synthesis, that are responsible for the precise regulation of intracellular ROS levels to prevent cells from undergoing apoptosis. Impairment of any of these anti-apoptotic components as well as alterations in stress

response and metabolic pathways lead to an increase of intracellular ROS and subsequent cell death.

1.2.2.2 Metacaspase Yca1p

Advanced pattern based sequence homology search led to the identification of metacaspase *YCA1* in *S. cerevisiae* . Yca1p has a central role in yeast apoptosis: under oxygen stress and during ageing, disruption of *YCA1* decreases cell death and the formation of an apoptotic phenotype (Madeo et al., 2002). Moreover, several publications have shown the dependency of apoptosis inducing processes on Yca1p. For example Mazzoni et al. showed that increased mRNA stability upon mutations in *lsm4*, a protein involved in mRNA decapping, led to apoptosis (Mazzoni et al., 2003). The same group showed that apoptosis induced by stabilized mRNA depends on Yca1p (Mazzoni et al., 2005). Deletion of *YCA1* in an *lsm4*-mutated background prevented mitochondrial fragmentation and rapid cell death during chronological ageing. In addition ROS accumulation and DNA breakage is diminished and resistance towards H_2O_2 and acetic acid is increased (Mazzoni et al., 2005). Another interesting cellular process connected to *YCA1* dependent apoptosis was described by Bettiga et al. (Bettiga et al., 2004). Loss of *UBP10*, which encodes a deubiquitinating enzyme that cleaves ubiquitin from histone H2B, led to a subpopulation of cells exhibiting typical apoptotic markers. This was suppressed upon *YCA1* deletion, whereas its overexpression strongly increased apoptosis in an *ubp10* background (Bettiga et al., 2004).

1.2.2.3 Bir1p

S. cerevisisae BIR1 is a gene encoding a ~108 kDa protein and based on sequence homology it appears to be the only member of the inhibitor-of-apoptosis protein family in this organism (Uren et al., 1998). Bir1p bears two type-II BIR domains at its N-terminus, while lacking a RING domain (Figs. 1 and 3). Bir1p localizes to the nucleus of cells due to a putative nuclear localization signal (NLS) and its C-terminal ~80 amino acids are sufficient for association with the anaphase spindle (Uren et al., 1999; Widlund et al., 2006). Until recently, the role of Bir1p in cell division rather than in apoptosis was

examined intensively (Bouck and Bloom, 2005; Cheeseman et al., 2002; Gillis et al., 2005; Li et al., 2000; Sandall et al., 2006; Silke and Vaux, 2001; Thomas and Kaplan, 2007; Uren et al., 1999; Widlund et al., 2006; Yoon and Carbon, 1999). By two-hybrid studies it was shown that Bir1p interacts with components of the kinetochores, i.e. Ndc10p, which is a subunit of the inner kinetochore subcomplex CBF3 (Yoon and Carbon, 1999). Bir1p is essential for spore formation and germination, but not for vegetative growth and deletion of the *BIR1* gene leads to a chromosome mis-segregation phenotype as shown by a colony color-sectoring assay (Yoon and Carbon, 1999). Moreover, a tandem affinity purification (TAP) approach identified an in vivo interaction of Bir1p with the homologues of human Aurora B kinase and inner centromere protein (INCENP), Ipl1p and Sli15p, respectively (Cheeseman et al., 2002), pinpointing to Bir1p's roles in chromosome bi-orientation, spindle stabilization, and cytokinesis (Bouck and Bloom, 2005; Gillis et al., 2005; Sandall et al., 2006; Thomas and Kaplan, 2007).

In addition to the well-known role of Bir1p as a chromosomal passenger protein, it recently became evident that Bir1p is involved in apoptosis regulation in *S.cerevisiae* as well (Walter et al., 2006). Bir1p is cleaved by the pro-apoptotic serine protease Nma111p, when over-expressed from an episomal plasmid, and both proteins are directly interacting in vitro (Walter et al., 2006). The molecular mechanism by which Bir1p exhibits its anti-apoptotic function, however, has remained elusive, since Bir1p does not bind the yeast caspase Yca1p (Walter et al., 2006). Nevertheless, cells lacking *BIR1* show typical hallmarks of apoptosis, such as chromatin condensation and fragmentation, DNA single strand breaks and accumulation of ROS, whereas over-expression of Bir1p protects cells against apoptosis induced by H_2O_2 treatment or during chronological ageing. Simultaneous over-expression of Nma111p reverses the protective effect of increased Bir1p levels, underlining the interaction between the two proteins in vivo (Walter et al., 2006).

Interestingly, Bir1p has recently been shown to be SUMOylated (Montpetit et al., 2006; Wohlschlegel et al., 2004; Zhou et al., 2004), which in turn is dependent on its localization to the anaphase spindle and SUMO modification of Ndc10p (Montpetit et al., 2006). Furthermore, SUMO modification of Bir1p is lost in a *bir1* variant lacking the BIR repeats and upon spindle checkpoint activation by nocodazole, implying a role of

Bir1p SUMOylation in apoptosis and/or spindle checkpoint regulation, respectively. Moreover, Bir1p levels have been shown to fluctuate during the cell cycle and Bir1p gets phosphorylated in a cell cycle-dependent manner (Widlund et al., 2006). Both, regulation of Bir1p levels and its diverse posttranslational modifications might play a role in coordinating the different functions of the protein including its anti-apoptotic activity.

1.2.2.4 Nma111p

Another key player of yeast apoptosis is the HtrA2/Omi-like protein Nma111p (nuclear mediator of apoptosis). Under cellular stress conditions (e.g. elevated temperature or H_2O_2 treatment) the serine protease aggregates in the nucleus and yeast cells lacking *NMA111* survive better under temperature stress conditions and show no apoptotic markers after treatment with H_2O_2 (Fahrenkrog et al., 2004). Unlike its human homologue, which is located in mitochondria, Nma111p has been found only in the nucleus and it proapoptotic activity depends on its nuclear localization and on its serine protease activity (Belanger et al., 2009; Fahrenkrog et al., 2004). Its human homologue HtrA2/Omi antagonizes XIAP, an X-linked IAP in human cells, which in turn inhibits downstream caspases. Similarly, Bir1p is antagonized by Nma111p. Bir1p is cleaved by Nma111p, when over-expressed from an episomal plasmid, and both proteins are directly interacting in vitro (Walter et al., 2006).

1.2.2.5 Cytochrome *c*

Release of cytochrome *c*, another hallmark of human apoptosis, also occurs in *S. cerevisiae*. During acetic acid induced apoptosis release of cytochrome *c* from the mitochondria was observed (Ludovico et al., 2002). As in mammals, this release serves as an essential apoptotic signal, as respiratory deficient strains and cytochrome *c* deleted strains show diminished apoptosis upon acetic acid treatment (Ludovico et al., 2002).

1.2.2.6 Aif1p

The existence and apoptotic function of an AIF homologue in yeast is another proof for the conservation of elements of the apoptotic machinery from yeast to man (reviewed in

(Modjtahedi et al., 2006)). The mode of action of *S. cerevisiae* Aif1p closely resembles that of mammalian AIF. Upon apoptosis induction by H_2O_2 or acetate, as well as in chronologically aged cultures, Aif1p translocates from the mitochondria to the nucleus (Wissing et al., 2004). Consistently, in an *AIF1* knockout strain H_2O_2 and acetate induced apoptosis is abolished and age-induced apoptosis is delayed (Wissing et al., 2004). Interestingly the apoptotic function of Aif1p seems to be in part Yca1p-dependent, as cell survival during overexpression of Aif1p together with mild H_2O_2 stress was elevated from 10 to 70% when *YCA1* was deleted (Wissing et al., 2004).

1.2.2.7 EndoG/Nuc1p

EndoG is another apoptotic mammalian mitochondrial protein for which a yeast orthologue has been identified. EndoG is a mitochondrial nuclease first identified in rat (Li et al., 2001) and *C. elegans* (Parrish et al., 2001). During apoptosis it is released from mitochondria and transferred to the nucleus where it causes DNA fragmentation (Li et al., 2001). Buttener et al extended these results to yeast by demonstrating that the yeast EndoG (Nuc1p) can efficiently trigger apoptotic cell death when excluded from mitochondria (Buttner et al., 2007). Nuc1p induces apoptosis in yeast independently of metacaspase Yca1p or of apoptosis inducing factor Aifp. Instead, the permeability transition pore, karyopherin Kap123p, and histone H2B interact with Nuc1p and are required for cell death upon Nuc1p overexpression (Buttner et al., 2007), suggesting a pathway in which mitochondrial pore opening, nuclear import, and chromatin association are successively involved in EndoG-mediated death.

1.2.2.8 Ste20p

Phosphorylation of histone H2B at serine 14 (H2BS14ph), catalyzed by the Mst1 kinase, has been linked to chromatin compaction during mammalian apoptosis (Cheung et al., 2003). Ahn and colleagues extended these results to yeast by demonstrating that Ste20 kinase, a yeast orthologue of Mst1, directly phosphorylates H2B at serine 10 (H2BS10ph) in a hydrogen peroxide-induced cell death pathway (Ahn et al., 2005). Unlike Mst1, Ste20 translocates into the nucleus in a caspase-independent fashion to mediate

phosphorylation of H2B (Ahn et al., 2005). Ahn et al. recently described an undirectional crosstalk relationship between two residues of the histone H2B tail, namely lysine 11 (K11) acetylation and serine (S10) phosphorylation (Ahn et al., 2006). They propose that, after addition of H_2O_2 histone deacetylase, Hos3p catalyses deacetylation of H2BK11, which mediates phosphorylation of H2BS10 catalysed by Ste20 kinase.

1.2.2.9 Fis1p

Fragmentation of mitochondria is an early event of apoptosis in nematode and mammalian cells (Desagher and Martinou, 2000; Frank et al., 2001; Jagasia et al., 2005; Mancini et al., 1997). Accordingly, Fannjiang and colleagues recently showed a link between the mitochondrial fission machinery and apoptosis in yeast (Fannjiang et al., 2004). Fis1p is a highly conserved protein, which plays a role in fission of mitochondria in yeast and mammals, i.e. it correctly distributes proteins required for fission within the outer mitochondrial membrane, namely Dnm1p and Mdv1p (Bleazard et al., 1999; Mozdy et al., 2000; Okamoto and Shaw, 2005; Otsuga et al., 1998). However, Fis1p has also been shown to inhibit apoptosis in *S. cerevisiae* as its deletion drastically enhances cell death in H_2O_2 treated cells in an Yca1p-dependent manner (Fannjiang et al., 2004). The mechanism by which Fis1p protects against apoptosis in *S. cerevisiae* remains unclear. However, Fis1p, like the mammalian Bcl-2 and Bcl-x_L proteins (Gonzalez-Garcia et al., 1994; Kaufmann et al., 2003; Lithgow et al., 1994; Mozdy et al., 2000; Nguyen et al., 1993), is anchored to the cytosolic side of the outer mitochondrial membrane and shares some biophysical properties with these anti-apoptotic proteins (Fannjiang et al., 2004). Moreover, the anti-apoptotic function of Fis1p can be functionally replaced by either Bcl-2 or Bcl-xL, implying that Fis1p acts in a Bcl-2-like manner (Fannjiang et al., 2004).

1.2.2.10 Porin1

In the mitochondrial apoptotic pathway of mammals and yeast, outer mitochondrial membrane permeabilization (MOMP) and the release of pro-apoptotic proteins such as cytochrome *c* from the inter-membrane space are crucial for programmed cell death. In

mammals, opening of a mitochondrial pore called permeability transition pore (PTP) has been considered one of the key mechanisms underlying MOMP (Kinnally and Antonsson, 2007). Yet, the nature of the pore that releases these proteins is still unknown and the identity of the proteins involved in its formation is controversial (Kinnally and Antonsson, 2007; Lawen, 2007; Ly et al., 2003; Zoratti et al., 2005). However, yeast possesses homologues of putative core PTP proteins like the yeast VDACs 1 and 2 (*POR1* and *2*), the yeast mitochondrial cyclophilin (*CPR3*) and three ADP/ATP carrier proteins (*AAC1*, *AAC2* and *AAC3*) that are believed to function in a similar manner, forming a yeast PTP (Manon et al., 1998). Pereira and colleagues analyzed the role of these proteins in apoptosis and suggested that Por1p protects against apoptosis (Pereira et al., 2007), since a *por1* deletion strain shows enhanced apoptosis when treated with various death stimuli including acetic acid, H_2O_2, or diamide, another pro-oxidant compound. In contrast, deletion of *CPR3* has no effect on cell death induced by any of these stimuli. However, Liang and colleagues showed recently that deletion of *CPR3* confers resistance to copper-induced apoptosis (Liang and Zhou, 2007). Furthermore the loss of all three ADP/ATP carrier proteins leads to enhanced death induced by H_2O_2, but confers protection against acetic acid (Pereira et al., 2007). Therefore Cpr3p as well as AAC proteins play different roles during cell death depending on the death-triggering cellular context. Whether or not the effects observed with the yeast strains lacking Por1p, *Cpr3p* and the AAC proteins are due to their association with the yeast PTP remains to be clarified. As the mammalian homologue of Por1p, VDAC, is also localized to the plasma membrane, where it can regulate apoptosis, Por1p might have functions in addition to those in the mitochondrial outer membrane.

1.2.2.11 Ef2p

An important level on which regulation of apoptosis inhibition can occur is by controlling translation, which became evident from studies in fission yeast. *S. pombe* was used as a model system to study apoptosis induced by HIV-1 viral protein R (Vpr) (Zelivianski et al., 2006). Vpr induces apoptosis in mammalian cells and is believed to contribute to CD4+ lymphocyte depletion, a hallmark of acquired immunodeficiency syndrome (AIDS) (Poon et al., 1997; Somasundaran et al., 2002). Heterologous expression of Vpr

in fission yeast leads to rapid cell death accompanied by some characteristics of apoptotic cells (Zhao et al., 1996; Zhao et al., 1998). In a genome-wide search for multicopy suppressors of Vpr-induced apoptosis in *S.pombe*, *EF2* (elongation factor 2) was identified as anti-apoptotic Vpr suppressor (Zelivianski et al., 2006). Overproduction of *EF2* in fission yeast as well as in human cells abolishes Vpr-induced apoptosis (Zelivianski et al., 2006). The anti-apoptotic property of *EF2* in human cells is demonstrated by its ability to suppress caspase 9 and caspase 3-mediated apoptosis induced by Vpr (Zelivianski et al., 2006). Additionally, it reduces cytochrome c release induced by Vpr, staurosporine and TNFα (Zelivianski et al., 2006). Taken together these data suggest that EF2 acts as a highly conserved anti-apoptotic protein by a yet unknown molecular mechanism. However, *EF2* is an evolutionarily conserved monomeric GTPase involved in protein synthesis and translation elongation and its activity is regulated by several post-translational modifications including phosphorylation and ribosylation (Rhoads, 1999), suggesting that *EF2* may confer its anti-apoptotic effect through its regulatory role in protein synthesis.

1.2.3 Caspase-dependent and -independent yeast apoptosis

Saccharomyces cerevisiae can undergo cell death accompanied by diagnostic features of apoptosis, such as phosphatidylserine externalization, DNA fragmentation, chromatin condensation, cytochrome *c* release from mitochondria, and dissipation of the mitochondrial transmembrane potential. Both caspase-dependent and caspase-independent cell death executors participate in yeast apoptosis (Figure 1.6).

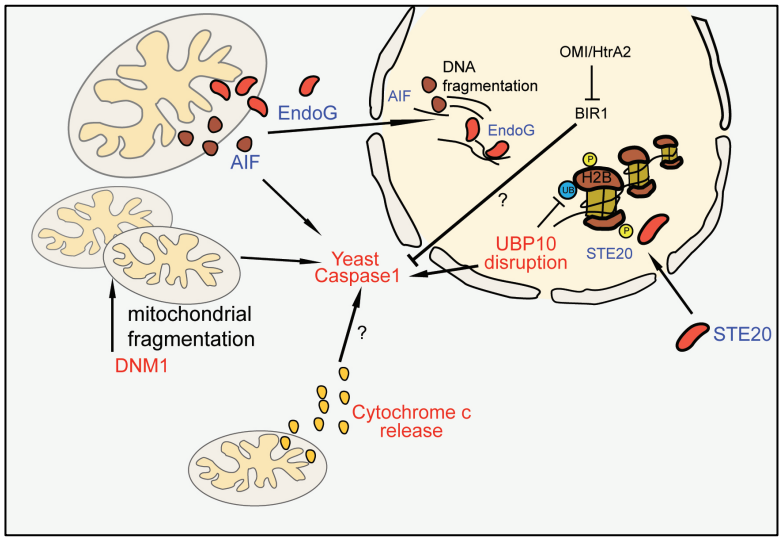

Figure 1.6: Caspase-dependent and caspase-independent cell death.
Exogenous and endogenous induction of yeast apoptosis leads to the activation of the basic molecular machinery of cell death. Both caspase-dependent and caspase-independent cell death scenarios exist. Mitochondrial fragmentation, disruption on the ubiquitin specific protease *UBP10* and cytochrome c release from the mitochondria are implicated in caspase dependent apoptosis, whereas the endonucleases EndoG/Nuc1p and Aifp as well as Ste20p mediated H2B phosphorylation can mediate caspase independent apoptosis. Adapted from (Madeo et al., 2009).

1.2.3.1 Caspase-dependent yeast apoptosis

Deletion of the yeast metacaspase *YCA1* can protect yeast cells against multiple distinct forms of lethal insult. For instance, yeast cells exposed to salt (NaCl) (Wadskog et al., 2004) or low doses of valproic acid, a short chained fatty acid with anti-tumor activity, undergo *YCA1*-dependent apoptosis (Mitsui et al., 2005).

Exposure to toxins produced by virus-carrying killer yeast strains also leads to apoptosis in yeast (Reiter et al., 2005) and deletion of *YCA1* in the attacked strain leads to reduced toxin sensitivity (Reiter et al., 2005). Similarly, heterologous expression of expanded polyglutamine domains, which cause protein aggregation and neurodegeneration in human Huntington's disease, leads to apoptosis in yeast (Sokolov et al.), and this is again inhibited by *YCA1* deletion (Bocharova et al., 2008).

Recently, the involvement of *ISC1*, the gene encoding the inositol-phosphosphingolipid phospholipase C, in apoptosis has been reported (Almeida et al., 2008). Isc1p translocates to mitochondria in the post-diauxic phase and plays a role in the regulation of cellular redox homeostasis through modulation of iron levels. However,

deletion of *ISC1* has been shown to shorten chronological lifespan and to enhance H_2O_2 sensitivity, which is *YCA1*-dependent and can be suppressed by iron chelation (Almeida et al., 2008).

Moreover, yeast death triggered by defects in ubiquitination, reduced mRNA stability, mitochondrial fragmentation or ageing can occur at least partly in a caspase-dependent fashion (see chapter 1.2.2.2 and 1.2.2.9)(Bettiga et al., 2004; Fannjiang et al., 2004; Herker et al., 2004; Mazzoni et al., 2005).

1.2.3.2 Caspase-independent yeast apoptosis

Yca1p-independent apoptosis in yeast occurs during long-term development of yeast multicellular colonies (Vachova and Palkova, 2005) or by defective N-glycosylation in cells lacking Ost2p, the yeast homolog of the mammalian defender of apoptosis-1 (*DAD1*) protein (Hauptmann et al., 2006). Moreover, upon defective N-glycosylation in the temperature-sensitive *wbp1-1* mutant or after treatment with tunicamycin, yeast apoptosis depends on the protease activity of *KEX1* (but not *YCA1*) (Hauptmann et al., 2006). This recently identified apoptotic protease also plays a role in cell death induced by acetic acid or chronological ageing (Hauptmann and Lehle, 2008). Finally, yeast apoptosis triggered by Nuc1p, Aifp and Ste1p-mediated phosphorylation of histone H2B occur at least partly in a caspase-independent fashion (see chapter 1.2.2.6, 1.2.2.7 and 1.2.2.8) (Ahn et al., 2005; Buttner et al., 2007; Wissing et al., 2004).

1.2.4 Physiological role of yeast apoptosis

1.2.4.1 Ageing yeast

Ageing is considered as a physiological trigger of apoptosis in yeast (Fabrizio et al., 2004; Herker et al., 2004; Laun et al., 2001). Two forms of ageing exist in yeast: the replicative life span (RLS) is defined as the number of daughter cells produced by a mother cell before senescence. The chronological life span (CLS) in turn is defined as the time a yeast cell can survive in a nondividing state (Fabrizio and Longo, 2003). Apoptotic cell death is present in both ageing processes (reviewed in (Rockenfeller and Madeo, 2008)).

1.2.4.1.1 Replicative ageing yeast

Accumulation of ROS, which is causally linked to yeast apoptosis, is observed in replicative old cells when dying (Laun et al., 2001). Consistently, additional phenotypes of apoptotic death such as PS exposure to the outer membrane leaflet, nuclear DNA fragmentation, and chromatin condensation occur in replicative old yeast mother cells (Laun et al., 2001), indicating that replicative old yeast cells die in an apoptotic fashion.

The formation and accumulation of extrachromosomal rDNA circles (ERCs) is a major cause of yeast replicative ageing (Sinclair and Guarente, 1997). The yeast rDNA is a tandem array of several dozen copies of a 9.1 kb repeat, and ERCs can be formed by homologous recombination between adjacent rDNA repeats (Sinclair and Guarente, 1997). At each cell division, ERCs replicate and segregate asymmetrically to the mother cell during mitosis (Murray and Szostak, 1983). Why their accumulation contributes to the ageing of the mother is unclear. However, mutations that accelerate the rate of ERC formation shorten replicative lifespan (Falcon and Aris, 2003), whereas those that reduce ERC accumulation enhance longevity (Defossez et al., 1999).

The major genetic determinant of replicative life span in yeast is *SIR2*; a loss-of-function mutation in *SIR2* shortens life span while increased gene dosage extends it (Kaeberlein et al., 1999). Sir2p is a histone deacetylase that is required for silencing gene transcription at selected loci, i.e. the silent mating type cassettes *HMR* and *HML (HM)*, telomeres, and the ribosomal DNA (rDNA) (reviewed in (Moazed, 2001a)). To date, the role that Sir2 plays in modulating replicative longevity in yeast has been assumed to be by affecting the rate of rDNA recombination and thus ERC formation (Guarente, 2000). Deletion of *SIR2* increases rDNA recombination by 5–10-fold (Gottlieb and Esposito, 1989), increases ERC levels (Kaeberlein et al., 1999), and reduces life span by about 50% (Kennedy et al., 1995).

At the time Sir2p was first implicated in yeast longevity, little was known about the mechanism by which Sir2p protein was able to promote transcriptional silencing (Kennedy et al., 1995). Later, two groups reported that Sir2p catalyzes an NAD-dependent histone deacetylation reaction (Imai et al., 2000; Landry et al., 2000). The NAD-dependent nature of Sir2p catalysis suggested a potential link between Sir2p

activity and the metabolic state of the cell (Guarente, 2000). Lin and colleagues found that reducing the glucose concentration of the media from 2 to 0.5% increased replicative life span by 20–30% (Lin et al., 2000). The magnitude of life span extension from this calorie restriction protocol is comparable to that observed upon overexpression of Sir2p. Furthermore, in cells lacking Sir2p life span is shortened by approximately 50%, and reducing the glucose concentration fails to increase life span in this short-lived mutant (Lin et al., 2000). This latter finding suggests that life span extension by calorie restriction is mediated through activation of Sir2p (Lin et al., 2000). As replicative old cells die in an apoptotic fashion, these data suggest a potential role for Sir2p in antagonizing yeast apoptosis by preventing ERCs accumulation and imply that ERCs accumulation induces apoptosis in yeast.

1.2.4.1.2 Chronological ageing

Chronological ageing is defined by survival rates during long-term cultivation in a non-dividing, quiescent-like state of yeast cells (Fabrizio and Longo, 2003). Wild type yeast ageing chronologically show features of apoptotic death, such as DNA condensation/fragmentation, phosphatidylserine exposure, and caspase activation (Herker et al., 2004). Reactive oxygen species formation is enhanced in agreement with a central role for ROS in the activation of yeast apoptosis during ageing. ROS accumulation is decreased in a population that overexpresses *YAP1* (Herker et al., 2004), a functional homologue to the human apoptosis regulator AP-1 (Moye-Rowley et al., 1989). As a consequence, survival in chronologically aged cultures is increased, suggesting that cell death depends on ROS accumulation. A few genetic interventions with key yeast apoptotic regulators have been described that delayed chronological ageing and the appearance of the apoptotic features associated to it. Among these are the disruption of the yeast caspase *YCA1* gene, the Omi homologue (Nma111p), the AIF homologue (*AIF1*), and *NDE1* (coding for the yeast homologue of the AIF-homologous mitochondrion associated inducer of death, AMID) and overexpression of the inhibitor-of-apoptosis protein *BIR1 (see chapters 1.2.2.2, 1.2.2.6, 1.2.2.4 and 1.2.2.3)* (Belanger et al., 2009; Herker et al., 2004; Madeo et al., 2002; Walter et al., 2006; Wissing et al., 2004). However, chronological ageing in yeast is largely regulated by nutrients such as

glucose (Kaeberlein et al., 2007). Calorie restriction or mutation in RAS2, CYR1/PKA, TOR or SCH9, which are all encoding downstream effectors of glucose signalling, extend the yeast chronological life span up to 300% (Fabrizio et al., 2004; Fabrizio and Longo, 2003; Powers et al., 2006). This life span extension is mediated through transcription factors involved in stress resistance (Msn2, Msn4), heat shock proteins or scavenger enzymes for oxidative stress such as, mitochondrial superoxide dismutase (SOD) and catalases (Fabrizio and Longo, 2003; Fabrizio et al., 2001). Msn2 and Msn4 represent transcription factors stimulating the expression of stress resistance proteins (Gorner et al., 2002). Up-regulation of these transcription factors therefore leads to increasing SOD and catalase levels thereby minimizing oxidative stress and cellular damage (Gorner et al., 1998). A recent study by Wei et al. showed that the chronological life span extension in yeast caused by deficiencies in either the nutrient-responsive proteins Ras2p, Tor1p and Sch9p, or by calorie restriction is dependant on the serine/threonine kinase Rim15 (Wei et al., 2008). Furthermore, the deletion of Msn2/4 and Gis1, which are positively regulated by Rim15, cause a major reversion of the life span extending effect of calorie restriction (Wei et al., 2008).

The observation that apoptotic cell death is present in both replicative and chronologically aged cells may be an indication that the ultimate cause of ageing is similar in both dividing and nondividing yeast cells. This would be consistent with the finding that chronologically aged cells have a reduced RLS (Ashrafi et al., 1999) and that some interventions (e.g., DR or reduced target-of-rapamycin (TOR) signaling) increase both RLS and CLS (Kaeberlein et al., 2005; Powers et al., 2006; Reverter-Branchat et al., 2004).

1.2.4.2 Cell death in yeast colonies

During yeast colony development, regulated cell death is essential for the long-term survival of the colony population. Ammonia release serves as the signal for differentiation within the colony, adapting the population to the environment and reprogramming cell metabolism (Vachova et al., 2004). After the ammonia signal, cells displaying a complete apoptotic phenotype (ROS, chromatin condensation, TUNEL staining and PS exposition) are mainly located in the inner area of giant colonies

(Vachova et al., 2004). A *sok2* deletion strain, unable to produce the ammonia signal, shows apoptotic markers throughout the whole colony during prolonged colony growth. Removal of the inner part of giant colonies after the ammonia signal led to decreased colony growth in the outer regions, arguing for an active role of PCD in the colony core to provide nutrients for outer colony regions (Vachova et al., 2004). Interestingly, PCD in giant colonies seems to be independent of both *YCA1* and *AIF1* as respective deletion strains behave similar to a wild type strain (Vachova and Palkova, 2005).

1.3 Histones and post-translational modifications of histones

In eukaryotes, genomic DNA is packaged into chromatin, a nucleoprotein complex whose basic repeating unit is the nucleosome (Kornberg, 1977). The nucleosome is made up of 146 bp of DNA wrapped around a histone octamer consisting of two copies each of H2A, H2B, H3, and H4 (McGhee and Felsenfeld, 1980). Histones are subject to multiple covalent posttranslational modifications (Figure 1.7), some of which alter intrinsic chromatin properties, others present or hinder binding modules for non-histone, chromatin-modifying complexes (Berger, 2002; Fischle et al., 2003a; Shilatifard, 2006). The addition/removal of chemical moieties is a dynamic process that can influence cellular processes including transcription, cell division, differentiation, DNA repair and apoptosis (Cheung et al., 2000). These well-conserved modifications, including acetylation, methylation, ubiquitination, and many others, generally occur on the N-terminal and C-terminal tails of histones that extend out from the nucleosome (Figure 1.7) (Kouzarides, 2007). The presence or absence of many of these modifications was first associated with either transcriptionally active or inactive regions of chromatin, leading to speculation that these chemical alterations can alter transcriptional activity (Allfrey and Mirsky, 1964). However, only in the late 1980's genetic evidence for the importance of histones in transcription was first elucidated (Clark-Adams et al., 1988; Han and Grunstein, 1988). The identification of enzymes responsible for histone acetylation and methylation and the requirement of these modifications for transcriptional activity (Sterner and Berger, 2000; Zhang and Reinberg, 2001) culminated in the establishment of the histone code hypothesis as the dominant paradigm in the chromatin field (Strahl and Allis, 2000). The authors of this hypothesis postulated that histone modifications served as specific marks that can be recognized and bound by effector proteins to promote downstream outputs such as activation or repression of gene expression, cell division, differentiation, DNA repair and apoptosis. Subsequent studies have identified protein domains on effector proteins, such as bromodomains and chromodomains, which bind to acetylated and methylated histone tails, respectively, allowing for the "reading" of the histone code (Kouzarides, 2007). As with any code, the inputs (i.e. the histone modifications) can be combined in a variety of configurations to generate novel outputs. Some combinations elicit species-specific outputs but most are

conserved from yeast to humans, allowing lessons learned from yeast to be applied across evolution.

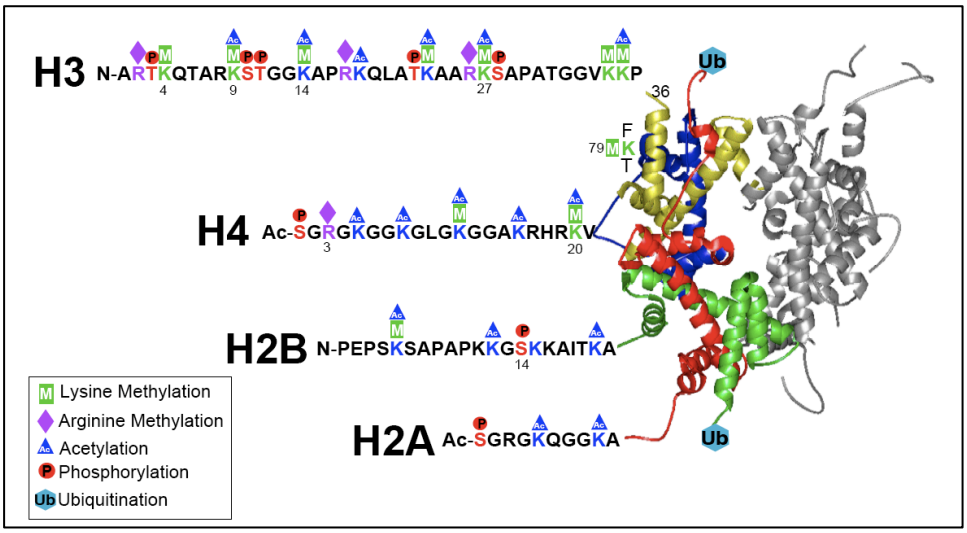

Figure 1.7: Posttranslational modifications on histones.
Specific amino acid sites of posttranslational modifications (acetylation, phosphorylation, ubiquitination and methylation) that are known to occur on histones are indicated by colored symbols. Half of the structure of the nucleosome core particle H3 (yellow), H4 (blue), H2A (red) and H2B (green) are shown in color. The other half is represented in grey. Adapted from: http://www.ag.purdue.edu/biochem/Pages/sdbriggs.aspx

1.3.1 Histone ubiquitination

In addition to small methyl and acetyl groups, histones can also be modified by much larger molecules, such as the 76 amino acid ubiquitin protein. The mechanism of ubiquitination starts with the activation of ubiquitin by the E1 ubiquitin-activating enzyme (Deshaies and Joazeiro, 2009). This activated ubiquitin is subsequently passed along to the E2 ubiquitin conjugating enzyme that ultimately catalyzes the final transfer to the target substrate with the assistance of a specific E3 ubiquitin ligase. Subsequent work has established ubiquitination as a crucial protein modification usually associated with the destruction of proteins when multiple ubiquitin moieties are added in a chain, allowing for recognition and degradation of the modified substrate by the proteasome (Glickman and Ciechanover, 2002). However, the addition of a single ubiquitin, termed monoubiquitination, can be used for signaling and the modulation of protein activity (Sun

and Chen, 2004). Two core histones can be modified by monoubiquitination at their C-termini, i.e. histone H2A and H2B. The existence of ubiquitin on these histones has been known for a long time; in fact, histones were the first proteins that were identified to be ubiquitinated over thirty years ago (Ballal et al., 1975; Goldknopf and Busch, 1975). However, the machinery that catalyzes this reaction has only been identified within the last decade. Monoubiquitinated histone H2B was identified along with its cognate E2 enzyme, Rad6p (Robzyk et al., 2000). The E3 ubiquitin ligase for this modification, however, remained elusive until 2003 when Bre1p was identified as the conserved E3 ubiquitin ligase for H2B ubiquitination (Hwang et al., 2003; Wood et al., 2003a).

The mechanism as to how Rad6p and Bre1p promote ubiquitination of histone H2B is highly regulated. Additional factors besides Bre1p and Rad6p are required to achieve ubiquitination of histone H2B in vivo, including the transcription elongation/processing factor PAF and the Bur1/Bur2 cyclin-dependent protein kinase complex (BUR complex) (Kao et al., 2004; Wood et al., 2003b; Xiao et al., 2005). As these factors are crucial for transcription initiation and elongation, it is assumed that ubiquitin is conjugated to histone H2B through a co-transcriptional mechanism that involves the association of Rad6p–Bre1p with components of the transcription initiation and elongation machinery (Wood et al., 2003b; Xiao et al., 2005).

The identification of Bre1p as the E3 ubiquitin ligase for ubiquitination of histone H2B in yeast has spurred many groups to identify and characterize Bre1p homologs in higher eukaryotes. Mutant versions of the *Arabidopsis thaliana* homologue of Bre1p display defects in progression through the cell cycle and the developmental transition into a flowering state (Fleury et al., 2007; Gu et al., 2009). Developmental defects were also observed for mutants of the *Drosophila melanogaster* homolog of Bre1p, similar to those observed in Notch signaling mutants (Bray et al., 2005), indicating that Bre1p regulates Notch signaling. Unlike in plant and *Drosophila*, *C. elegans* Bre1p is involved in apoptosis regulation. Upon knockdown of *C.elegans* Bre1p germ cell apoptosis is induced in a p53-independent manner (Lettre et al., 2004). Finally, there are two homologues of Bre1p in humans, RNF20 and 40, which have been identified to exist in a complex, although only RNF20 appears to be necessary for H2B ubiquitination in vivo (Kim et al., 2005; Zhu et al., 2005). RNF20 can interact with the tumor suppressor p53

and can be recruited to genes in a p53-dependent manner (Kim et al., 2005). RNF20 can also affect the expression of HOX genes (Zhu et al., 2005), suggesting that similar to the Bre1p homologs in *Drosophila* and *Arabidopsis*, RNF20 is also important for development in humans.

The ubiquitin moiety on histone H2B can be removed by the action of deubiquitinating enzymes, of which there are two in yeast, Ubp8p and Ubp10p (Emre et al., 2005; Gardner et al., 2005; Henry et al., 2003). Ubp8p is a novel member of the SAGA transcriptional coactivator complex that is recruited to active genes (Henry et al., 2003). Mutants deleted for Ubp8p, or other members of the SAGA complex responsible for Ubp8p association with the complex, display an increase in H2B ubiquitination (Henry et al., 2003). This deubiquitinating activity appears to be required for efficient transcriptional activity, as *ubp8Δ* mutants block the recruitment of Ctk1p, the kinase responsible for the transcriptional elongation promoting phosphorylation of pol II (Wyce et al., 2007). Deletion of the second deubiquitinating enzyme in yeast, Ubp10p, also causes increased H2B ubiquitination levels and when combined with a deletion of Ubp8p shows an additive increase in H2B ubiquitination levels, suggesting that they act on separate populations of H2B ubiquitination. Interestingly, loss of *UBP10* leads to a subpopulation of cells exhibiting typical apoptotic markers. This was suppressed upon metacaspase *YCA1* deletion, whereas its overexpression strongly increased apoptosis in a Δ*ubp10* background, providing a tantalizing link between H2B ubiquitination and apoptosis (Bettiga et al., 2004; Orlandi et al., 2004), which will be discussed in detail in Chapter 2 and 5.

1.3.2 Histone Methylation

The ability of histones to be modified with a methyl group is known since the 1960s (Murray, 1964) and subsequent studies identified many examples of this modification in vivo, mostly on histone H3 (Strahl et al., 1999). Each methylated residue on histones can be modified by as many as three methyl groups (mono-, di-, and tri-methylation). The identification of the first histone methyltransferase, the nuclear receptor co-activator-interacting protein CARM1/PRMT4 (Chen et al., 1999), provided the initial direct link between this well conserved histone mark with the active regulation of transcription.

Since then, subsequent studies in the past decade have identified numerous enzymes that are responsible for the methylation of known residues and have discovered novel sites of methylation on both histone H3 and H4 that function both in promoting and repressing gene expression (Kouzarides, 2007). In particular, the functions of H3 methylation on lysine 4 in the promotion of gene expression have been well dissected by research performed in the budding yeast *S. cerevisiae*. The initial characterization of the functional significance of methylated H3 K4 was performed in *Tetrahymena*, where the modification was associated with transcriptionally active macronuclei but not with inactive micronuclei (Strahl et al., 1999). Identification of the enzyme responsible for this conserved modification occurred a couple years later, when the yeast protein Set1p was demonstrated to be required for H3 K4 methylation *in vivo* (Briggs et al., 2001a; Miller et al., 2001; Roguev et al., 2001). Set1p exists in a complex with seven other proteins (Miller et al., 2001; Roguev et al., 2001), two of which (Swd2p and Spp1p) are only responsible for trimethylation of H3 K4 and are dispensable for mono- or dimethylation of H3 K4 (Schneider et al., 2005). High resolution chromatin immunoprecipitation linked to tiling microarray (ChIPCHIP) experiments examining H3 K4 methylation across genes on *S. cerevisiae* chromosome III display an orderly transition from tri- to di- to monomethylation of H3 K4 from the 5' to the 3' end of genes (Liu et al., 2005), pointing to a functional role of methylated H3 K4 in transcription (Table 1). Many reports have demonstrated an association of H3 K4 methylation, especially trimethylation of H3 K4, with transcriptionally active genes (Bernstein et al., 2002; Santos-Rosa et al., 2002) and Set1p has been shown to bind specifically to pol II that has been phosphorylated on Ser5 of its C-terminal domain (CTD) to initiate transcription (Ng et al., 2003b). Given that *MLL1*, the gene encoding for the human homolog of Set1p, is subject to loss of function rearrangements in more than 70% of infant leukemias, research into the regulation and downstream functions of methylated H3 K4 will hopefully yield new insights into the mechanism of carcinogenesis.

Dot1p, the methyltransferase responsible for H3 K79, was originally identified in a genetic screen looking for genes that, when overexpressed, disrupted telomeric silencing (Singer et al., 1998). Its function as a methyltransferase was established a few years later when Dot1p was shown to be necessary for H3 K79 methylation both *in vivo* and *in vitro*

(Lacoste et al., 2002; Ng et al., 2003a; van Leeuwen et al., 2002). Dot1p is unique amongst the other characterized yeast histone methyltransferases because of its lack of the SET domain, which is characteristic of and required for the activity of Set1p and Set2p, the methyltransferase for H3K36. In addition, H3 K79 methylation lies within the globular domain of histone H3, suggesting not only a novel mechanism of action for Dot1p but potentially novel downstream effects as H3 K79 methylation resides in a structured region of histone H3, unlike the modification of H3 K4 and K36 on the N terminal tail of H3. Methylation of H3 K79 could potentially alter the structure of histone H3 and/or the entire nucleosome to enact its functions.

In addition to its requirement for silencing, Dot1p has been characterized to be necessary for multiple functions in the cell; including meiotic checkpoint control and DNA damage response (Table 1)(Giannattasio et al., 2005; San-Segundo and Roeder, 2000; Wysocki et al., 2005). As the DNA damage response machinery is closely linked to apoptosis in yeast and higher eukaryotes (Burhans et al., 2003) a relation between H3 K79 methylation and apoptosis may exist and will be discussed in Chapter 3 and 5. However, recently, the human homolog of Dot1p, hDot1, has been demonstrated to interact with AF10, a protein causing leukemic transformation when fused to MLL (human Set1). Surprisingly, hDot1 can directly induce transformation when fused to the leukemia-translocated fragment of MLL in a methyltransferase activity-dependent manner (Okada et al., 2005). Furthermore, a recent study has observed increased levels of histone H3 K79 methylation at MLL-fusion protein binding loci in the chromatin of human leukemia cells, validating hDot1-mediated H3 K79 methylation as an marker of leukemic transformation (Guenther et al., 2008).

1.3.3 Histone H2B ubiquitination promotes methylation on histone H3 K4 and K79

The discovery of H2B ubiquitination in yeast brought about a bulk of research into the function of this modification, culminating in the elucidation of a novel requirement for H2B ubiquitination in promoting methylation of H3 K4 and K79 (Figure 1.8) (Briggs et al., 2002; Sun and Allis, 2002). This was the first identification of a dependence of one

histone modification for another on separate histones and added an additional layer of complexity to the histone code hypothesis.

Since the initial identification of this novel link between different histone modifications, the mechanism of how this link is established has been studied in more detail. Further refinement of the requirement for H2B ubiquitination to promote H3 methylation came from studies that demonstrated that H2B ubiquitination is not necessary for mono-methylation of H3 K4 and K79, suggesting that H2B ubiquitination promotes the processivity of the methyltransferases and not their recruitment (Dehe et al., 2005; Shahbazian et al., 2005).

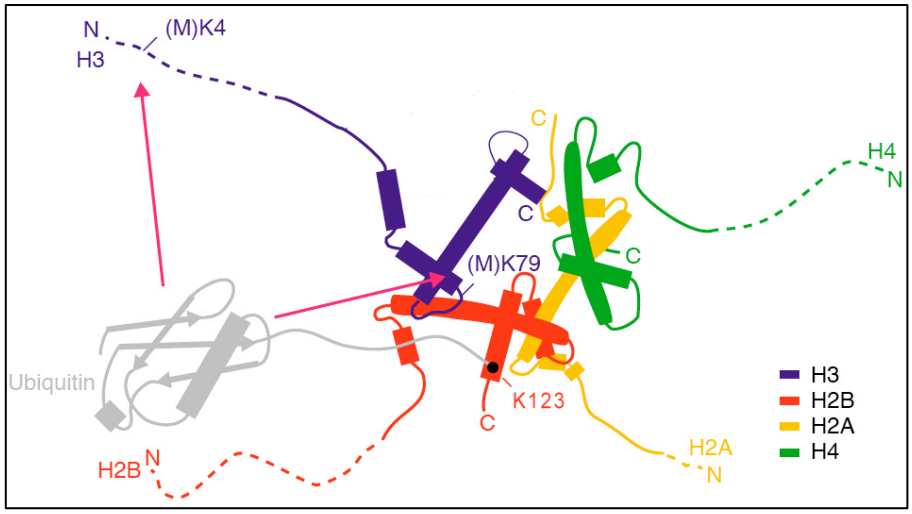

Figure 1.8: Cross-talk between histone H2B and H3.
Schematic representation of four core histones (one copy of each H2A, H2B, H3 and H4) as seen in the context of a nucleosome. The dashed lines represent the unstructured tails. Mono-ubiquitination (gray) of histone H2B carboxy-terminal tail at lysine K123 in H2B is shown. In a "trans-tail" pathway, this modification is necessary for methylation of histone H3 on lysine K4 and K79 (pink arrows). Adapted from: (Fischle et al., 2003b).

1.3.4 Functions of H3 K4 and H3 K79 methylation in chromatin silencing

Histone modifications can impact on many aspects of chromatin biology, including the maintenance of euchromatin and heterochromatin (Table 1) (Moazed, 2001a). Heterochromatin in yeast is generated by the action of the histone deacetylase Sir2p, which is necessary to maintain silencing of the three main regions of heterochromatin in yeast; the silent mating type cassettes *HMR* and *HML (HM)*, telomeres, and the ribosomal

DNA (rDNA) locus (Huang, 2002). Sir2p is recruited to telomeres and *HM* loci by association with DNA binding proteins bound to sequences called silencers. By the removal of acetylation on histones, Sir2p can promote the binding of its interaction partners Sir3p and 4 to deacetylated histones, which in turn recruit more Sir2p (Moazed, 2001b). Multiple iterations of this event allow for spread of the Sir complex away from silencers to generate heterochromatin (Moazed, 2001b). Sir2p intrusion into euchromatin is blocked by the presence of DNA sequences called boundary elements, whose mechanism of action still remains mysterious (Oki and Kamakaka, 2002). Before their identification as the modifying enzymes for the euchromatic methylation of H3 K4 and K79, *SET1* and *DOT1* were identified as genes that when deleted displayed defects in the silencing of a reporter gene located at the telomere, leading the authors to conclude that they encoded for factors that promoted heterochromatic silencing (Table 1) (Nislow et al., 1997; Singer et al., 1998). This presented a paradox when Set1p and Dot1p were found to promote two marks that were associated with euchromatin and active transcription. An explanation for this inconsistency was resolved when an increased Sir2 protein level in euchromatic regions was found when Set1p and Dot1p were deleted (Tompa and Madhani, 2007; van Leeuwen et al., 2002; Venkatasubrahmanyam et al., 2007). Because Sir2p levels are limiting in the cell, it was hypothesised that the ectopic spread of heterochromatin in strains missing the euchromatic methylation on histone H3 titrates away Sir2p in telomeric regions and thus indirectly cause a decrease in silencing (Smith et al., 1998).

Silencing at the third site of heterochromatin in yeast, the rDNA locus located in the nucleolus, is also mediated by Sir2p but via recruitment through a different set of proteins, i.e. the chromatin associated protein Net1p, which associates with a third protein, Cdc14p, to form the RENT (regulator of nucleolar silencing) complex (Straight et al., 1999). The mechanism of Sir2p mediated silencing at rDNA is largely unknown. However, studies that initially identified Set1p as the methyltransferase for H3 K4 also reported defects in rDNA silencing in strains missing Set1p, similar to the defects observed for Δ*set1* mutants in telomeric silencing (Briggs et al., 2001a; Bryk et al., 2002). Unlike at telomeres, however, these silencing defects at rDNA are not due to the redistribution of Sir2p away from silenced regions, as no change in Sir2p levels at rDNA

was observed in *Δset1* mutants (Bryk et al., 2002). Notably, *Δdot1* mutants do not display silencing defects at rDNA (Ng et al., 2003a), pointing to a different role of Set1p in silencing - at least at rDNA - as compared to Dot1p that may or may not account for the increased apoptosis sensitivity of *Δset1* cells (see chapter 3 and 5).

Table 1: Impact of H2B ubiquitination and H3 methylations on yeast cellular functions

Histone modification	**Functions regulated**
H2B K123 ubiquitination	• Transcription (Kao et al., 2004; Wood et al., 2003a) • Telomeric silencing (Hwang et al., 2003) • Silencing at rDNA (Sun and Allis, 2002) • DNA damage response (Giannattasio et al., 2005) • Apoptosis (Bettiga et al., 2004)
H3 K4 methylation	• Transcription (Liu et al., 2005) • Telomeric silencing (Nislow et al., 1997) • Silencing at rDNA (Briggs et al., 2001a; Bryk et al., 2002)
H3 K79 methylation	• Telomeric silencing (Singer et al., 1998) • DNA damage response (Giannattasio et al., 2005; Wysocki et al., 2005) • Meiotic checkpoint control (San-Segundo and Roeder, 2000)

1.4 Histone modifications in apoptosis

Chromosome condensation is one of the characteristics found in cells undergoing apoptosis. The mechanism by which chromosomes reorganize during apoptosis is still unsolved, but several lines of evidences suggest that histone modifications are crucial in this process. In mammalian cells, the histone modification that has been uniquely associated with apoptosis, is histone H2B phosphorylation in the N-terminal tail as shown by *in vivo* labeling of apoptotic cells (Ajiro, 2000). In addition, a cell-free *Xenopus* chromatin condensation system shows that the H2B N-terminus, but not other histone tails, is essential for chromatin condensation (de la Barre et al., 2001). Moreover, it has been shown that histone H2B is phosphorylated at Ser 14 (H2B S14) by caspase-3-activated Mst1p during apoptosis (Cheung et al., 2003). Ahn and colleagues extended these results to yeast by demonstrating that Ste20p kinase, a yeast orthologue of Mst1, directly phosphorylates H2B at serine 10 (H2B S10ph) in a hydrogen peroxide-induced cell death pathway (Ahn et al., 2005). Furthermore, yeast H2B S10ph plays a direct role in mediating apoptotic chromatin compaction. Accordingly, yeast H2B S10A mutants are resistant to cell death elicited by H_2O_2; in contrast, H2B S10E phosphosite mimics promote cell death and induce "constitutive" condensed chromatin (Ahn et al., 2005). Ahn and colleagues recently described an undirectional crosstalk relationship between two residues of the histone H2B tail, namely lysine 11 (K11) acetylation and serine (S10) phosphorylation (Ahn et al., 2006). They propose that, after addition of H_2O_2, histone deacetylase Hos3p catalyses deacetylation of H2B K11, which mediates phosphorylation of H2B S10 catalysed by Ste20 kinase.

A rapid and extensive deubiquitination of nucleosomal H2A occurs in Jurkat T-cells undergoing apoptosis initiated by different apoptotic stimuli (Mimnaugh et al., 2001), pointing to a role of histone H2A ubiquitination in apoptosis. In this line, Bettiga and colleagues found another exciting link between histone ubiquitination and apoptosis in yeast (Bettiga et al., 2004). Loss of the ubiquitin specific protease that cleaves ubiquitin from histone H2B leads to a subpopulation of cells exhibiting typical apoptotic markers. This was suppressed upon metacaspase *YCA1* deletion, whereas overexpression of Yca1p strongly increased apoptosis in an *ubp10* background (Bettiga et al., 2004; Orlandi et al., 2004). Recently, H2B ubiquitination 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) the notion of a relation between histone H2B ubiquitination and apoptosis is further supported and will be discussed in Chapter 2 and 5.

2

Bre1p mediated histone H2B ubiquitination regulates metacaspase-dependent apoptosis in *S. cerevisiae*

David Walter, Anja Matter and Birthe Fahrenkrog*

M.E. Müller Institute for Structural Biology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

Short title: Bre1p inhibits Yca1p-dependent apoptosis

* Present address: Institute for Biology and Molecular Medicine, Free University of Brussels, 6041 Gosselies, Belgium

Key words: apoptosis/histone H2B/S. cerevisiae/ubiquitin/YCA1

2.1 Abstract

BRE1 encodes an E3 ubiquitin protein ligase that is required for the ubiquitination of histone H2B at lysine 123 (K123). Histone H2B K123 ubiquitination is involved in a variety of cellular processes including gene activation and gene silencing. Abolishing H2B ubiquitination also confers X-ray sensitivity and abrogates checkpoint activation after DNA damage. Here we show that the *S. cerevisiae* Bre1p exhibits anti-apoptotic activity in yeast and that this is linked to H2B ubiquitination. 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 ubiquitination mediated by the E3 ligase activity of Bre1p. Furthermore, we found that the death of *Δbre1* cells depends on the yeast caspase Yca1p, since *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.

2.2 Introduction

Apoptosis is a form of programmed cell death that plays a central role in development and cellular homeostasis in higher eukaryotes. Knowledge about apoptotic regulation is particularly important for medical research, since apoptotic misregulation is implicated in many human diseases, such as Alzheimer's and Huntington's disease, immunodeficiency and cancer (Fadeel and Orrenius, 2005). Recent studies have established yeast as 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 deubiquitination (Bettiga et al., 2004) are able to 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., 1999b; 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., 2006; Ahn et al., 2005).

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). Ubiquitination controls the level of proteins by targeting them for proteasomal degradation. Along this line, members of the inhibitor-of-apoptosis protein (IAP) family are targeted for degradation, but they also contain a RING domain with ubiquitinating activity, by which they are able to mark other proteins such as caspases for degradation (Wilson et al., 2002). Additionally, mono-ubiquitination and nonclassical poly-ubiquitination of components of the apoptotic

pathway further is regulating apoptosis on a molecular level beyond degradation (Huang et al., 2000; Lee et al., 2002; Mimnaugh et al., 2001).

Histone proteins are well-known substrates for numerous covalent posttranslational modifications and these modifications are known to regulate a number of cellular processes including apoptosis (Ahn et al., 2006; Ahn et al., 2005; Cheung et al., 2003). Histone H2B is mono-ubiquitinated at Lys 123 by the ubiquitin conjugase Rad6p and the E3 ligase Bre1p (Hwang et al., 2003; Robzyk et al., 2000; Wood et al., 2003a). *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., 2001b; Dover et al., 2002; Mutiu et al., 2007; Sun and Allis, 2002). Moreover, the *C.elegans BRE1* was identified in a screen for anti-apoptotic proteins (Lettre et al., 2004). Only recently, histone H2B ubiquitination 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 relation between histone H2B ubiquitination and apoptosis may exist. This possibility is further 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 get more insight into the role of histone H2B ubiquitination in apoptosis and found that *S. cerevisiae* Bre1p exhibits anti-apoptotic activity in yeast and that this is linked to H2B ubiquitination. Enhanced expression of Bre1p protects yeast cells from hydrogen peroxide-induced cell death, whereas deletion of *BRE1* potentiates cell death. Moreover, cells lacking Bre1p show shortened lifespan during chronological ageing, a physiological apoptotic condition in yeast. Importantly, the resistance against apoptosis is conferred by histone H2B ubiquitination mediated by the E3 ligase activity of Bre1p. Furthermore, we found that the death of *Δbre1* cells depends on the yeast metacaspase Yca1p, since *Δbre1* cells exhibit increased caspase activity when compared to wild type cells and deletion of *YCA1* leads to reduced apoptosis sensitivity of cells lacking Bre1p.

2.3 Results

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

The E3 ubiquitin ligase Bre1p is required for histone H2B ubiquitination (Hwang et al., 2003; Robzyk et al., 2000; Wood et al., 2003a), which in turn is implicated in transcriptional regulation and DNA repair. Moreover, H2B ubiquitination appears to play a role in apoptosis regulation since 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 ubiquitination in apoptosis regulation.

To explore a role for Bre1p mediated histone H2B ubiquitination 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 and 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 Figure 2.1A, yeast cells lacking *BRE1* exhibited an increased sensitivity to H_2O_2 (31% ± 4% cell viability) as compared to wild type cells (53% ± 6% cell viability). This increase in sensitivity to H_2O_2 of *Δbre1* cells was accompanied by enhanced ROS production (58% of *Δbre1* versus 25% of wild type cells were DHE positive; Figure 2.1B and C) and by an increase in apoptotic DNA fragmentation and TUNEL positive cells as compared to wild type cells (31% versus 14% TUNEL positive cells; Figure 2.1B and D). In contrast, cells overexpressing ProtA-Bre1p showed resistance to H_2O_2 (78% ± 7% cell viability; Figure 2.1A) and a decrease in ROS accumulation (15% DHE positive cells; Figure 2.1B and C) and DNA fragmentation (4% TUNEL positive cells; Figure 2.1B and D). Taken together, our data indicate that Bre1p exhibits anti-apoptotic activity.

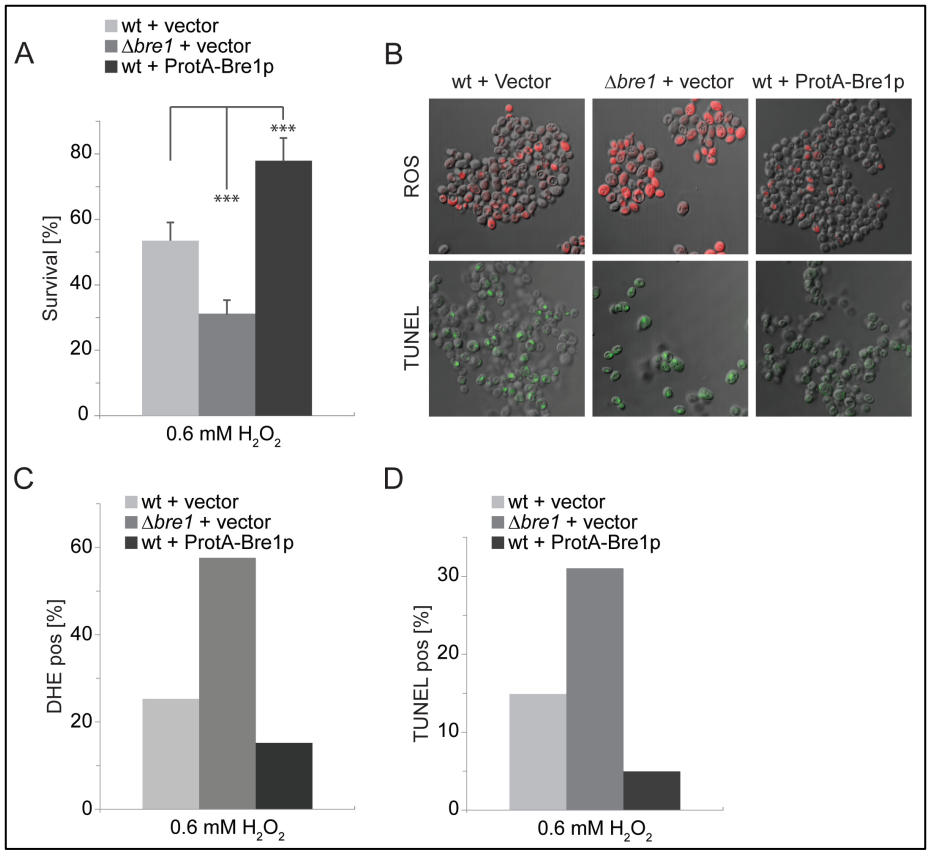

Figure 2.1: *S. cerevisiae* Bre1p confers resistance to apoptosis induced by H_2O_2.
(A) Wild type (wt) and *Δbre1* yeast cells harbouring the empty vector control, and ProtA-Bre1p over-expressing cells were treated with 0.6 mM H_2O_2 for 8 hours and survival was determined by clonogenicity. Data represent mean ± SD ($n = 6$; ***$P < 0.001$). **(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.

2.3.2 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 to wild type cells (Figure 2.2A). After 2 days in culture, *BRE1* lacking cells showed survival rates of 23% ± 4% as compared to 75% ± 5% of wild type

cells (Figure 2.2A). When, after two 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 (73% of *Δbre1* versus 13% of wild type cells were DHE positive; Figure 2.2B and C) and an increase in apoptotic DNA fragmentation as detected by TUNEL labelling (30% of *Δbre1* versus 6% of wild type cells were TUNEL positive; Figure 2.2B and D). These data further support the notion that Bre1p acts as inhibitor of apoptosis in yeast. Constitutive over-expression of ProtA-Bre1p, however, did not significantly influence chronological lifespan of yeast cells (data not shown).

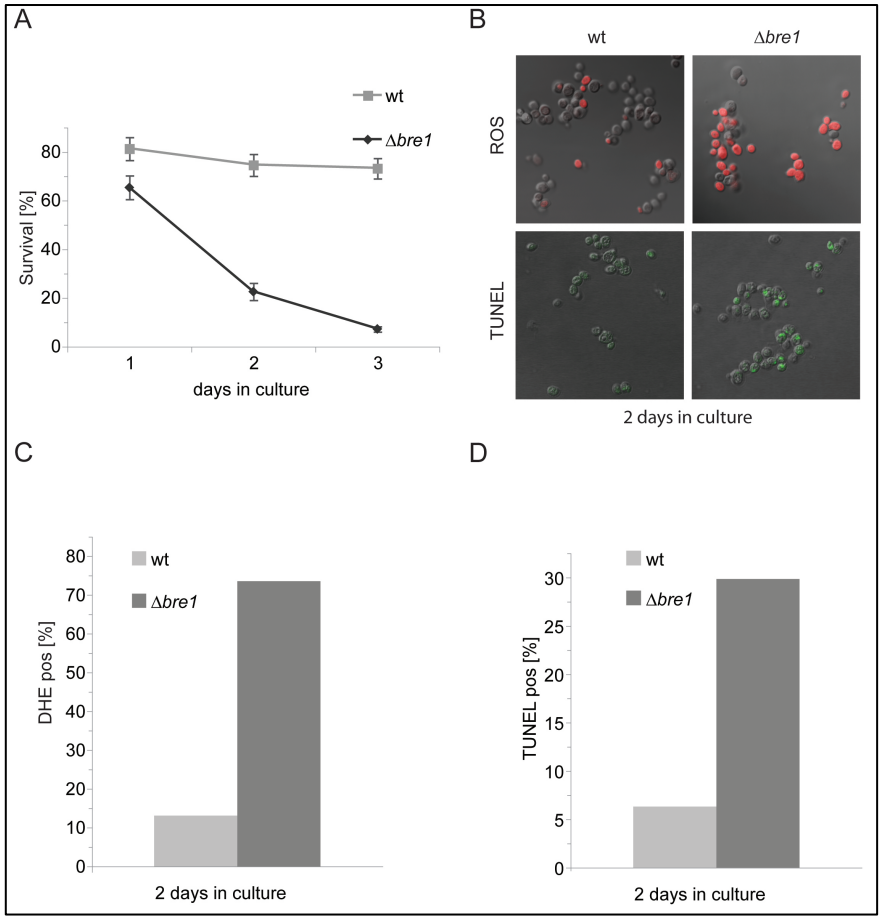

Figure 2.2: Disruption of *BRE1* causes an early onset of apoptosis during chronological ageing.
(A) Survival of wild type (wt) and *Δbre1* cells determined by clonogenicity during chronological ageing. Data represent mean ± SD (n = 9). **(B)** ROS accumulation and DNA fragmentation in wild

type and *Δbre1* cells after two days in culture determined by DHE staining and by 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. **(D)** TUNEL positive cells were quantified by manual counting of at least 500 cells.

2.3.3 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 critical 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 ubiquitination 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 mutated two conserved cysteins (i.e. C648 and C651) within the RING domain to glycins and complemented *Δbre1* and *Δbre1* cells expressing H2B-GFP (*Δbre1 HTB1-GFP),* respectively, with plasmid-borne ProtA-Bre1p or ProtA-Bre1p(C648G, C651G). As the mutation of these cysteins might affect the overall folding of the protein, we additionally created a mutant where leucin L650 is mutated. This hydrophobic residue likely mediates the E2 interaction and does not affect the zinc-coordination and hence stability of the protein.

We first tested the functionality of the ProtA-Bre1p and ProtA-Bre1p(C648G, C651G) and ProtA-Bre1p(L605A) as a measure of their ability to ubiquitinate histone H2B. Mono-ubiquitination 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 ubiquitinated species was absent in *Δbre1* cells harbouring a GFP-tagged allele of the *HTB1* (Figure 2.3A), 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 mono-ubiquitination, whereas *Δbre1 HTB1-GFP* cells complemented with ProtA-Bre1p(C648G, C651G) and ProtA-Bre1p(L605A) lacked ubiquitination of histone H2B similar to *Δbre1 HTB1-GFP* cells (Figure 2.3A). We

conclude that the ProtA-Bre1p fusion protein is functional and that Bre1p requires the conserved cysteins C648, C651 and leucin L650 for its E3 ligase activity.

To explore the contribution of the E3-ligase activity of Bre1p to apoptosis resistance, we analyzed the survival of *Δbre1* cells complemented with ProtA-Bre1p, ProtA-Bre1p(C648G, C651G) and ProtA-Bre1p(L650E) during chronological ageing. *BRE1* lacking cells complemented with ProtA-Bre1p showed no significant difference in cell survival when compared to wild type cells (89% ± 5% cell viability versus 92% ± 5% cell viability after 2 days in culture; Figure 2.3B). Consistently, *Δbre1* cells complemented with ProtA-Bre1p and wild type cells showed similar amounts of apoptotic markers with ~ 10% DHE and 5-7% TUNEL positive cells, respectively (Figure 2.3D-F). In contrast, *Δbre1* cells complemented with ProtA-Bre1p(C648G, C651G) or ProtA-Bre1p(L650E) showed an early onset of cell death during chronological ageing similar to *Δbre1* cells (21% ± 2% and 23% ± 1% cell viability vs. 23% ± 4% cell viability after 2 days in culture; Figure 2.3B). About 74% of *Δbre1* cells complemented with Bre1p(C648G, C651G) showed ROS accumulation and 35% were TUNEL positive, similar to *Δbre1* cells (74% ROS positive, 30% TUNEL positive cells; Figure 2.3D - F).

Consistent with the chronological ageing experiments, plasmid-borne ProtA-Bre1p but not ProtA-Bre1p(C648G, C651G) or ProtA-Bre1p(L650E) rescues *Δbre1* cells from H_2O_2 induced cell death (Figure 2.3C). H_2O_2 treated cells lacking *BRE1* that are complemented with ProtA-Bre1p showed a slightly better cell survival than wild type cells (67% ± 5% cell viability vs. 54% ± 6% cell viability; Figure 2.3C) and less apoptotic markers (16% versus 25% DHE positive cells as well as 5% versus 14% TUNEL positive cells; Figure 2.3D, G and H). On the other hand, *Δbre1* cells complemented with ProtA-Bre1p(C648G, C651G) or ProtA-Bre1p(L650E) showed H_2O_2 sensitivity similar to *Δbre1* cells (35% ± 2% and 33% ± 3% cell viability versus 31% ± 4% cell viability; Figure 2.3C). About 48% of *Δbre1* cells complemented with Bre1p(C648G, C651G) showed ROS accumulation and 28% were TUNEL positive, similar to *Δbre1* cells (58% ROS positive, 31% TUNEL positive; Figure 2.3D, G and H). We conclude that the E3 ligase activity of Bre1p is required for its ability to grant resistance to apoptosis.

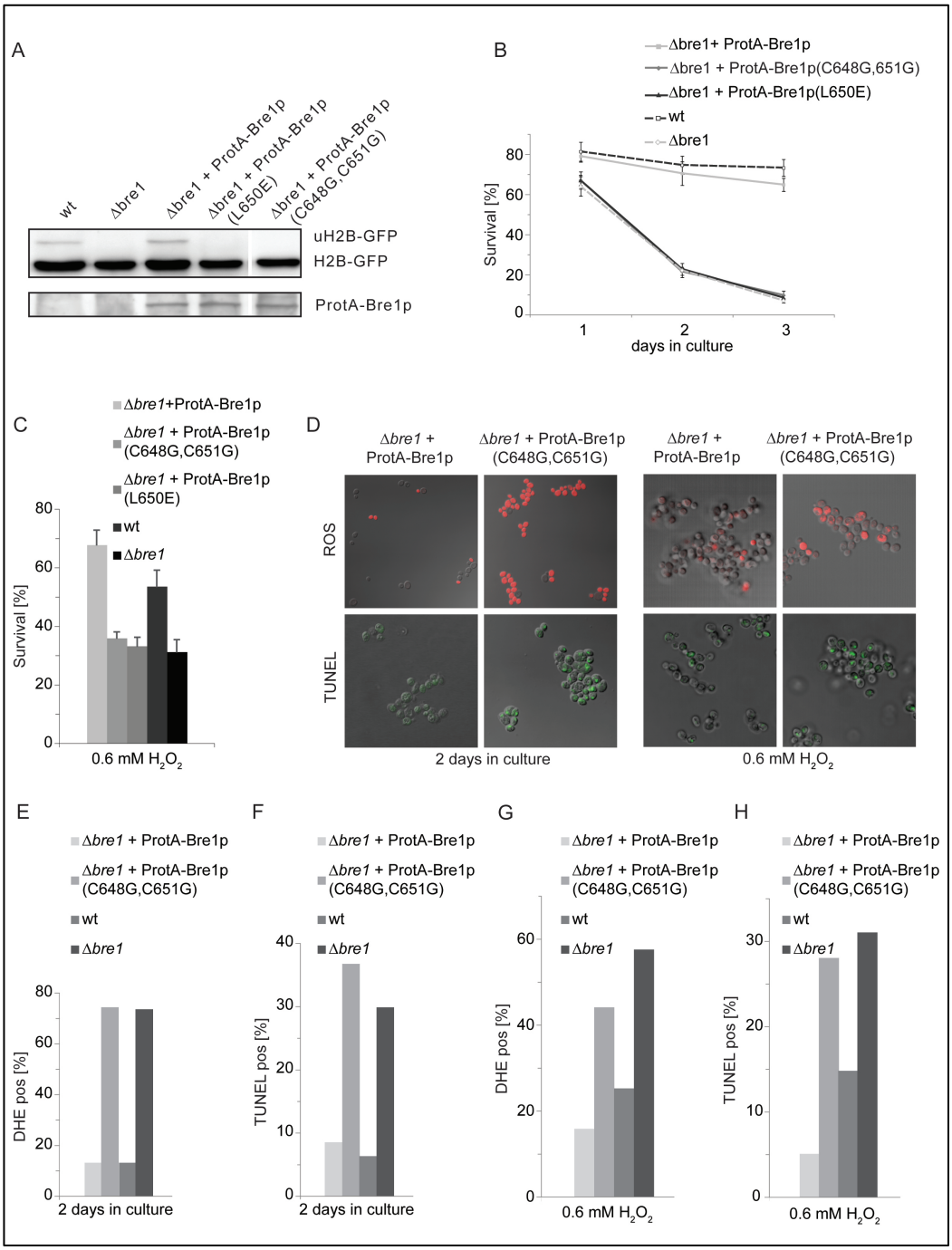

Figure 2.3: The E3 ligase activity of Bre1p is required for apoptosis inhibition.
(A) Functionality of ProtA-Bre1p, ProtA-Bre1p(C648,651G) and ProtA-Bre1p(L650E) was tested as a measurement of the ability to ubiquitinate histone H2B. Wild type (wt), *Δbre1* and *Δbre1* cells complemented with ProtA-Bre1p, ProtA-Bre1p(C648G, C651G) and ProtA-Bre1p(L650E) and harboring a functional GFP-tagged allele of the HTB1 gene (encoding histone H2B) were grown in synthetic complete medium (SC) over night. 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 (Sigma; St Louis, US). **(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 mean ± SD (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 mean ± SD (n = 9). **(D)** ROS accumulation and DNA fragmentation in *Δbre1* cells complemented with ProtA-Bre1p and ProtA-Bre1p(C648G, C651G) after two 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. **(F)** TUNEL positive cells during chronological ageing were quantified after 2 days in culture by manual counting at least 500 cells. **(G)** DHE-positive cells were quantified after H_2O_2 treatment by manually counting at least 500 cells. **(H)** TUNEL positive cells were quantified after H_2O_2 treatment by manually counting at least 500 cells.

2.3.4 Bre1p confers apoptosis resistance by histone H2B ubiquitination

Bre1p targets Lys123 in histone H2B for ubiquitination. We therefore asked whether the ubiquitination site of histone H2B is required for the anti-apoptotic property of Bre1p. To address this question we analyzed the chronological lifespan of the yeast strain *FLAG-htb1K123R*, which expresses a FLAG tagged histone H2B variant containing a lysine-to-arginine substitution at lysine 123 and therefore fails to be ubiquitinated H2B (Sun and Allis, 2002). We found that these cells showed an early onset of cell death during chronological ageing, similar 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; Figure 2.4A).

We next determined whether the lack of Bre1p and the lack of histone H2B ubiquitination 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 analyzed the chronological lifespan of the resulting double mutant *Δbre1 FLAG-htb1K123R*. An additive phenotype for the double mutant is expected in the case that 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 as compared to either single mutant (35% ± 5% cell viability versus 36% ± 7% and 32% ± 5% cell viability, respectively, after 3 days in culture; Figure 2.4A), indicating that both mutations affect the same pathway. Moreover, these

data suggest that Bre1p confers resistance to age induced apoptosis by mediating histone H2B ubiquitination. Likewise, FLAG-*htb1-K123R* mutant cells showed H_2O_2 sensitivity similar to *Δbre1 FLAG-HTB1* cells (66% ± 7% cell viability vs. 69% ± 7% cell viability; Figure 2.4B), whereas the double mutant strain *Δbre1 FLAG-htb1K123R* exhibited no further decrease in survival as compared to either single mutant (70% ± 6% cell viability versus 66% ± 7% and 68% ± 7% cell viability, respectively; Figure 2.4B). Together, our data indicate that Bre1p diminishes apoptotic death by mediating histone H2B ubiquitination.

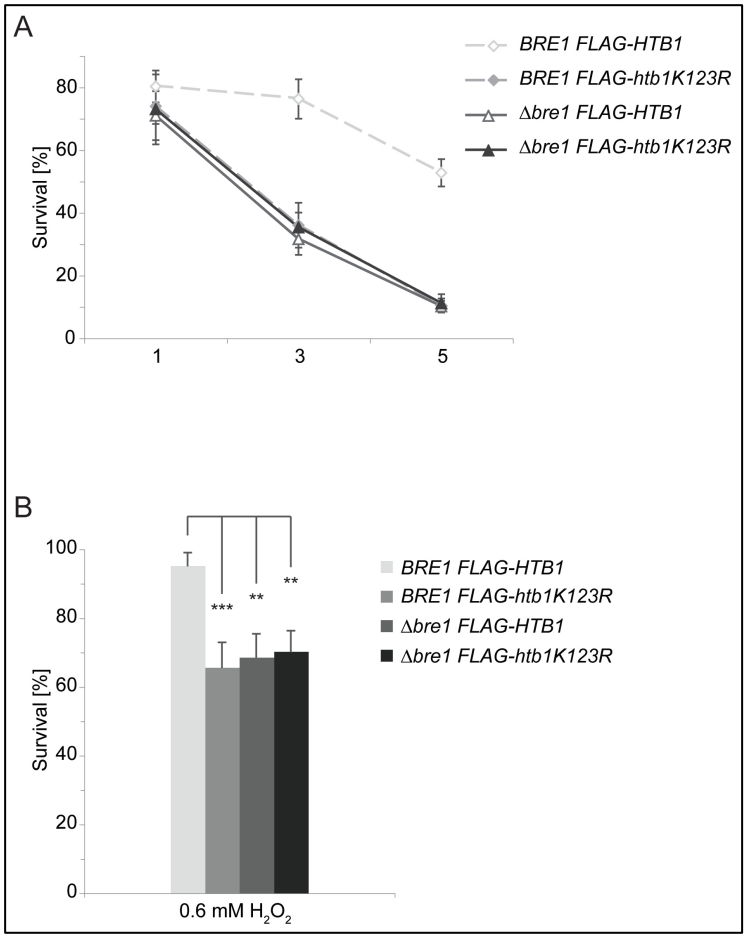

Figure 2.4: Histone H2B ubiquitination confers apoptosis resistance.
(A) Survival determined by clonogenicity of wild-type cells (*BRE1 FLAG-HTB1*) and its derivates i.e. *BRE1 FLAG-htb1K123R*, *Δbre1 FLAG-HTB1* and *Δbre1 FLAG-htb1K123R* during chronological ageing. Data represent mean ± SD (n = 6). **(B)** The same strains were treated with 0.6 mM H_2O_2 and survival was determined by clonogenicity. Data represent mean ± SD (n = 6; $^{***}P < 0.001$, $^{**}P < 0.01$).

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

Yeast cells lacking *BRE1* are sensitive to apoptotic stimuli and display morphological marker of apoptosis upon H_2O_2 treatment and during chronological ageing (Figure 2.1 and Figure 2.2). 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 when compared to *Δbre1* cells (47% ± 3% cell viability versus 21% ± 2% cell viability after 2 days in culture; Figure 2.5A), 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; Figure 2.5A). In addition, *Δbre1Δyca1* cells displayed less ROS accumulation during chronological ageing when compared to *Δbre1* cells (38% versus 74% cells were DHE positive after 2 days in culture; Figure 2.5C), but more than wild type cells (38% versus 13% cells were DHE positive after 2 days in culture; Figure 2.5C). Therefore, the death of *Δbre1* cells during chronological ageing is partially Yca1p-dependent.

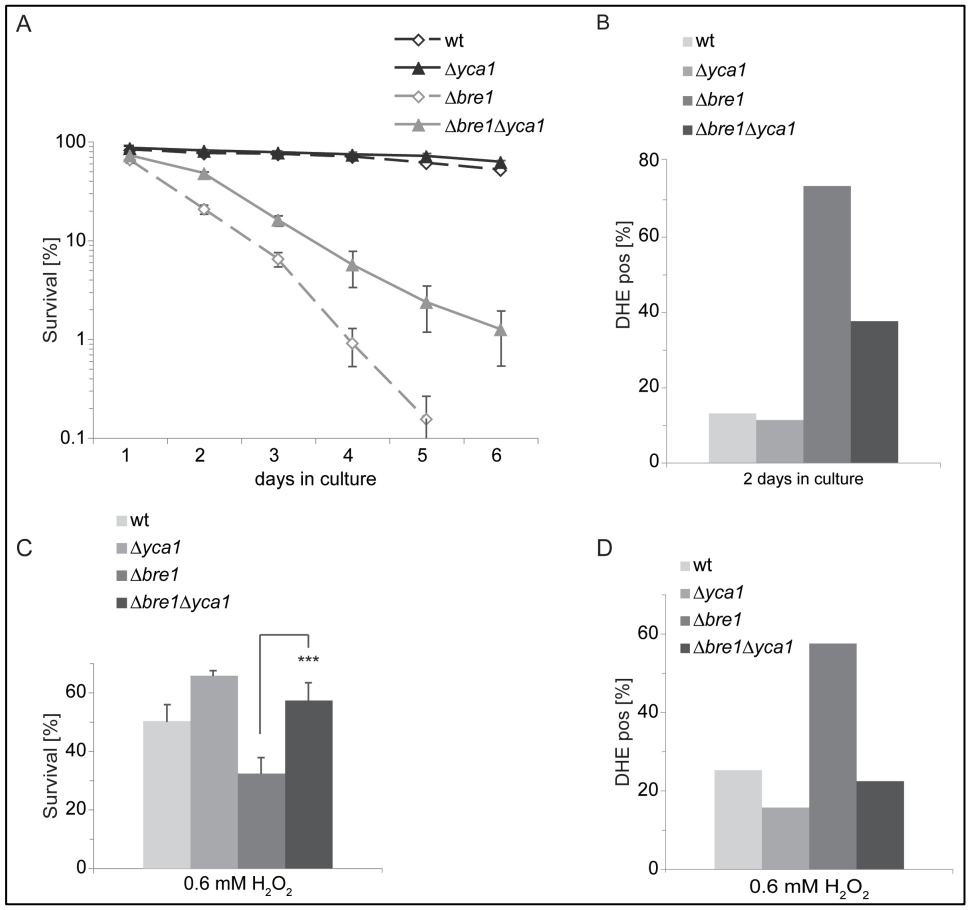

Figure 2.5: Death of *Δbre1* cells depends on the yeast caspase-like protein Yca1p.
(A) Survival determined by clonogenicity during chronological ageing of wild type (wt), *Δyca1*, *Δbre1* and *Δbre1Δyca1* cells. Data represent mean ± SD (n = 6). **(B)** DHE-positive wild type (wt), *Δyca1*, *Δbre1* and *Δbre1Δyca1* cells were quantified after 2 days in culture using flow cytometry. In each experiment, 10.000 cells were evaluated. **(C)** Wild type (wt), *Δyca1*, *Δbre1* and *Δbre1Δyca1* cells were exposed to 0.6 mM H_2O_2 for 8h and survival was determined by clonogenicity. Data represent mean ± SD (n = 6). **(D)** DHE-positive positive cells were quantified after H_2O_2 treatment by manually counting at least 500 cells.

Notably, unlike *Δbre1* cells, *Δbre1Δyca1* cells did not display apoptotic DNA fragmentation during chronological ageing as detected by TUNEL labelling (Figure 2.6B), suggesting that *Δbre1Δyca1* cells dye in a necrotic rather than apoptotic fashion. In this line, Annexin V/propidium iodide (PI) costaining was further used to analyze apoptotic externalization of phosphatidylserine and necrotic membrane permeabilization. *Δbre1* cells unlike *Δbre1Δyca1* cells displayed externalization of phosphatidylserine as

detected by Annexin V staining (Figure 2.6A), further supporting the notion that *Δbre1* cell dye in an apoptotic manner whereas the death of *Δbre1Δyca1* is of necrotic nature.

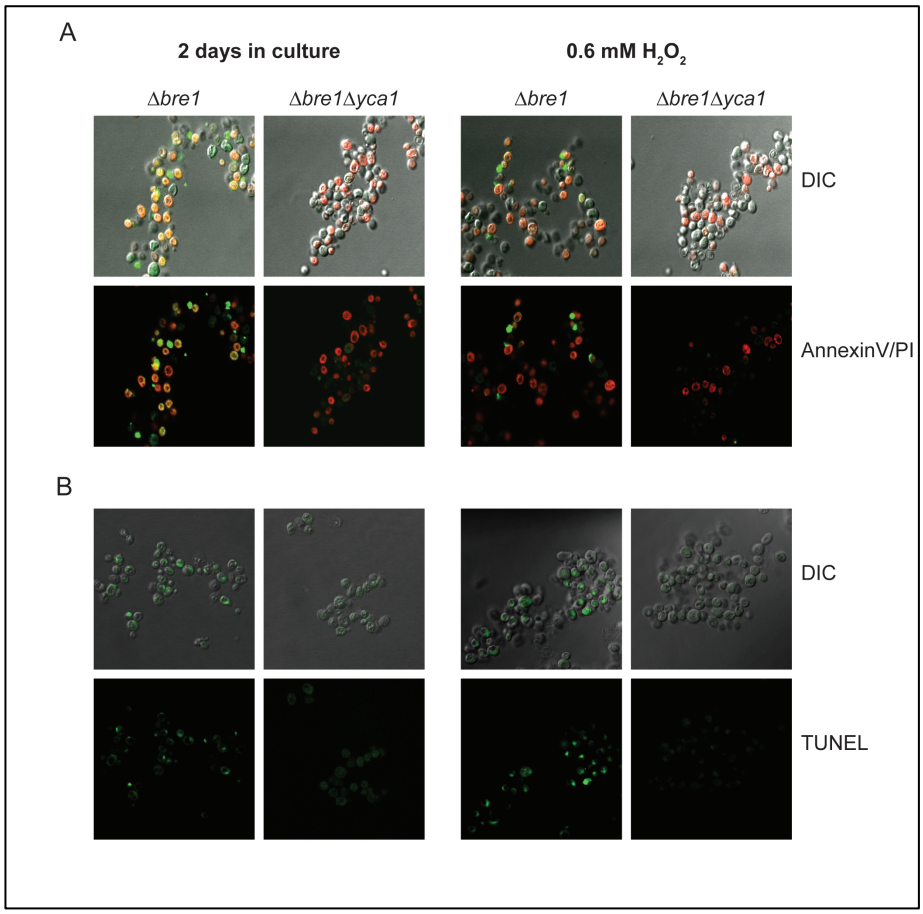

Figure 2.6: *Δbre1Δyca1* cells do not show apoptotic markers.
(A) Annexin V/PI costaining of *Δbre1* and *ΔbreΔyca1* cells after two days in culture and after H_2O_2 treatment to determine phosphatidylserine externalization and membrane integrity. **(B)** DNA fragmentation in *Δbre1* and *ΔbreΔyca1* cells after two days in culture and after H_2O_2 treatment determined by TUNEL staining.

Next we tested the response of *Δbre1Δyca1* cells to H_2O_2 exposure. As shown in Figure 2.5B, the death rate after H_2O_2 treatment is decreased in *Δbre1Δyca1* cells when compared to *Δbre1* cells (57% ± 5% cell viability versus 32% ± 5% cell viability; Figure 2.5B). Unlike during chronological ageing, the death of *Δbre1* cells seems to be exclusively Yca1p-dependent after H_2O_2 treatment as the double mutant cells *Δbre1Δyca1* show survival rates similar to wild type cells (57% ± 5% cell viability versus

50% ± 6% cell viability; Figure 2.5C). In addition, the population of *Δbre1Δyca1* cells with ROS accumulation after H_2O_2 treatment is smaller when compared to *Δbre1* cells but similar to wild type cells (22% DHE positive *Δbre1Δyca1* cells as compared to 57% DHE positive *Δbre1* and 25% DHE positive wild type cells; Figure 2.5D). 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 occur Yca1p-independent. Along this line, the mitochondria localised apoptosis inducing factor Aif1p (Wissing 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 if these pro-apoptotic factors are also involved in the Bre1p pathway, we generated distinct double mutant strains, i.e. *Δbre1Δnuc1*, *Δbre1Δaif1*, *Δbre1Δnma111*. As shown in Figure 2.7, none of these strains displayed better survival during chronological ageing or upon H_2O_2 treatment as compared to *Δbre1* cells, indicating that these other pro-apoptotic factors do not contribute to the Bre1p pathway.

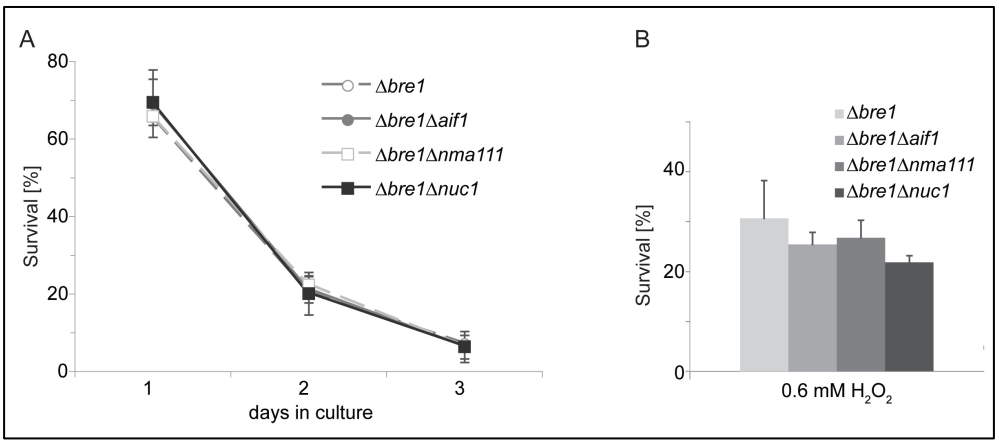

Figure 2.7: Death of *Δbre1* cells does not depend on Nuc1p, Aif1p and Nma111p
(A) Survival determined by clonogenicity during chronological ageing of *Δbre1Δnuc1*, *Δbre1Δaif1*, *Δbre1Δnma111* and *Δbre1* cells. Data represent mean ± SD (n = 6). **(B)** *Δbre1Δnuc1*, *Δbre1Δaif1*, *Δbre1Δnma111* and *Δbre1* cells were treated with 0.6 mM H_2O_2 for 8 hours and survival was determined by clonogenicity. Data represent mean ± SD (n = 3).

2.3.6 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 if *Δbre1* cells exhibit higher caspase activity after induction of apoptosis when compared to wild type cells. In order 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 may have the limitation that it stains dead cells unspecific (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 Figure 2.8A, after treatment with 0.6 mM H_2O_2 34% of *Δbre1* cells showed caspase activity (FITC positive, PI negative), 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 (Figure 2.8A). 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 Figure 2.8B, after 1 day of chronological ageing and after treatment with 0.6 mM H_2O_2, *Δbre1* cells exhibit higher caspase activity as compared to wild type cells. Taken together, our data show that *Δbre1* cells have higher caspase activity in comparison to wild type cells, further supporting the notion that Bre1p acts in an Yca1p-dependent manner.

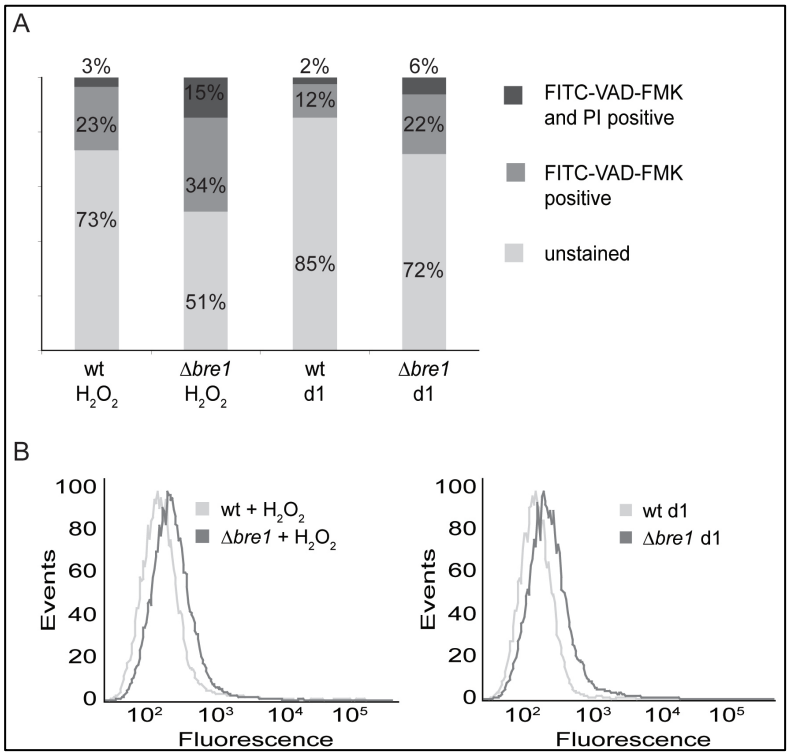

Figure 2.8: Cells lacking *BRE1* show increased caspase activity.
(A) Chronological aged wild type and *Δbre1* cells and cells treated with 0.6 mM H_2O_2 were labelled with FITC-VAD-fmk and propidium iodide (PI) and analyzed by flow cytometry. One representative experiment from three independent experiments 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 analyzed by flow cytometry. One representative experiment from three independent experiments with similar results is shown.

2.4 Discussion

BRE1 encodes an evolutionary conserved E3 ubiquitin ligase that, in yeast, catalyses monoubiquitination of histone H2B at lysine 123 (K123). Histone H2B K123 ubiquitination is involved in a variety of cellular processes, i.e. gene activation, gene silencing and checkpoint activation after DNA damage (Briggs et al., 2001b; 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 novel role for Bre1p and show that *S. cerevisiae* Bre1p protects yeast cells from hydrogen peroxide-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 monoubiquitination. Further we show that Bre1p protects yeast cells from death in a Yca1p-dependent manner.

2.4.1 A novel function for Bre1p in programmed cell death

Enhanced levels of Bre1p protect yeast from hydrogen peroxide-induced cell death and diminish the development of apoptotic hallmarks, i.e. ROS accumulation and DNA single strand breaks (Figure 2.1). In contrast, cells lacking Bre1p are more sensitive to H_2O_2 treatment (Figure 2.1) and show decreased lifespan during chronological ageing (Figure 2.2A), which coincided with the appearance of apoptotic markers (Figure 2.2B-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 likely evolutionary conserved and it will be interesting to see if, for example, the human homologues of Bre1p i.e. RNF20 and RNF40, are also implicated in apoptosis regulation.

Bre1p's ability to reduce cell death is conferred by its E3 ubiqutin ligase activity and histone H2B ubiquitination (Figure 2.3 and Figure 2.4). Bre1p harbours a C-terminal zinc-binding motif known as RING finger domain (Hwang et al., 2003) that is frequently found in E3 ubiquitin ligases and required for catalysing the transfer of ubiquitin from the E2 to the substrate (Deshaies and Joazeiro, 2009). RING domains appear critical for apoptosis regulation, as, for eample, members of the inhibitor-of-apoptosis protein (IAP)

family comprise RING domains, which enable IAPs to mark other proteins such as caspases for proteosomal degradation (Feng et al., 2003). Our data indicate that besides polyubiquitination, monoubiquitination may play a role in apoptosis. We show that *Δbre1* cells complemented with the RING finger mutants ProtA-Bre1p(C648G, C651G) and ProtA-Bre1p(L659E) are lacking monoubiquitinated histone H2B (Figure 2.3A) and are exhibiting increased apoptosis sensitivity similar to *Δbre1* cells, whereas *Δbre1* cells complemented with a functional ProtA-Bre1p behave like wild type cells (Figure 2.3). These findings suggest that Bre1p requires its E3 ligase activity to confer H2B monoubiqitination and apoptosis resistance and support the notion that RING domains play a major role in apoptosis regulation.

The importance of H2B monoubiquitination for the anti-apoptotic activity of Bre1p is further supported by our observation that *htb1-K123R* mutant cells, which fail to be ubiquitinated histone H2B, also exhibit increased apoptosis sensitivity, similar to *Δbre1* cells (Figure 2.4A and B). Interestingly, yeast cells that exhibit enhanced levels of ubiquitinated histone H2B due 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), Therefore, the lack of histone H2B ubiquitination as well as high levels of histone H2B ubiquitination appear to predisposes yeast to apoptotic stimuli, indicating that H2B monoubiquitination needs to be tightly regulated to assure 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, as *YCA1* disruption leads to reduced apoptosis sensitivity of cells lacking Bre1p (Figure 2.5). Furthermore the death of *Δbre1* cells depends neither on other pro-apoptotic factors, such as EndoG, Nma111p nor Aif1p (Figure 2.7), indicating that Bre1p's anti-apoptotic activity is exclusively caspase-dependent. Consistently, we show that *Δbre1* cell exhibit higher caspase activity as compared to wild type cells (Figure 2.8), suggesting that the caspase-like activity of Yca1p is implicated in the death of *Δbre1* cells.

2.4.2 Possible mechanisms

Our data suggest that lack of histone H2B monoubiquitination leads to Yca1p-dependent apoptosis during chronological ageing and after H_2O_2 treatment. Histone H2B ubiquitination plays a role in the DNA damage response as well as in transcriptional control (Briggs et al., 2001b; 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 ubiquitination may undergo Yca1p-dependent apoptosis either because of accumulated DNA damage or because of alterations in transcription. While H2B ubiquitination has been implicated in transcriptional silencing (Briggs et al., 2001a; 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 ubiquitination 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 Δ*bre1* cells. Furthermore, comparison of global mRNA transcripts between wild type and *Δbre1* cells could help to identify novel regulators of apoptosis.

Histone H2B ubiquitination 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 ubiquitination may undergo Yca1p-dependent apoptosis because of accumulated DNA damage. However, neither *Δrad9* nor *Δrad51* cells exhibit apoptosis sensitivity similar to *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 ubiquitination 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 ubiquitination. Future studies in yeast likely provide more details on the connection between transcription, DNA repair and apoptosis.

2.5 Materials and Methods

2.5.1 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 NcoI and BamHI and cloned into pNOPATA1L (Hellmuth et al., 1998). Plasmid pBF346 coding for ProtA-Bre1p(C648G, C651G) was constructed by site-directed polymerase chain reaction (PCR) mutagenesis using pBF326 as template according to the manufacturer's instructions (Stratagene, QuickChange™ Site-Directed Mutagenesis Kit).

BY4742 (MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) and derivative strain *Δbre1* were obtained from Euroscarf. Construction of the *Δbre1Δyca1*, *Δbre1ΔendoG*, *Δbre1Δnma111* and *Δbre1Δaif1* double mutant strains was performed according to (Gueldener et al., 2002). Yeast strains YZS276 (*FLAG-HTB1*) and YZS277 (*FLAG-htb1K123R*) were a gift from C.D. Allis (Rockefeller University, NY 10065) (Sun and Allis, 2002). *Δbre1 FLAG-HTB1* and *Δbre1 FLAG-htb1K123R* were derived from YZS276 and YZS277, respectively and constructed according to (Longtine et al., 1998). *HTB1-GFP Δbre1* was derived from *Δbre1* strain and constructed according to (Longtine et al., 1998).

Survival plating was conducted on YPAD (1% yeast extract, 2% peptone, and 2% glucose, 40mg/ml Adenine) media 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 by (Gietz et al., 1992).

2.5.2 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 (OD600~2) and exposed to 0.6 mM hydrogen peroxide (H_2O_2) for 8 h.

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 / 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.

2.5.3 In vivo staining of caspase activity by flow cytometric analysis

5x10^6 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 1μg/ml propidium iodide (PI) and analyzed 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 manufacturer's instructions.

2.5.4 Immunoblotting

Protein lysates were prepared using a lysis buffer containing 100mM NaCl, 50mM Tris/HCl pH 7.5, 50mM NaF, 5mM EDTA and 0.1% IGEPAL. Lysates were electrophoresed on 12% acrylamide-Tris HCl gels and proteins were transferred to an

Immobilon PVDF mambrane (Millipore; Billerica, MA). The filters were hybridized with anti GFP antibody (Dianova, clone: MA1-26343; Hamburg, Germany) and anti-protein-A antibody (Sigma; St Louis, US), respectively and the peroxidase-conjugated secondary anti-mouse antibody (Sigma; St Louis, US).

2.6 Acknowledgements

We thank C.D. Allis for providing plasmids YZS276 and YZS277 and Yvonne Lussi for critically reading the manuscript. This work was supported by grants from the Swiss National Science Foundation (to B.F.) as well as by the Kanton Basel Stadt and the M. E. Müller Foundation.

3

The histone H3 methyltransferase Dot1p is required for apoptosis in budding yeast

David Walter, Anja Matter and Birthe Fahrenkrog*

M.E. Müller Institute for Structural Biology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

Short title: Dot1p is required for yeast apoptosis

* Present address: Institute for Molecular Biology and Medicine, Free University of Brussels, 6041 Gosselies, Belgium

3.1 Abstract

Histone H2B ubiquitination at lysine K123 is a prerequisite for subsequent methylation of histone H3 at lysine K4 and K79 by the two methyltransferases Set1p and Dot1p, respectively. Histone H2B K123 ubiquitination and its downstream effects on histone H3 methylation are involved in a variety of cellular processes including transcriptional regulation and DNA damage response. We have previously shown that cells lacking histone H2B ubiquitination undergo metacaspase Yca1p-dependent apoptosis. To test whether apoptosis in these cells is caused by defects in histone H3 methylation, we analyzed the apoptosis sensitivity of *Δset1* and *Δdot1* cells. We found that *Δset1* cells are prone to Yca1p-dependent apoptosis, whereas *DOT1* disruption confers apoptosis resistance. Moreover, we found that Dot1p is required for Yca1p-dependent cell death of *Δset1* cells. Together, these studies highlight the requirement of Dot1p mediated histone H3 K79 methylation for an Yca1p-dependent cell death scenario and point to a novel role of the conserved histone H2B/H3 crosstalk in apoptosis regulation.

3.2 Introduction

Apoptosis is a form of programmed cell death that plays a central role in the development and cellular homeostasis in multicellular organisms. Deregulation of apoptosis contributes to the pathogenesis of multiple diseases including neoplastic and neurodegenerative disorders (Fadeel and Orrenius, 2005). Recent studies have established the unicellular yeast *Saccharomyces cerevisiae* as model to study the mechanisms of apoptotic regulation. It is well established that *S. cerevisiae* undergoes apoptosis when treated with various agents including hydrogen peroxide, acetic acid and pheromone (Ludovico et al., 2001; Madeo et al., 1999b; Severin and Hyman, 2002). Other than this, ageing is to date the best-studied physiological scenario of apoptosis induction in yeast. Two forms of ageing exist in *S. cerevsiae*, i.e. replicative and chronological ageing and apoptotic cell death is present in both ageing processes (reviewed in (Rockenfeller and Madeo, 2008)). The replicative life span is defined as the number of daughter cells produced by a mother cell before senescence. The formation and accumulation of extrachromosomal rDNA circles (ERCs), which depends on silencing gene transcription at the ribosomal DNA (rDNA), is a major cause of yeast replicative ageing (Sinclair and Guarente, 1997). The chronological life span (CLS) is defined as the length of time a yeast cell can survive in a nondividing state (Fabrizio and Longo, 2003). Few genetic interventions with key yeast apoptotic regulators have been described that delay chronological ageing and the appearance of the apoptotic features associated to it (Belanger et al., 2009; Herker et al., 2004; Madeo et al., 2002; Walter et al., 2006; Wissing et al., 2004). Particularly, disruption of the yeast metacaspase *YCA1* gene delays cell death and the formation of an apoptotic phenotype during chronological ageing (Madeo et al., 2002).

We have previously uncovered a link between histone H2B ubiquitination and Yca1p-dependent apoptosis in yeast (Walter et al., manuscript in revision for Journal of Cell Science). Cells lacking the E3 ubiquitin ligase Bre1p, which monoubiquitinates histone H2B at lysine 123 (H2B K123), are prone to Yca1p dependent apoptosis. Consistent with the idea that Bre1p acts through H2B ubiquitination, cells containing catalytically inactive Bre1p or a histone H2B variant containing a lysine-to-arginine substitution at lysine 123 and therefore fails to be ubiquitinated H2B mimic the apoptotic

phenotypes similar to a *BRE1* deletion strain (Walter et al., manuscript in revision for Journal of Cell Science).

Histone H2B ubiquitination is required in promoting methylation of histone H3 lysine K4 and K79 (Briggs et al., 2002; Sun and Allis, 2002). These methylations are brought about by the conserved methyltransferases Set1p and Dot1p, respectively. Dot1p is required for telomeric silencing (Singer et al., 1998) and was shown to be necessary for methylation of H3 K79 both *in vivo* and *in vitro* (Lacoste et al., 2002; Ng et al., 2003a; van Leeuwen et al., 2002). In addition to its requirement for telomeric silencing, Dot1p has been characterized to be necessary for multiple functions in the cell, including DNA damage response (DDR) (Giannattasio et al., 2005; San-Segundo and Roeder, 2000; Wysocki et al., 2005). Set1p, the methyltransferase responsible for H3 K4 methylation, is required for telomeric silencing, similar to Dot1p (Nislow et al., 1997; Singer et al., 1998) and plays an additional role in transcriptional activation (Bernstein et al., 2002; Ng et al., 2003b; Santos-Rosa et al., 2002) particularly, of genes involved in DNA replication and repair, such as replication factor C or ATP-dependent nuclease Dna2p (Nislow et al., 1997). However, unlike Dot1p, Set1p is required for silencing at rDNA (Briggs et al., 2001a; Bryk et al., 2002; Ng et al., 2003a), which is a major regulator of ERC formation and replicative ageing in yeast.

In this study, we asked whether the apoptosis sensitivity of cells lacking H2B ubiquitination depends on a lack of histone H3 K4 and/or H3 K79 methylation. To do so we analyzed the apoptosis sensitivity as a measurement of the chronological lifespan of *Δset1* and *Δdot1* cells. We found that *Δset1* cells are prone to Yca1p-dependent apoptosis, whereas *DOT1* disruption confers apoptosis resistance. Moreover, we found that Dot1p is required for Yca1p-dependent cell death of *Δset1* cells. Together, these studies highlight the requirement of Dot1p mediated histone H3 K79 methylation for an Yca1p-dependent cell death scenario and point to a novel role of the conserved histone H2B/H3 crosstalk in apoptosis regulation.

3.3 Results and Discussion

Histone H2B ubiquitylation is a prerequisite for histone H3 methylation at lysine K4 and K79. These methylations are brought about by the conserved methyltransferases Set1p and Dot1p, respectively. We found previously that cells lacking histone H2B ubiquitylation are prone to Yca1p-dependent apoptosis (Walter et al., manuscript in revision for Journal of Cell Science). In this study, we asked whether the apoptosis sensitivity of cells lacking H2B K123 ubiquitination depends on a lack of histone H3 K4 and/or H3 K79 methylation.

3.3.1 *SET1* disruption causes Yca1p-dependent cell death during chronological ageing

Histone H3 K4 methylation is conferred by the methyltransferase Set1p (Nislow et al., 1997). To test whether a lack of H3 K4 methylation predisposes yeast to apoptotic stimuli, we analyzed the apoptosis sensitivity of *Δset1* cells. Chronological ageing is to date the best-studied physiological scenario of apoptosis induction in yeast. Therefore, we analyzed the chronological lifespan wild type and *Δset1* cells. We observed that *Δset1* cells showed an early onset of cell death during chronological ageing when compared to wild type cells. After 2 days in culture *Δset1* cells showed survival of 35% ± 5%, whereas 73% ± 6% of wild type cells were viable (Figure 3.1A). Next, we asked whether the death *SET1* disrupted cells is of apoptotic nature. Staining with dihydroethidium (DHE) was used to visualize accumulation of reactive oxygen species (ROS), which are causally linked to apoptosis in yeast. DNA fragmentation was detected by using TUNEL staining. After two days in culture 72% of *Δset1* cells were DHE positive, but only 17% of wild type cells (Figure 3.1B-C). Consistently, *Δset1* cells unlike wild-type cells show apoptotic DNA fragmentation, as determined by TUNEL staining (Figure 3.1B).

Apoptosis in yeast can occur in a caspase-dependent or caspase-independent manner (Madeo et al., 2009). We showed previously that cells lacking *BRE1*, which is required for H2B ubiquitination are prone to Yca1p-dependent apoptosis (Walter et al., manuscript in revision for Journal of Cell Science). To investigate whether the yeast metacaspase Yca1p is also involved in the death of *Δset1* cells, we generated a *Δset1Δyca1* double mutant strain and analyzed its chronological lifespan. The death rate

during chronological ageing was decreased in *Δset1Δyca1* cells when compared to *Δset1* cells (60% ± 8% cell viability versus 35% ± 5% cell viability after 2 days in culture; Figure 3.1A). When, after two days in culture, these yeast cells were tested for ROS accumulation, 45% of *ΔsetΔyca1* cells were DHE positive, whereas 72% of *Δset1* cells showed ROS accumulation (Figure 3.1C). Moreover, unlike *Δset1* cells, *Δset1Δyca1* cells did not exhibit apoptotic DNA fragmentation as detected by TUNEL labelling (Fig 1C). Thus, these data indicate that Yca1p is implicated in the cell death of *Δset1* cells during chronological ageing.

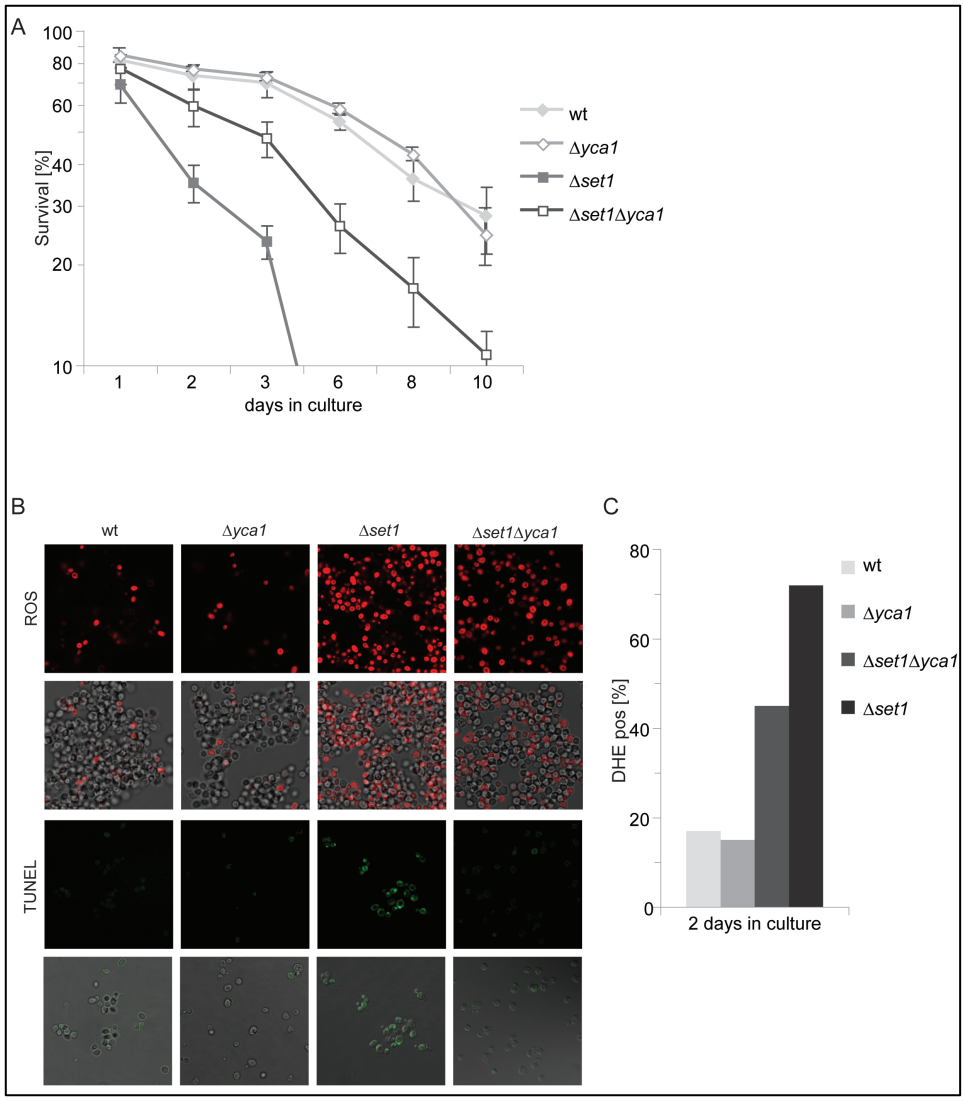

Figure 3.1: *SET1* disruption causes Yca1p-dependent cell death during chronological ageing. (A) Survival of wild type (wt), *Δyca1*, *Δset1* and *Δset1Δyca1* cells was determined by clonogenicity during chronological ageing. Data represent mean ± SD (n = 3). (B) ROS accumulation and DNA fragmentation in wt, *Δyca1*, *Δset1* and *Δset1Δyca1* cells cells after two days in culture determined by DHE staining and by 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.

SET1 disruption results in a complex phenotype that includes failures in gene activation and defects in transcriptional silencing at telomeres (Bryk et al., 2002; Krogan et al., 2002; Nagy et al., 2002; Nislow et al., 1997). Previous work has also shown that Set1p is required for the repression of genes placed within the rDNA locus (Bryk et al.,

2002; Mueller et al., 2006). As silencing gene transcription at the rDNA is regulating the replicative lifespan of yeast (Sinclair and Guarente, 1997) and the replicative and chronological lifespan are interrelated (Ashrafi et al., 1999), it is possible that failures in rDNA silencing trigger Yca1p dependent apoptosis in chronologically aged *Δset1* cells. This would be conflicting with the fact that cells lacking the histone deacelylase Sir2p, which are also defective in rDNA silencing do not show a early onset of age induced cell death during chronological ageing (Fabrizio et al., 2005). However, Set1p mediated rDNA silencing is independent of Sir2p mediated rDNA silencing (Bryk et al., 2002). Therefore, Sir2p-independent silencing defects at rDNA may account for the apoptosis sensitivity of *Δset1* cells. Alternatively, as transcription of genes involved in DNA replication and repair, such as replication factor C or the ATP-dependent nuclease *DNA2* (Nislow et al., 1997), is impaired in *Δset1* cells, DNA damage is likely accumulated in chronologically aged *Δset1* cells. Genome instability and DNA damage is an important determinant of chronological ageing in budding yeast (Fabrizio et al., 2005; Weinberger et al., 2007). Therefore *Δset1* cells may undergo Yca1p-dependent apoptosis during chronological ageing because of accumulated DNA damage.

3.3.2 *DOT1* disruption protects against Yca1p-dependent cell death during chronological ageing

Histone H2B ubiquitylation is not only a prerequisite for histone H3 K4 methylation but also for K79 methylation. Therefore, we asked whether the lack of histone H3 K79 methylation has also an impact on the chronological lifespan of *S. cerevisiae*. To address this question, we analyzed the chronological lifespan of cells lacking the methyltransferase Dot1p and therefore lack histone H3 K79 methylation. Unexpectedly, we found that disruption of *DOT1* confers apoptosis resistance. We observed that *Δdot1* cells exhibit enhanced survival during chronological ageing when compared to wild type cells. After 5 days in culture the *Δdot1* strain showed a survival rate of 69% ± 4%, whereas 56 ± 4% of wild type cells were viable (Figure 3.2A). When, after 3, 5, 7, 10 and 13 days these yeast cells were tested for ROS accumulation as detected by DHE staining, more wild type cells showed ROS accumulation as compared to *Δdot1* cells at these time points (Figure 3.2B-C). Moreover, after 5 days in culture wild type unlike *Δdot1* cells

exhibit apoptotic DNA fragmentation as detected by TUNEL labelling (Figure 3.2B). These data suggest that Dot1p in contrast to Set1p has a pro-death role during chronological ageing.

Next, we asked whether Dot1p-mediated cell death depends on Yca1p. To address this question, we generated a *Δdot1Δyca1* double mutant and compared its survival during chronological ageing with *Δdot1* and *Δyca1* cells. We expected better survival of the double mutant as compared to the single mutant cells, if Dot1p and Yca1p would act independently. However, we found that *Δdot1Δyca1*, *Δdot1* and *Δyca1* cells show similar survival rates during chronological ageing. After 5 days in culture the *Δdot1Δyca1* strain showed a survival rate of 64% ± 4%, whereas 69 ± 4% of *Δdot1* and 68 ± 5% of *Δyca1* cells but only 56 ± 4% of wild type cells were viable (Figure 3.2D). When, after five days in culture, these yeast cells were tested for ROS accumulation, 39% of *Δdot1Δyca1* showed ROS accumulation, whereas 35% of *Δdot1* and 36% of *Δyca1* but 45% of wild-type cells were DHE positive (Figure 3.2E). Together these data suggest that Dot1p and Yca1p act within the same pathway during chronological ageing as pro-apoptotic proteins.

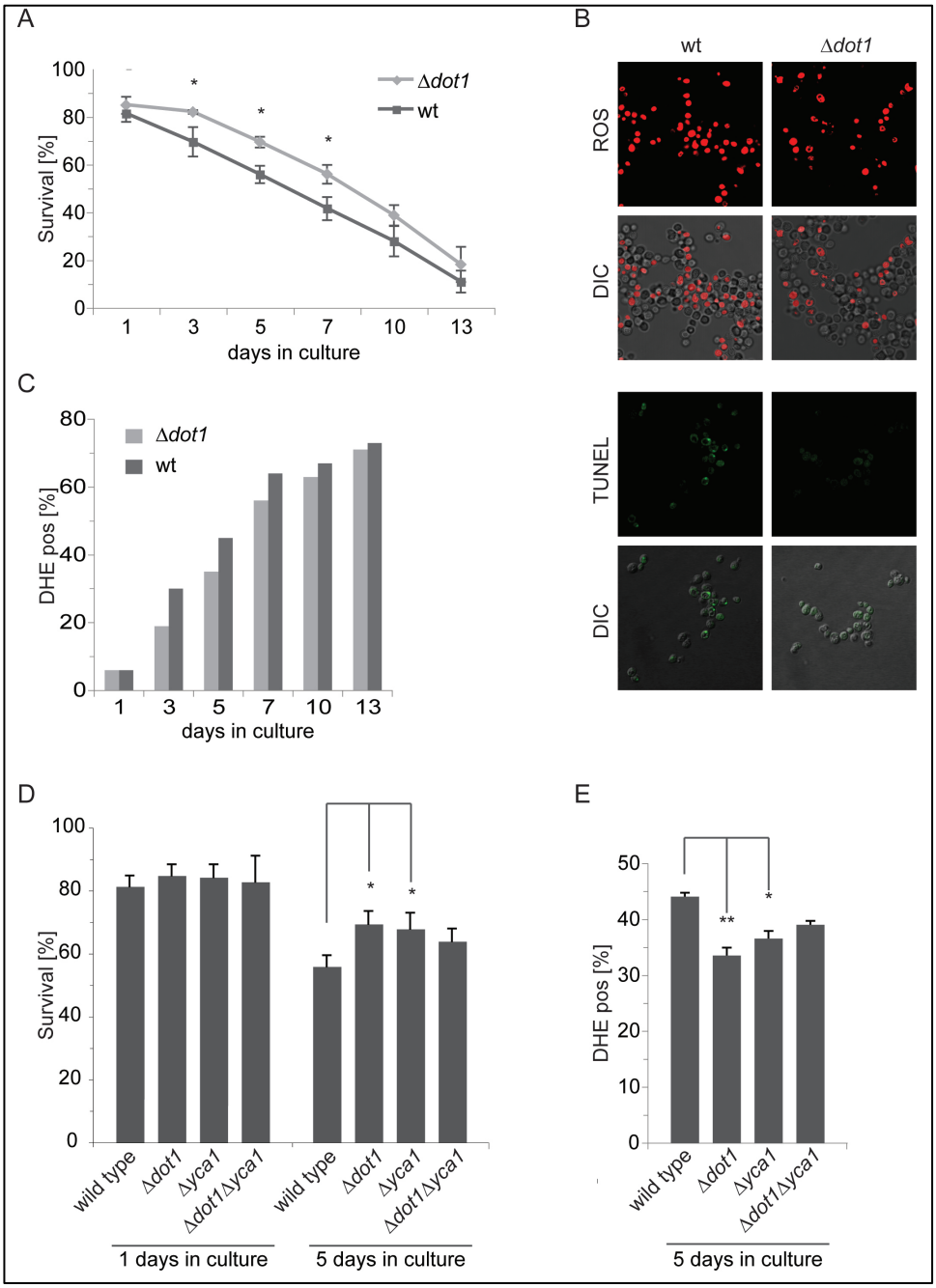

Figure 3.2: *DOT1* disruption protects against Yca1p dependent cell death during chronological ageing.
(A) Survival of wt and *Δdot1* cells was determined by clonogenicity during chronological ageing. Data represent mean ± SD ($n = 3$, $^*P<0.05$). **(B)** ROS accumulation and DNA fragmentation in wt and *Δdot1* cells after two days in culture determined by DHE staining and by TUNEL staining, respectively. **(C)** DHE-positive wt and *Δdot1* cells were quantified after 1, 3, 5, 7, 10 and 13 days in culture using flow cytometry. In each experiment, 10.000 cells were evaluated. **(D)** Survival of wild type (wt), *Δdot1*, *Δyca1* and *Δdot1Δyca1* cells was determined by clonogenicity after 1 and 5

days of chronological ageing. Data represent mean ± SD (n = 3, *P<0.05). (E) DHE-positive wt, *Δdot1*, *Δyca1* and *Δdot1Δyca1* cells were quantified after 5 days in culture using flow cytometry. In each experiment, 10.000 cells were evaluated. Data represent mean ± SD (n = 3, *P<0.05, **P<0.005)

Dot1p lacking cells are defective in gene silencing at telomers similar to *Δset1* cells (Singer et al., 1998), but unlike *SET1* disrupted cells *Δdot1* cells are not defective in gene silencing at rDNA (Ng et al., 2003a). However, in addition to its requirement for telomeric silencing, Dot1p has been characterized to be necessary for DDR (Giannattasio et al., 2005; Wysocki et al., 2005). Particularly Dot1p mediated H3 K79 methylation is required for Rad9p-dependent checkpoint activation after DNA damage (Wysocki et al., 2005). The DNA-damage response is an evolutionarily conserved signaling cascade crucial for sensing DNA damage and activating cellular responses such as cell-cycle arrest, DNA repair and apoptosis. Accordingly, DNA damage response genes have also been implicated in yeast apoptosis. Notably, deletion of the budding yeast *RAD9* gene can partially suppress the lethal effects of the apoptotic *orc2-1* mutation in the origin recognition complex (Watanabe et al., 2002), suggesting that Rad9p-dependent checkpoint function is required for apoptosis induction in *orc2-2* cells. As Dot1p is required for Rad9p-dependent checkpoint activation, we consider it likely that *Δdot1* cells fail to activate apoptosis as a result of a defective checkpoint function.

3.3.3 Dot1p is required for Yca1p-dependent cell death of *Δset1* cells

As shown in Figure 3.2A, *Δdot1* cells exhibit enhanced survival during chronological ageing when compared to wild type cells, suggesting that Dot1p-mediated methylation of histone H3 K79 induces cell death during chronological ageing. To further analyze the pro-death role of histone H3 K79 methylation, we asked whether the cell death of cells lacking histone H3 K4 methylation depends on histone H3 K79 methylation. To address this question, we constructed a *Δset1Δdot1* double mutant, which lacks both, histone H3 K4 and K79 methylation. We observed a rescue in cell survival during chronological ageing for the double mutant as compared to *Δset1* cells. After 2 days in culture the *ΔsetΔdot1* strain showed a survival rate of 69% ± 3%, whereas 35% ± 5% of *Δset1* cells were viable (Figure 3.3A). When, after 2 days in culture, these yeast cells were tested for ROS accumulation, 41% of *Δset1Δdot1* cells were DHE positive, whereas 72% of *Δset1*

cells accumulated ROS (Figure 3.3B). These data confirm the pro-death role of Dot1p and suggest that Dot1p is implicated in the death of *Δset1* cells.

We found that *Δset1* cells die in a partially Yca1p-dependent manner during chronological ageing (Figure 3.1A). As Dot1p is required for cell death of *Δset1* cells, we asked whether or not Dot1p is required specifically for Yca1p-dependent cell death of *Δset1* cells. To address this question we generated the triple mutant *Δset1Δdot1Δyca1* and analyzed its survival during chronological ageing. We expected a better survival of the triple mutant as compared to *Δset1Δdot1* cells, if Dot1p would act in an Yca1p-independent manner. However, the triple mutant *Δset1Δdot1Δyca1* did not show better survival during chronological ageing as compared to *Δset1Δdot1* cells. After two days in culture the *ΔsetΔdot1* strain showed a survival rate of 69% ± 3%, whereas 52% ± 5% of *Δset1Δdot1Δyca1* cells were viable (Figure 3.3A). When, after two days in culture, these yeast cells were tested for ROS accumulation, 41% of *Δset1Δdot1* cells were DHE positive and 57% of *Δset1Δdot1Δyca1* cells (Figure 3.3B). Thus, the deletion of *YCA1* in the *Δset1Δdot1* background provoked an additional decrease in viability, indicating that *SET1DOT1* disruption and *YCA1* disruption might impact identical vital functions and hence can mutually compensate for vitality. However, these data suggest that Dot1p is specifically required for Yca1p-dependent cell death of *Δset1* cells.

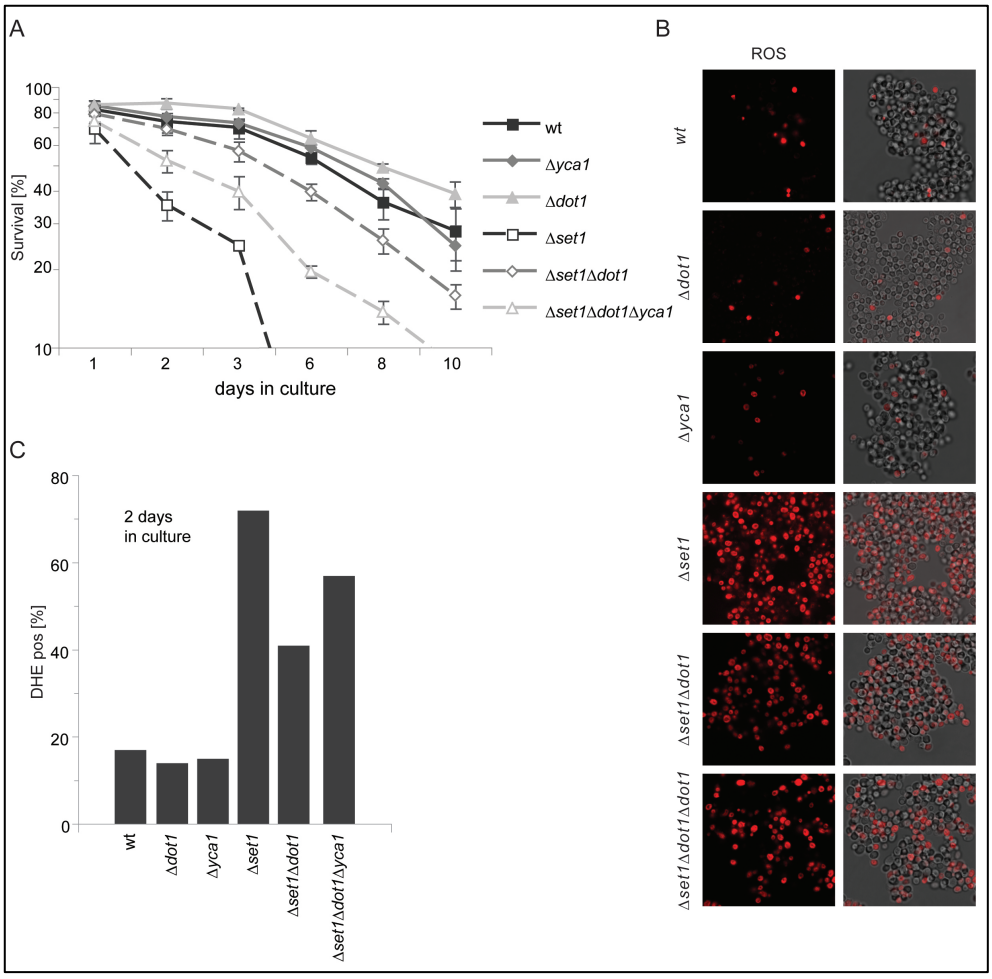

Figure 3.3: Dot1p is required for Yca1p-dependent cell death of *Δset1* cells.
(A) Survival of wt, *Δyca1*, *Δdot1*, *Δset1*, *Δset1Δdot1* and *Δset1Δdot1Δyca1* cells was determined by clonogenicity during chronological ageing. Data represent mean ± SD (n = 3). **(B)** ROS accumulation in wt, *Δyca1*, *Δdot1*, *Δset1*, *Δset1Δdot1* and *Δset1Δdot1Δyca1* cells after two days in culture was determined by DHE staining. **(C)** DHE-positive cells were quantified after 2 days in culture using flow cytometry. In each experiment, 10.000 cells were evaluated.

In conclusion we identified a new Yca1p-dependent cell death scenario in yeast, which is caused by *SET1* disruption. Moreover, we identified Dot1p as a potential pro-apoptotic protein since *DOT1* disruption confers apoptosis resistance and Dot1p is specifically required for Yca1p-dependent cell death of *Δset1* cells. We consider it likely that Dot1p exhibits pro-apoptotic activity through its role in the DNA damage response, as DDR is activating cellular responses including apoptosis in higher eukaryotes. Genome instability and DNA damage is an important determinant of chronological ageing in

budding yeast (Fabrizio et al., 2005) and Set1p has been proposed to be a general transcriptional regulator that positively regulates genes involved in DNA replication and repair, such as replication factor C or the ATP-dependent nuclease *DNA2* (Nislow et al., 1997). Therefore, it is plausible that accumulated DNA damage in chronologically aged *Δset1* cells triggers apoptosis via a Dot1p-mediated DDR.

3.4 Materials and Methods

3.4.1 Plasmids, yeast strains, and culture conditions

BY4742 (MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) and its derivatives *Δdot1, Δyca1* were obtained from Euroscarf. *Δset1* was derived from BY4742 and constructed according to (Gueldener et al., 2002). *Δset1Δdot1* and *Δset1Δdot1Δyca1* were derived from *Δdot1* and constructed according to (Gueldener et al., 2002). *Δset1Δyca1* and *Δdot1Δyca1* were derived from *Δyca1* and constructed according to (Gueldener et al., 2002).

Survival plating was conducted on YPAD (1% yeast extract, 2% peptone, and 2% glucose, 40mg/ml adenine) media supplemented with 2% agar. For experiments testing the 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 by (Gietz et al., 1992).

3.4.2 Chronological ageing and test for apoptotic markers

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. 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 after indicated time points.

Apoptotic tests using DHE-staining and TUNEL-staining were performed as described previously (Buttner et al., 2007). For quantification of DHE-staining, in each sample 10.000 cells were evaluated using flow cytometry (FACS-Aria, BD) and processed using BD FACSDiva software.

3.4.3 Statistical analysis

All statistical analyzes were performed using Students T-Test (one-tailed, unpaired), with $*P<0.05$ and $**P<0.005$, respectively.

4

Nuclear localisation is critical for the proapoptotic activity of the HtrA-like serine protease Nma111p

Kenneth D. Belanger[1], David Walter[2], Tracey A. Henderson[1,3], Allison L. Yelton[1,4], Travis G. O'Brien[1,3], Karyn G. Belanger[1], Susan J. Geier[1], and Birthe Fahrenkrog[2,5]

[1]Department of Biology, Colgate University, 13 Oak Dr., Hamilton, NY 13346, USA
[2]M. E. Muller Institute for Structural Biology, Biozentrum, University of Basel, Klingelbergstr. 70, 4056 Basel, Switzerland
[3]Current address: University of Rochester School of Medicine, 601 Elmwood Ave., Rochester, NY 14642 USA
[5]Current address: Institute for Biology and Molecular Medicine, Free University of Brussels, 6041 Gosselies, Belgium

Running title: Nuclear Nma111p and yeast apoptosis.

4.1 Abstract

Programmed cell death is induced by the activation of a subset of intracellular proteins in response to specific extra- and intra-cellular signals. In the yeast *S. cerevisiae*, Nma111p functions as a nuclear serine protease that is necessary for apoptosis under cellular stress conditions, such as elevated temperature or treatment of cells with hydrogen peroxide to induce cell death. We have examined the role of nuclear protein import in the function of Nma111p in apoptosis. Nma111p contains two small clusters of basic residues toward its amino terminus, both of which are necessary for efficient translocation into the nucleus. Nma111p does not shuttle between the nucleus and cytoplasm during either normal growth conditions or under environmental stresses that induce apoptosis. The amino-terminal half of Nma111p is sufficient to provide the apoptosis-inducing activity of the protein, and both the NLS sequences and catalytic serine 235 are necessary for this function. We provide compelling evidence that intranuclear Nma111 activity is necessary for apoptosis in yeast.

4.2 Introduction

Higher organisms make use of programmed cell death or apoptosis during development, morphogenesis and homeostasis, to sculpt and maintain distinct tissues and organs. Cell death programs also exist in lower eukaryotes and the yeast *S. cerevisiae*, for example, undergoes cell death upon viral infection as well as during chronological and replicative ageing (Herker et al., 2004; Laun et al., 2001; Reiter et al., 2005). Moreover, apoptosis in yeast can be stimulated by oxidative and osmotic stress, acetic acid, nitrogen oxide, the yeast mating type factor alpha or decreased actin dynamics (Almeida et al., 2007; Gourlay and Ayscough, 2006; Ludovico et al., 2001; Madeo et al., 1999a; Severin and Hyman, 2002; Silva et al., 2005) In addition, the basic molecular machinery executing cell death is evolutionarily conserved and orthologues of caspases (Yca1p), the serine protease Omi/HtrA2 (Nma111p), the apoptosis-inducing factor (Aif1p), endonuclease G (Nuc1p) and the inhibitor of apoptosis protein (IAP) survivin (Bir1p) have been identified (Buttner et al., 2007; Madeo et al., 2002; Walter et al., 2006; Wissing et al., 2004). Yeast apoptotic death comes along with the typical diagnostic features of apoptosis, such as phosphatidylserine externalisation, DNA condensation and fragmentation, production of reactive oxygen species (ROS), cytoskeletal perturbations, histone H2B phosphorylation, cytochrome c release from mitochondria, and dissipation of the mitochondrial transmembrane potential (Ahn et al., 2005; Fannjiang et al., 2004; Gourlay and Ayscough, 2005; Ludovico et al., 2002; Ludovico et al., 2001; Madeo et al., 1997).

The serine protease Nma111p belongs to the family of HtrA (high temperature requirement A) proteins that are defined by a characteristic combination of a catalytic serine protease domain with at least one PDZ (postsynaptic density 95/disc large/zona occludens) domain (Clausen et al., 2002; Pallen and Ponting, 1997; Ponting, 1997; Vande Walle et al., 2008). Nma111p harbours an internal duplication of the HtrA-like sequence, with the N-terminal repeat retaining the catalytic triade residues of HtrA-like serine proteases (Clausen et al., 2002; Pallen and Ponting, 1997; Ponting, 1997). The C-terminal repeat, in contrast, contains an incomplete serine protease site and is supposed to be non-functional. Bacterial HtrA family members have been implicated in stress tolerance and pathogenicity, while human and *Drosophila* Omi/HtrA2 are mitochondrial proteins that contribute to apoptosis through caspase-dependent and -independent processes (Challa et

al., 2007; Hegde et al., 2002; Igaki et al., 2007; Suzuki et al., 2001a; Suzuki et al., 2004; Vande Walle et al., 2008; Verhagen et al., 2002). Nma111p, also known as Ynm3p, is able to promote apoptotic cell death by, at least in part, degradation of Bir1p, the only identified IAP in *S. cerevisiae* (Fahrenkrog et al., 2004; Walter et al., 2006). In addition, it is implicated in lipid homeostasis and exhibits chaperone activity (Padmanabhan et al., 2009; Tong et al., 2006). In this context, both the apoptotic and the chaperone activity of Nma111p depends on its serine protease activity, although the actual catalytic serine has remained controversial (Fahrenkrog et al., 2004; Padmanabhan et al., 2009).

Nma111p is a nuclear protein that interacts with nuclear pore complexes (Fahrenkrog et al. 2004). Both Yca1p, the yeast caspase, and Bir1p, the only identified substrate for Nma111p, are also nuclear proteins (Walter et al., 2006). Therefore, some of the processes that occur in the cytoplasm during mammalian apoptosis seem to occur in the nucleus during yeast cell death. The importance of the nucleus for the yeast apoptotic program is further supported by the notion that Aif1p and Nuc1p translocate from mitochondria to the nucleus upon induction of apoptosis (Buttner et al., 2007; Wissing et al., 2004).

Here, we identify and characterize the nuclear localisation signal (NLS) of Nma111p and show that nuclear localisation is a prerequisite for Nma111p's apoptotic activity. Nma111p exhibits a bipartite NLS and its nuclear import is mediated by the nuclear import receptor Kap95p. In heterokaryon assays, we found that Nma111p is not shuttling between the nucleus and the cytoplasm. Mutations in the NLS of Nma111p lead to reduced sensitivity of the mutant cells to hydrogen peroxide treatment as well as to prolonged life span during chronological ageing. Moreover, the N-terminal HtrA-repeat of Nma111p, which localises to the nucleus, is sufficient to promote apoptosis, whereas the C-terminal HtrA-repeat, which lacks a NLS and an active catalytic site, fails to trigger cell death. Together, our data show that nuclear localisation, the N-terminal HtrA-repeat and serine protease activity are required for the pro-apoptotic activity of Nma111p.

4.3 Results

Nma111p contains an HtrA-like serine protease domain and is required for efficient apoptosis in yeast in response to several stresses, including elevated temperature and oxidative stress (Fahrenkrog et al., 2004). Interestingly, Nma111p is the only known HtrA-like protein that is localised primarily to the cell nucleus. In this study, we seek to determine if Nma111p is an exclusively nuclear protein, how Nma111p nuclear localisation is mediated, and if changes in nuclear localisation are important for Nma111p function in response to oxidative stress and/or during chronological ageing.

4.3.1 Nma111p does not undergo nucleocytoplasmic shuttling

To determine the subcellular localisation of Nma111p under normal and apoptotic conditions, we expressed GFP fusion proteins in cells grown to log phase and observed fluorescence after exposure to the oxidising agent hydrogen peroxide (H_2O_2). A positive control for nuclear localisation, the SV40 "classical NLS" fused to GFP (cNLS-GFP), is primarily nuclear in the absence of H_2O_2 (Figure 4.1A). However the cNLS-GFP becomes more cytosolic after exposure to the oxidising agent, possibly as a result of diffusion out of the nucleus due to the increased nuclear pore size in apoptotic cells (Mason et al., 2005). A different localisation pattern was observed for Nma111p. In the absence of H_2O_2, Nma111-GFP fluorescence is nuclear (Figure 4.1A; see also (Fahrenkrog et al., 2004)). After exposure to 3 mM H_2O_2, Nma111-GFP localisation remains unchanged, exhibiting intense fluorescence within the nucleus and lacking any detectable cytosolic staining. Thus Nma111p is predominantly present within the nucleus under steady state conditions in both the absence and presence of a concentration of H_2O_2 that induces apoptosis in yeast.

While the steady state localisation of Nma111-GFP fluorescence is within the nucleus in both the presence and absence of H_2O_2, it is possible that Nma111p undergoes nucleocytoplasmic shuttling under either or both conditions. The appearance of nuclear localisation under steady state conditions may simply be due to a higher rate of Nma111p protein import compared to export, resulting in a much greater protein concentration within the nucleus than the cytoplasm (DeLotto et al., 2007; Feng and Hopper, 2002; Selitrennik et al., 2006). To determine if Nma111p undergoes nucleocytoplasmic

shuttling, we performed a heterokaryon shuttling assay (Feng and Hopper, 2002) on cells expressing an Nma111-GFP fusion protein. Briefly, we expressed Nma111p under control of the *GAL1* promoter in a wild type haploid yeast strain until a detectable amount of Nma111-GFP was visible in the nucleus. We then repressed further Nma111-GFP transcription by adding glucose and allowed the existing Nma111-GFP to equilibrate within the cells. Next, we introduced haploid cells of the opposite mating type that contain a *kar1-1* allele. Cells with this *kar1* mutation are able to undergo cytoplasmic fusion with cells of the opposite mating type as an early stage of diploid zygote formation, but are unable to complete karyogamy with a partner cell (Conde and Fink, 1976), thus generating a heterokaryon with two distinct nuclei derived from two distinct populations of haploid cells. Since one nucleus is from a cell expressing Nma111-GFP and no new Nma111-GFP is being synthesised, the only way the second nucleus can become fluorescent is by importing Nma111-GFP that was exported or diffused out of the first. Thus, shuttling is detected through the observation of fluorescence in both nuclei of the zygote. Indeed, examination of heterozygotes in which one donor nucleus harbours Cca1-GFP, which shuttles to assist in tRNA export (Feng and Hopper, 2002), reveals the appearance of fluorescence in both nuclei, indicating that Cca1-GFP has been exported from one nucleus and some Cca1-GFP protein has been imported into the second (Figure 4.1B). Conversely, the histone protein H2B does not shuttle (Mosammaparast et al., 2001), so we observe fluorescence in only one nucleus of the heterokaryon. When we observe the pattern of fluorescence generated by Nma111-GFP in heterokaryons, we only see Nma111p in a single nucleus (Figure 4.1B, top row). Thus Nma111-GFP does not shuttle between the nucleus and cytoplasm under steady-state conditions.

Nma111p is essential for efficient apoptosis in the presence of 3 mM H_2O_2 (Fahrenkrog et al., 2004). While we had observed that Nma111p does not shuttle under steady-state conditions, the possibility remained that the protein is selectively exported under conditions that induce apoptosis. To examine whether Nma111p shuttling is induced under apoptotic conditions, we repeated our shuttling assay using Nma111-GFP, this time exposing zygotes to 3 mM H_2O_2 for 2h (data not shown) and 4 h (Figure 4.1C) after initiation of mating. In both the presence and absence of H_2O_2, heterokaryons

containing Nma111-GFP retain fluorescence in only a single nucleus. These data indicate that Nma111p does not leave the nucleus, even under conditions that induce apoptosis.

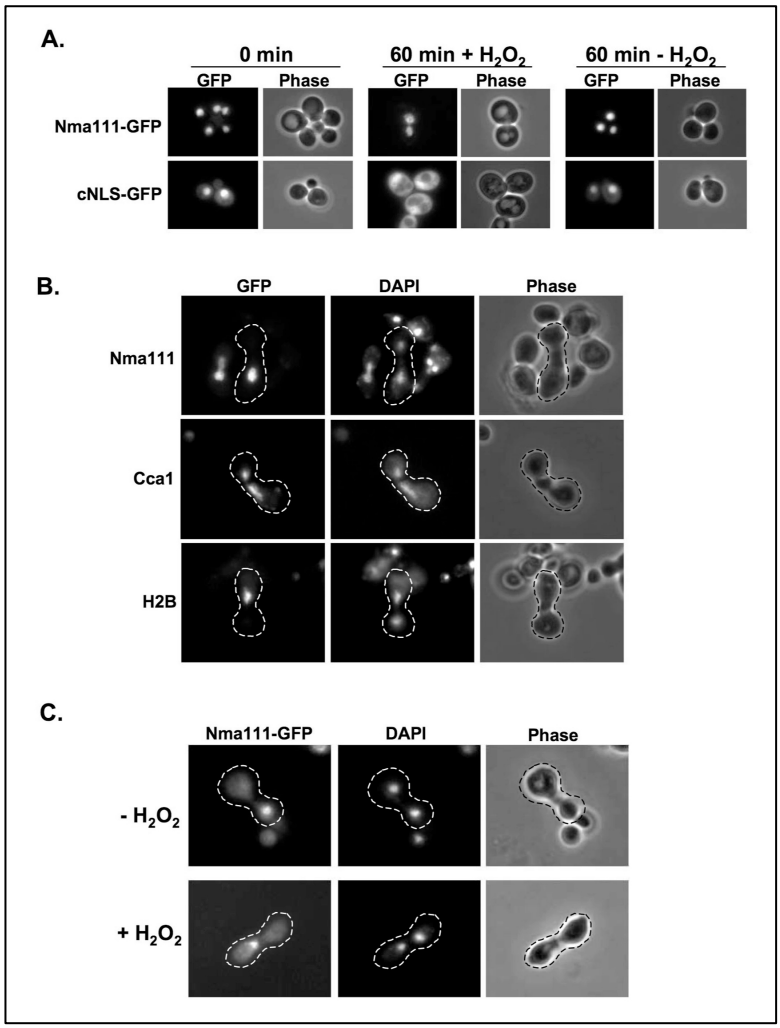

Figure 4.1: Nma111p does not shuttle between the nucleus at the cytoplasm under normal or apoptotic conditions.
(**A**) Steady-state localization of Nma111p in the presence and absence of oxidative stress. Yeast expressing Nma111p tagged at the C-terminus with GFP (Nma111-GFP) or a classical nuclear localization signal fused to GFP (cNLS-GFP) were grown in SD –Ura media, then exposed to 3 mM hydrogen peroxide for 1 h. Cells were examined by direct fluorescence (GFP) and phase contrast (phase) light microscopy. Nma111p is localized to the nucleus in cells grown in either the presence or absence of hydrogen peroxide. (**B**) Test for nucleocytoplasmic shuttling of Nma111p under normal growth conditions. Cells expressing Nma111p, Cca1p, or histone H2B fused to GFP and under control of the *GAL1* promoter were grown in media containing galactose to induce GFP fusion protein expression. The cells were then shifted to repressing conditions by the addition of glucose for 1 h and were mated with a *kar1-1* mutant strain (MS739) to generate heterokaryons.

Representative zygotes are depicted (dashed outline) expressing Nma111-GFP (top), the shuttling tRNA processing protein Cca1-GFP (middle), and a nuclear histone H2B-GFP fusion (bottom). GFP localization was determined by direct fluorescence. DNA was observed by DAPI staining. (**C**) Test for nucleocytoplasmic Nma111-GFP shuttling under oxidative stress conditions. Nma111-GFP shuttling was tested as described above, except that 3 mM hydrogen peroxide was added to cells 30 minutes after mating was initiated and cells were observed 4 h later. Images depict representative heterokaryons incubated in the absence (- H_2O_2) and presence (+ H_2O_2) of hydrogen peroxide.

4.3.2 The amino-terminal 35 amino acids of Nma111p are sufficient for nuclear targeting

In order to investigate the targeting of Nma111p to the nucleus, we examined the amino acid sequence of the protein for domains that might contain potential NLSs. *In silico* examination of the entire Nma111p sequence using the PSORT (Nakai and Horton, 1999) or PredictNLS (Cokol et al., 2000) algorithms failed to identify any potential cNLS sequences (data not shown). However, careful manual analysis of the amino terminal region of Nma111p revealed two short basic clusters of three residues each at amino acids 9 – 11 and 28 – 30 (Figure 4.2A). The close juxtaposition of these basic residues is similar to the organisation of a prototypical cNLS (Lange et al., 2007). To determine if these basic clusters are sufficient for mediating nuclear import of a reporter protein, we generated two chimeric polypeptides that respectively included the first 35 (Nma111$^{1-35}$-GFP) and first 83 (Nma111$^{1-83}$-GFP) amino acids of Nma111p fused to GFP. Expression of these chimeras in wild-type yeast resulted in predominantly nuclear fluorescence, with a low level of cytosolic staining that was excluded from the vacuole (Figure 4.2B and 2C). Thus, the first 35 amino acids of Nma111p containing the two short basic clusters are sufficient to function as an NLS.

In order to determine if either or both of the clusters of basic residues in the N-terminus of Nma111p are necessary for the NLS activity of this region, we performed site-directed mutagenesis on Nma111$^{1-83}$-GFP to generate chimeric proteins that contained the first 83 amino acids of Nma111p with the lysine (K) and arginine (R) residues at amino acids 9 – 11 (*nls1*) altered to alanines to make Nma111$^{1-83}$*nls1*Δ-GFP, with K and R residues 28 – 30 (*nls2*) replaced with alanines (Nma111$^{1-83}$*nls2*Δ-GFP), and with both basic clusters altered (Nma111$^{1-83}$-*nls1*Δ*nls2*Δ-GFP). Each Nma111p mutant was then expressed in wild type yeast and observed for localisation of fluorescence

(Figure 4.2B). Expression of Nma111$^{1\text{-}83}$*nls1*Δ-GFP results in substantially more cytosolic fluorescence than Nma111$^{1\text{-}83}$-GFP, but retains some nuclear accumulation of the fusion protein. Nma111$^{1\text{-}83}$*nls2*Δ-GFP is similarly found in the cytoplasm and nucleus, with a subtly greater accumulation within the nucleus. However, alteration of both basic clusters from this short region at the amino-terminus of Nma111p (Nma111$^{1\text{-}83}$-*nls1*Δ*nls2*Δ-GFP) results in almost no nuclear accumulation above that found within the cytosol. Thus, while the alteration of either basic cluster affects the efficiency of nuclear import of this region of Nma111p, altering the entire bipartite NLS is necessary to severely reduce the accumulation of the GFP fusion within the nucleus.

4.3.3 Kap95p is an importin for Nma111p

The two basic amino acid clusters in the amino terminus of Nma111p that are necessary for efficient nuclear import have the characteristics of being a classical NLS (cNLS) imported by the heterodimeric Kap60p-Kap95p importin complex. In order to determine if Kap95p is necessary for Nma111p import, we expressed Nma111$^{1\text{-}83}$-GFP in yeast cells expressing a temperature-sensitive *kap95-3* allele and assayed for nuclear fluorescence at the permissive and restrictive temperatures. At the permissive temperature, Nma111$^{1\text{-}83}$-GFP expressed in a *kap95-3* mutant is present both in the cytoplasm and the nucleus, with some accumulation in the nucleus (Figure 4.2C). However, after a 2 h shift to 37°C, Nma111$^{1\text{-}83}$-GFP in *kap95-3* cells is redistributed exclusively to the cytosol, with little detectable nuclear fluorescence. We did not observe this redistribution of Nma111$^{1\text{-}83}$-GFP in cells expressing mutant alleles of the karyopherins *msn5* (Figure 4.2C) or *crm1* (data not shown). These observations indicate that Kap95p is the primary karyopherin for importing the amino-terminal "cNLS" of Nma111p.

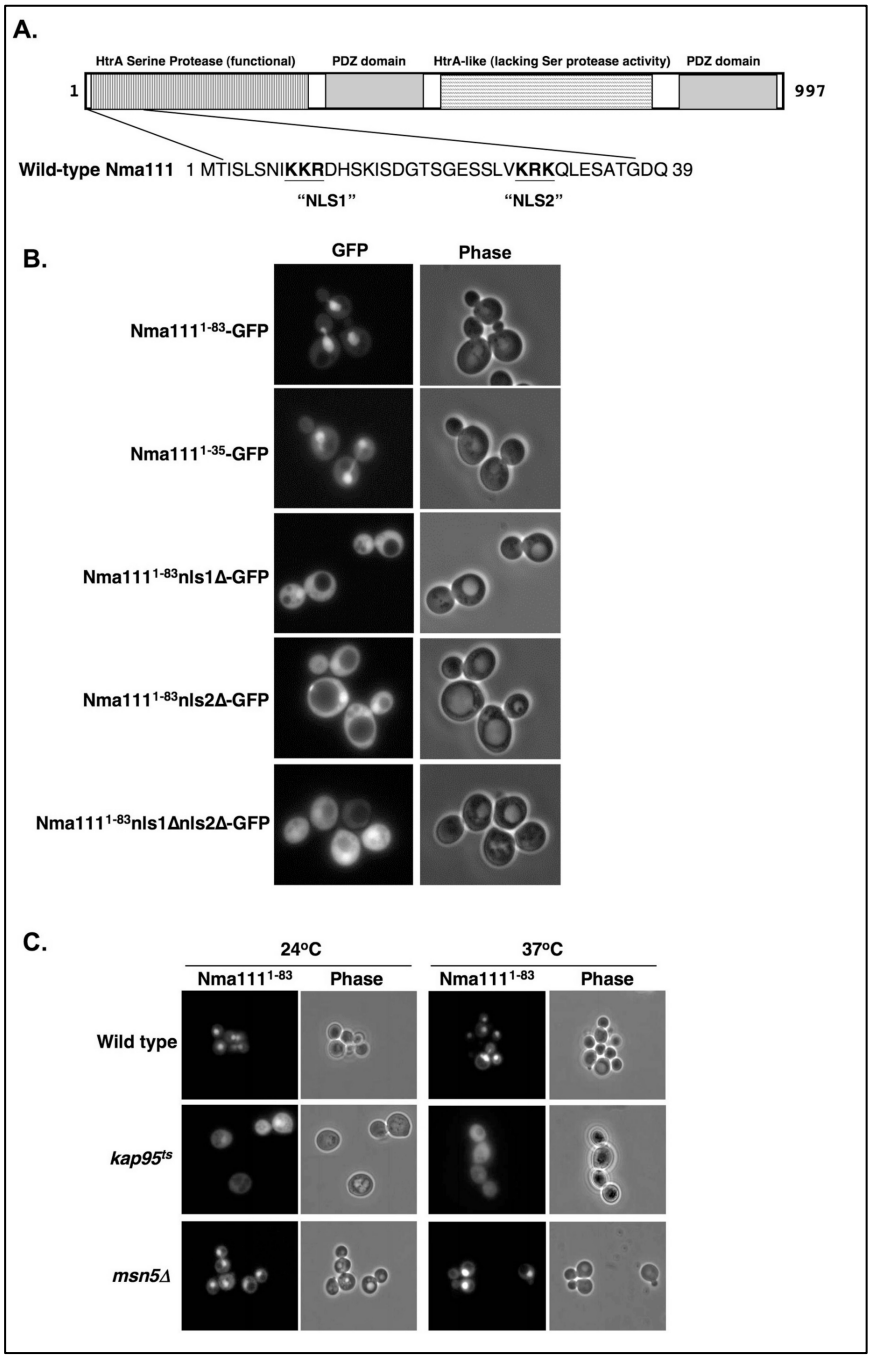

Figure 4.2: Nma111p contains a bipartite nuclear localization signal near its amino-terminus.

(**A**) Cartoon diagram of Nma111p depicting the predicted HtrA-like serine protease and C-terminal PDZ domains. The amino-terminal 39 amino acids are magnified, with the basic residues of the predicted bipartite NLS in bold and underlined. (**B**) The N-terminal 35 amino acids of Nma111p are sufficient for nuclear targeting. Cells expressing fusions of the N-terminus of Nma111p with GFP were observed by direct fluorescence (GFP) and phase contrast (phase) light

microscopy. The N-terminal 83 (Nma111$^{1-83}$, top row) or 35 (Nma111$^{1-35}$, second row) amino acids of Nma111p were fused in frame with GFP and expressed under control of the *NMA111* promoter. The Nma111$^{1-83}$-GFP construct was altered by site-directed mutagenesis so that either the upstream NLS ("NLS1"), downstream NLS ("NLS2"), or both NLSs were replaced with three alanine residues. Each mutagenised construct (Nma111$^{1-83}$*nls1*Δ-GFP, third row; Nma1-111$^{1-83}$*nls2*Δ-GFP, fourth row; Nma111$^{1-83}$*nls1*Δ*nls2*Δ-GFP, bottom row) was then expressed in yeast under control of the endogenous *NMA111* promoter. (**C**) Kap95p is essential for Nma111$^{1-83}$ nuclear import. A plasmid expressing full-length Nma111p fused to GFP was expressed in wild-type yeast and in yeast strains containing a temperature-sensitive *kap95* mutation (*kap95-3*) as well as a deletion of the karyopherin Kap142p/Msn5p (*msn5*Δ). Cells were grown to log phase at 24°C, shifted to 37°C for two hours, and Nma111$^{1-83}$-GFP localization was observed by direct fluorescence.

While these experiments suggest that the clusters of basic residues found in the amino-terminal 30 amino acids of Nma111p function as a bipartite NLS, we sought to determine if these sequences were necessary for import of the full-length Nma111p protein. To this end, we constructed a chimeric GFP fusion containing the entire 997 amino acids of the Nma111p protein. Expression of this protein in wild-type cells resulted in entirely nuclear fluorescence, with essentially no detectable Nma111-GFP visible in the cytoplasm (Figure 4.3A, top row). We then generated mutants with the three K and R residues in either *nls1* or *nls2* replaced with three alanines. Replacement of either basic cluster with three alanine residues results in a redistribution of the Nma111-GFP protein so that strong cytosolic fluorescence is observed with no detectable GFP accumulation within the nucleus (Figure 4.3A, second and third row). Removal of both basic clusters also results in exclusively cytosolic fluorescence (Figure 4.3A, bottom row). Thus, in the context of the full-length Nma111 protein, the loss of either basic cluster from the bipartite NLS results in a loss of nuclear Nma111p accumulation suggesting that both *nls1* and *nls2* are necessary for Nma111p nuclear import.

We also examined the localisation of full-length Nma111p in yeast lacking functional Kap95p and Msn5p. Wild type, *msn5Δ*, and *kap95-3* cells were transformed with a plasmid expressing Nma111-GFP from its endogenous promoter. Cells expressing Nma111-GFP were grown at 24°C and shifted to 37°C for up to 5 h, then observed for intracellular fluorescence (Figure 4.3B). As observed for the Nma111$^{1-83}$ fragment, full-length Nma111-GFP is predominantly nuclear in wild type cells and in cells lacking Msn5p at both 24°C and 37°C. A similar nuclear localisation is observed at both the permissive and restrictive temperatures in cells containing a *kap95-3* allele, indicating

that nuclear import and accumulation of full-length Nma111p is not solely dependent on Kap95p.

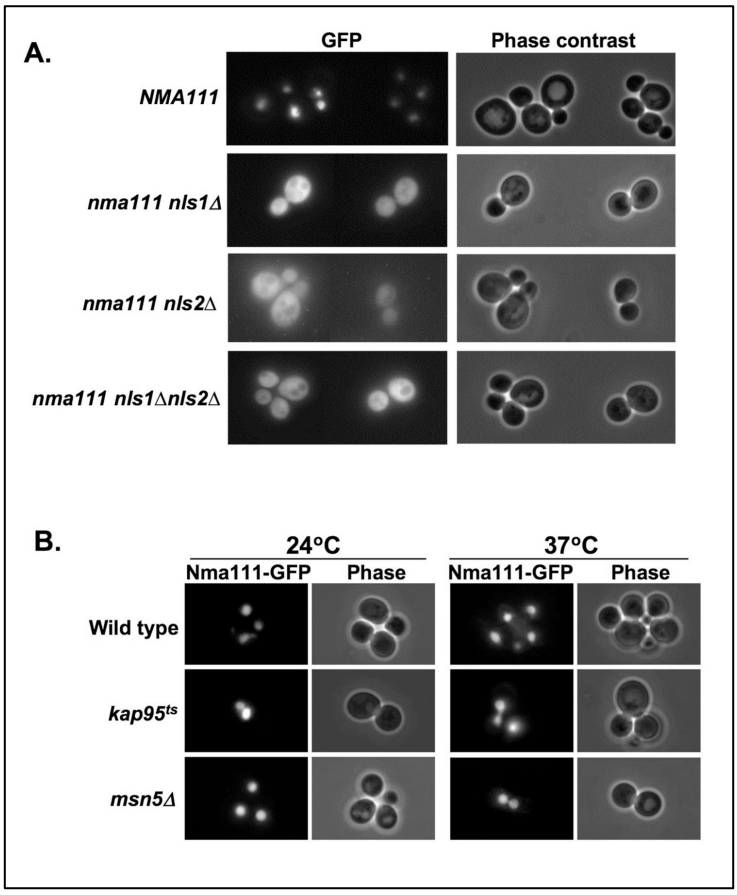

Figure 4.3: Both NLS sequences are necessary for efficient targeting of Nma111p to the nucleus.
(**A**) The entire coding region of *NMA111* was fused in-frame with GFP under control of the *NMA111* promoter and observed in yeast by direct fluorescence (top row). Nma111-GFP fusions in which *nls1*, *nls2*, or both *nls1* and *nls2* were replaced by alanines were also expressed and observed in wild-type cells. (**B**) Full length Nma111-GFP was expressed in wild-type, *kap95*^{ts}, and *msn5*Δ cells, grown to log phase at 24°C, and shifted to 37°C for 3 h. Cells expressing GFP fusion proteins were observed by direct fluorescence (GFP) and phase contrast (phase) microscopy.

4.3.4 NLS mutants of Nma111p lack pro-apoptotic activity

Previously we have shown that Nma111p is able to promote apoptosis and that this pro-apoptotic activity depends on its serine protease activity (Fahrenkrog et al., 2004). To

determine whether nuclear localisation of Nma111p is critical to trigger cell death, we next mutated the NLS1, NLS2 or both NLSs in the plasmid pNOPPATA1L-NMA111 (Fahrenkrog et al., 2004), respectively, as described for the GFP-fusion proteins and transformed the resulting plasmids into Δ*nma111* cells. First, we determined the subcellular localisation of the wild type and the resulting mutant ProtA-Nma111p fusion proteins by indirect immunofluorescence microscopy. ProtA-Nma111p is a nuclear protein in wild type and *kap95-3* cells, whereas the replacement of either or both NLSs with three alanine residues results in a redistribution of the ProtA-Nma111p protein with strong cytosolic fluorescence and no detectable accumulation of the fusion proteins within the nucleus (Figure 4.4).

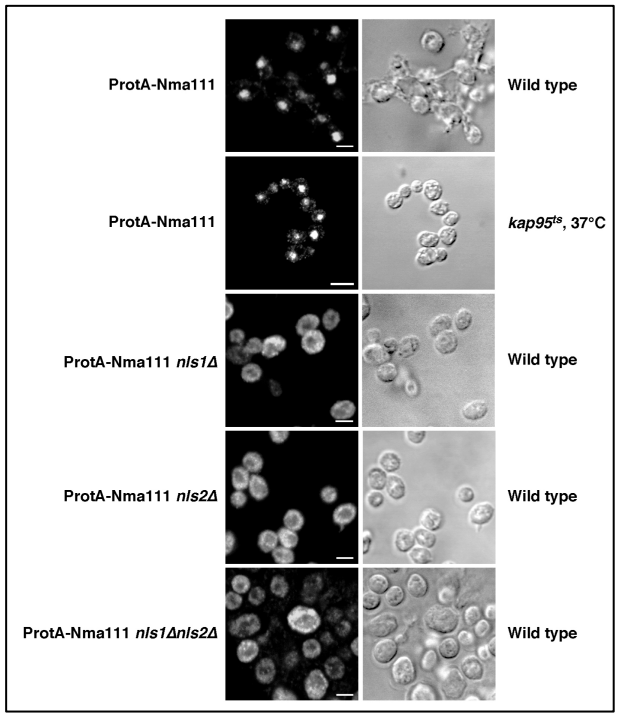

Figure 4.4: Localisation of ProtA-Nma111p as detected by immunofluorescence microscopy. Indirect immunofluorescence localisation of ProtA-Nma111p wild-type cells and *kap95-3* mutants as well as ProtA-Nma111p in which NLS1, NLS2 or both NLS1 and NLS2 were replaced by alanines observed in wild-type cells. Cells were stained with a primary rabbit anti-protein-A antibody and a secondary anti-rabbit IgG antibody labelled with Alexa Fluor 488. Shown are confocal fluorescence micrographs and differential interference contrast images. Scale bars: 5 μm.

Next, ProtA-Nma111p, ProtA-Nma111*nls1Δ*, ProtA-Nma111*nls2Δ* and ProtA-Nma111*nls1Δnls2Δ* cells were incubated with 0.4 mM H_2O_2 for 4 hours and analyzed for apoptotic hallmarks. Apoptotic features include chromatin condensation and fragmentation, single stranded DNA-breaks and accumulation of reactive oxygen species (ROS). As shown in Figure 4.5A, ProtA-Nma111p cells showed accumulation of ROS as indicated by dihydroxyethidium (DHE) staining after treatment with H_2O_2. DHE reacts with ROS and forms red fluorescent ethidium (Sharikabad et al., 2001). ProtA-Nma111*nls1Δ*, ProtA-Nma111*nls2Δ* and ProtA-Nma111*nls1Δnls2Δ* cells in contrast showed less DHE staining as compared to ProtA-Nma111p cells. Quantification of DHE staining revealed that about 11 % of ProtA-Nma111p cells were ROS positive, but only 3-4% of the NLS-mutant cells (Figure 4.5B).

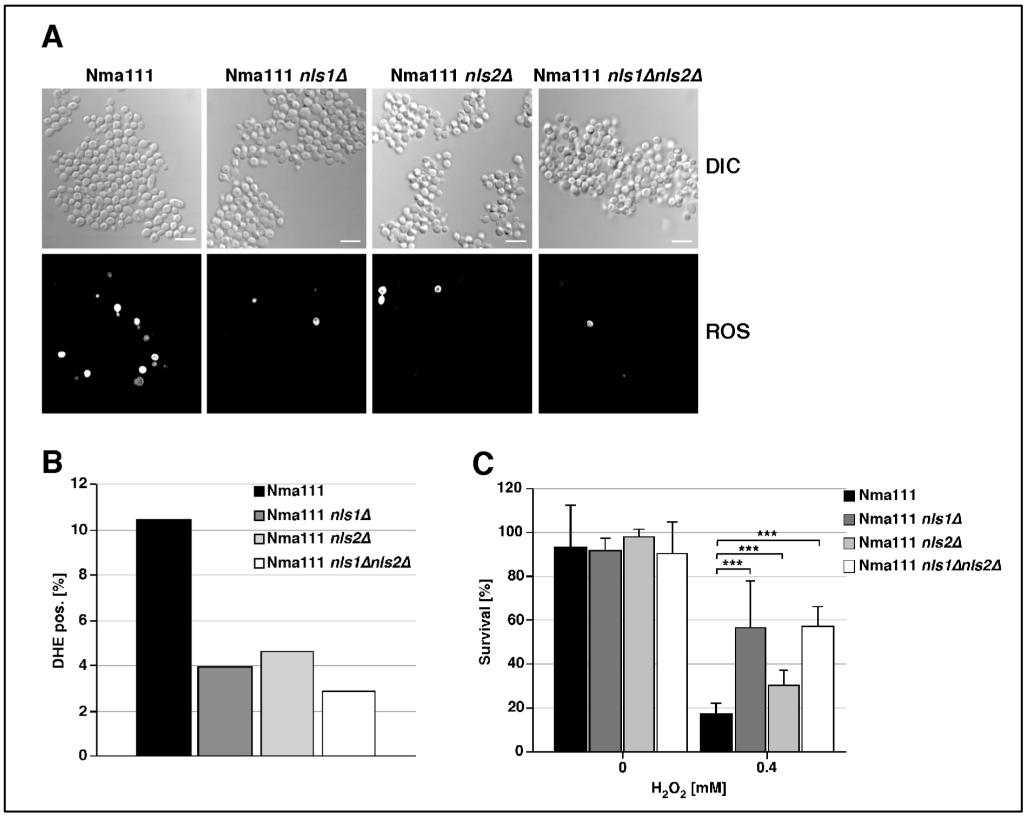

Figure 4.5: Mutations in the NLS sequences of Nma111p protect against apoptosis.
(**A**) Cells expressing protein A-tagged Nma111p, Nma111p *nls1Δ*, Nma111p *nls2Δ* and Nma111p *nls1Δnls2Δ,* respectively, were grown in selective medium, treated with 0.4 mM H_2O_2 and analyzed for apoptotic hallmarks. Reactive oxygen species (ROS) were detected by DHE staining. Shown are confocal micrographs and differential-interference contrast (DIC) images. Bars, 5 μm.

(**B**) Quantification of ROS accumulation using DHE staining after treatment with 0.4 mM H_2O_2. 500-1000 cells were counted. (**C**) Survival determined by clonogenicity of yeast cells expressing ProtA-Nma111p compared with NLS mutants without pretreatment or with incubation in 0.4 mM H_2O_2 for 4 hours. Bars present mean ±s.d.

Single-strand DNA breaks 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 chromosome fragmentation, by attaching labelled nucleotides with terminal deoxynucleotidyl transferase. Consistent with the ROS staining, ProtA-Nma111p cells were TUNEL positive, ProtA-Nma111*nls2Δ* cells were partially positive, whereas ProtA-Nma111*nls1Δ* and ProtA-Nma111*nls1Δnls2Δ* cells were all TUNEL-negative (Figure 4.6A, top two rows). Furthermore, AnnexinV/propidium iodide (PI) costaining was used to discriminate between early apoptotic (Annexin V positive, PI negative), late apoptotic state (Annexin V positive, PI positive), and necrotic (annexin V negative, PI positive) cell death. This AnnexinV/PI costaining revealed that ProtA-Nma111p cells mainly undergo apoptosis, whereas ProtA-Nma111*nls1Δ* and ProtA-Nma111*nls1Δnls2Δ* cells were neither apoptotic nor necrotic (Figure 4.6A, bottom two rows).

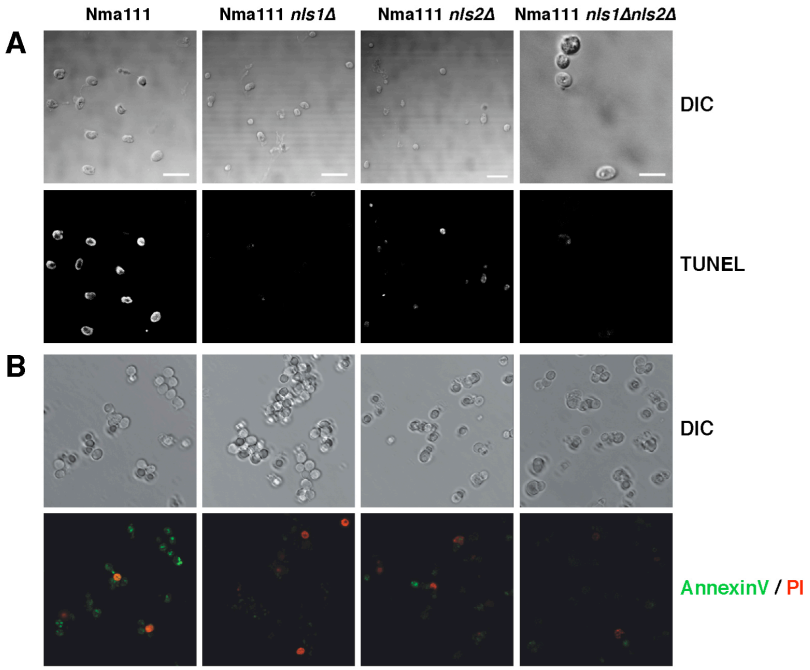

Figure 4.6: Mutations in the NLS sequences of Nma111p causes a lack of apoptotic hallmarks.
Cells expressing protein-A-tagged Nma111p, Nma111p-NLS1Δ, Nma111p-NLS2Δ and Nma111p-NLS1ΔNLS2Δ, respectively, were grown in selective medium, treated with 0.4 mM H_2O_2 and analyzed for apoptotic hallmarks. **(A)** Single-strand DNA breaks were detected by the TUNEL test and **(B)** phosphatidylserine externalisation by annexin-V/PI costaining. Shown are confocal micrographs and differential-interference contrast (DIC) images. Scale bars: 5 μm; 2.5 μm TUNEL Nma111p-NLS1ΔNLS2Δ.

Cell survival of ProtA-Nma111p cells was further tested in a clonogenicity assay (Buttner et al., 2007). Treatment with 0.4 mM H_2O_2 for 4 hours resulted in the death of yeast cells expressing ProtA-Nma111p (survival rate of less than 20%), whereas ProtA-Nma111*nls1Δ* and ProtA-Nma111*nls1Δnls2Δ* cells were largely unaffected (survival rates ~60 %; Figure 4.5C). Interestingly, ProtA-Nma111*nls2Δ* cells were more sensitive to H_2O_2 as compared to the other NLS-mutant cells with about 30% of surviving cells (Figure 4.5C), consistent with the stronger nuclear accumulation of the mutant proteins (Figure 4.2B). In the absence of H_2O_2, all ProtA-Nma111p show similar survival rates between 80-90% (Figure 4.5C). Together, these data indicate that the nuclear localisation of Nma111p is required for its function in response to oxidative stress.

4.3.5 Lack of nuclear localisation of Nma111p causes late onset of cell death during chronological ageing

Chronologically aged yeast cells show features of apoptotic death and are considered to have undergone physiologically induced apoptosis (Fabrizio and Longo, 2008). We therefore investigated whether Nma111p's nuclear localisation is relevant for cell death during chronological ageing. We observed that disruption of either NLS1 or NLS2 of Nma111p does not significantly delay the onset of cell death in chronologically ageing cells after 14 days in culture, whereas, by contrast, combined disruption of the two NLSs significantly increased survival of the cells (Figure 4.7A). The survival rates were reproduced in nine to twelve independent experiments. When these yeast cells were tested for apoptotic markers after 7 days in culture Nma111p and Nma111*nls2Δ* cells showed typical hallmarks of apoptosis, such as the production of ROS (as detected by DHE-staining) or DNA condensation (data not shown), whereas Nma111*nls1Δ* and Nma111*nls1Δnls2Δ* cells lack apoptotic markers (Figure 4.7B and C). Therefore, nuclear localisation of Nma111p is also fundamental for cell death during chronological ageing.

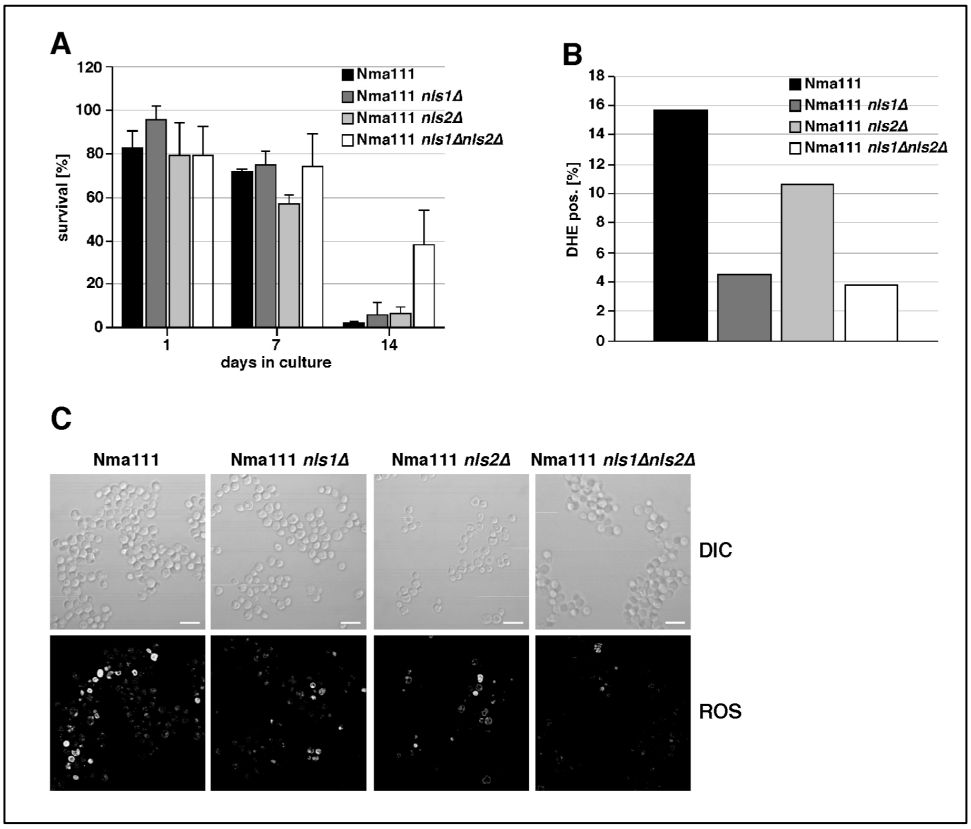

Figure 4.7: Chronological ageing of wild-type ProtA-Nma111p and NLS mutant cells.
(A) Survival rates. Error bars, mean ±s.d. (B) Quantification of ROS production, (C) ROS detection (DHE) and visualisation of DNA (DAPI) in Nma111p and Nma111p NLS mutants after 5 days of cultivation. Bars, 5 μm (ROS), 2.5 μM (DNA).

4.3.6 The N-terminal HtrA-repeat of Nma111p is required for apoptosis induction

Nma111p belongs to the HtrA-family of serine proteases and consists of two tandem HtrA-repeats, each completed with two PDZ domains (Clausen et al., 2002; Pallen and Ponting, 1997; Ponting, 1997). While the N-terminal HtrA-repeat harbours a complete catalytic triade characteristic for serine proteases, the second repeat is lacking two of the three active site residues (Pallen and Ponting, 1997). To test whether the N-terminal HtrA-repeat is required and sufficient to promote cell death, we generated two truncations of Nma111p that respectively expressed the N-terminal and the C-terminal HtrA-repeat fused to protein A. The resulting plasmids pNOPPATA1L-Nma111-N (residues 2-449) and pNOPPATA1L-Nma111-C (residues 450-997) were transformed into *Δnma111* cells

and indirect immunofluorescence microscopy revealed that ProtA-Nma111p-N was predominantly nuclear with some cytosolic staining, while ProtA-Nma111p-C showed no nuclear accumulation (Figure 4.8). Next, ProtA-Nma111-N and ProtA-Nma111-C cells were incubated with 0.4 mM H_2O_2 for 4 hours and analyzed for apoptotic markers. ProtA-Nma111-N cells produced ROS as determined by DHE staining (Figure 4.9A), showed single-strand DNA breaks as detected by TUNEL staining (Figure 4.10A) and were AnnexinV (Figure 4.10B), whereas ProtA-Nma111-C cells showed no apoptosis marks. Quantification of DHE staining revealed that about 9.5% of ProtA-Nma111-N cells were ROS positive, but only 2.5% of the ProtA-Nma111-C cells (Figure 4.9B).

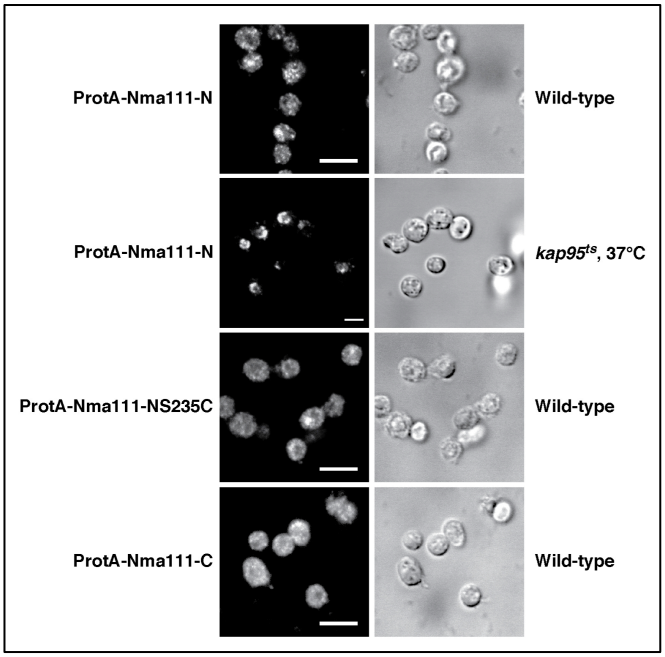

Figure 4.8: Localisation of ProtA-Nma111p variants as detected by immunofluorescence microscopy.
Indirect immunofluorescence localisation of ProtA–Nma111-N in wild-type cells and *kap95-3* mutants, as well as ProtA–Nma111-N(S235C) and ProtA–Nma111-C in wild-type cells. Cells were stained with a primary rabbit anti-protein-A antibody and a secondary anti-rabbit IgG antibody labelled with Alexa Fluor 488. Shown are confocal fluorescence micrographs and differential interference contrast images. Scale bars: 5 μm.

Next, ProtA-Nma111-N and ProtA-Nma111-C cells were tested for cell survival after H_2O_2 treatment. While about 20% (19.6% ± 6.9 %) of the ProtA-Nma111-N cells

survived, ~36% (35.6% ± 14.4%) of ProtA-Nma111-C cells were resistant to hydrogen peroxide (Figure 4.9C).

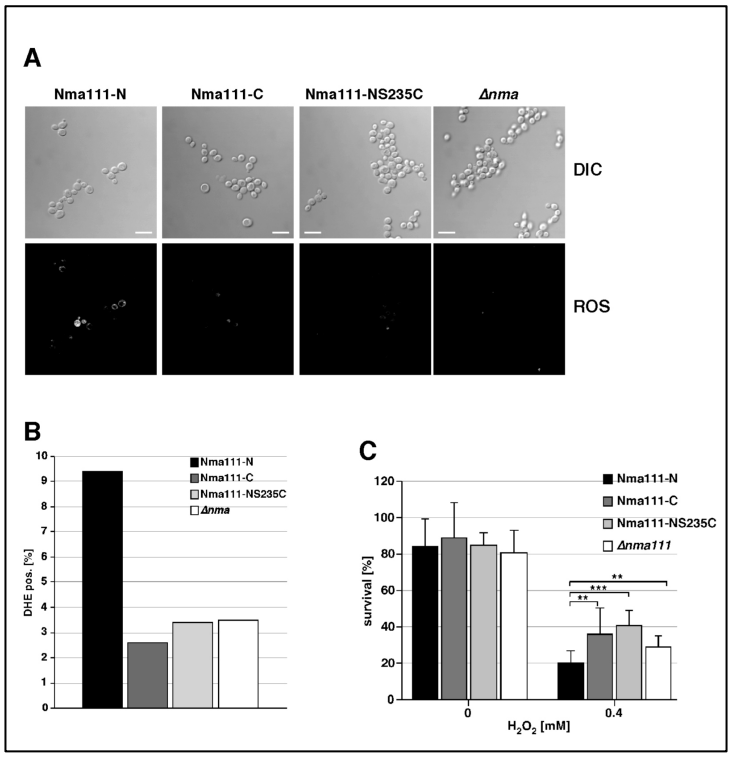

Figure 4.9: The N-terminal HtrA-repeat of Nma111p is mediating its pro-apoptotic activity. **(A)** ProtA-Nma111p-N, ProtA-Nma111p-C, ProtA-Nma111p-NS235C and *Δnma111* cells, respectively, were grown in selective medium, treated with 0.4 mM H_2O_2 and analyzed for apoptotic hallmarks. Reactive oxygen species (ROS) were detected by DHE staining and single strand DNA breaks by the TUNEL test. Shown are confocal micrographs and differential-interference contrast (DIC) images. Bars, 5 μm. **(B)** Quantification of ROS accumulation using DHE staining after treatment with 0.4 mM H_2O_2. 500-1000 cells were counted. **(C)** Survival determined by clonogenicity of yeast cells expressing ProtA-Nma111p HtrA-truncations compared with *nma111* mutants without pre-treatment or with incubation in 0.4 mM H_2O_2 for 4 hours. Data present mean ±s.d.

We have previously shown that serine235 is required for the death promoting activity of Nma111p (Fahrenkrog et al. 2004). To confirm that S235 is necessary for Nma111p activity and not simply localisation, we mutated this serine residue to a cysteine by oligonucleotide site directed mutagenesis in the plasmid pNOPPATA1L-NMA111-N. The resulting pNOPPATA1L-NMA111-NS235C was transformed into

Δnma111 cells and the subcellular localisation of the protein was found to be predominantly nuclear based on indirect immunofluorescence microscopy (Figure 4.8). ProtA-Nma111-NS235C cells that were treated with 0.4 mM H_2O_2 for 4 hours showed no production of ROS (Figure 4.9A) and were TUNEL and AnnexinV negative (Figure 4.10), similar to *Δnma111* cells. Quantification of DHE staining revealed ~3.5% of the ProtA-Nma111-NS235C and the *nma111* disrupted cells to be ROS positive (Figure 4.9B). In consistency with these results the clonogenicity assay revealed survival rates of about 40% (40.3 % ± 8.6 %) for ProtA-Nma111-NS235C cells and ~30% (28.7 % ± 6.0 %) for *Δnma111* cells (Figure 4.9C).

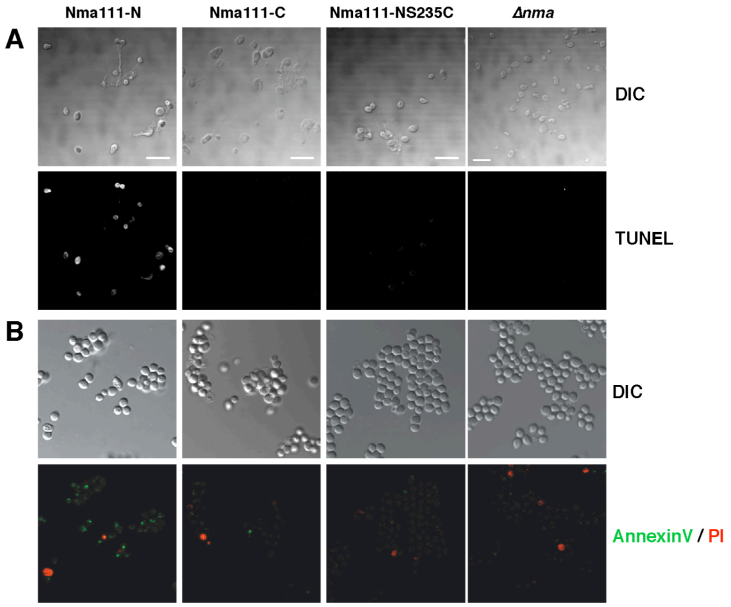

Figure 4.10: Expression of the N-terminal HtrA repeat of Nma111p causes apoptotic hallmarks.
ProtA–Nma111p-N, ProtA–Nma111p-C, ProtA–Nma111p-N(S235C) and Δ *nma111* cells, respectively, were grown in selective medium, treated with 0.4 mM H_2O_2 and analyzed for apoptotic hallmarks. Reactive oxygen species (ROS) were detected by DHE staining and single-strand DNA breaks were detected by the (A) TUNEL test and (B) phosphatidylserine externalisation by annexin-V/PI costaining. Shown are confocal micrographs and differential-interference contrast (DIC) images. Scale bars: 5 μm.

To elucidate the role of the Nma111p's two HtrA-repeats under more natural conditions, we performed chronological ageing assays over 14 days and found that

expressing ProtA-Nma111-C or ProtA-Nma111-NS235C, and to *Δnma111* cells (Figure 4.11A). When these yeast cells were tested for ROS accumulation after 7 days in culture 18% of ProtA-Nma111-N cells showed the production of ROS whereas only 5% of ProtA-Nma111-C cells, 4% of ProtA-Nma111-NS235C and 3% of *Δnma111* showed ROS accumulation (Figure 4.11B and C).

Taken together, the N-terminal HtrA-repeat of Nma111p is sufficient to promote apoptosis and its death promoting activity is reduced by a mutation of serine235 to cysteine.

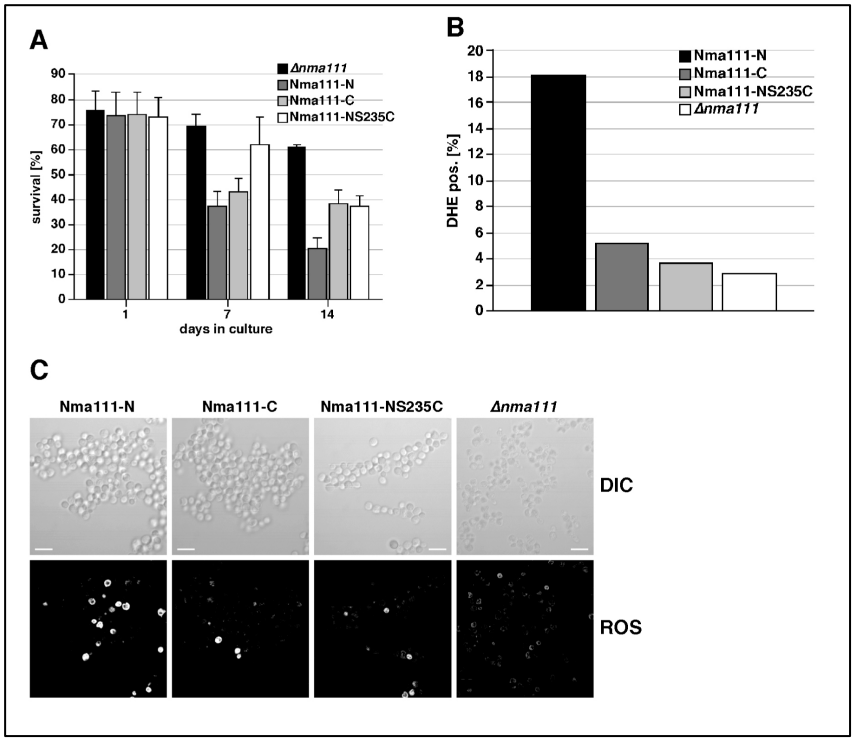

Figure 4.11: Chronological ageing of ProtA-Nma111p-mutant cells.
(A) Survival rates. Error bars, mean ±s.d. **(B)** Quantification of ROS production, **(C)** ROS detection (DHE) and visualisation of DNA (DAPI) after 5 days of cultivation. Bars, 5 μm (ROS), 2.5 μM (DNA).

4.4 Discussion

The HtrA-like serine protease Nma111p is a nuclear protein that is able to promote apoptosis in yeast in a serine protease-dependent manner. We show here that Nma111p harbours a classical bipartite NLS within the first 35 N-terminal amino acids and identified Kap95p as its nuclear import receptor. Also, we show that Nma111p is a non-shuttling protein that even under oxidative stress conditions remains nuclear. The pro-apoptotic activity of Nma111p requires its nuclear localisation, as mutations of any critical residue in the bipartite NLS reduce Nma111p's ability to mediate apoptosis. We show further that Nma111p's death-promoting activity is restricted to its N-terminal HtrA-repeat, which harbours the NLSs and the active catalytic site of the protein.

4.4.1 Nma111p is a nuclear protein that does not undergo nucleocytoplasmic shuttling

Nma111p is a nuclear protein under steady-state conditions in the absence and presence of hydrogen peroxide added to induce apoptosis. In contrast, a nuclear reporter protein (i.e. cNLS-GFP) is primarily nuclear in the absence of H_2O_2, but the cNLS-GFP becomes more cytosolic after exposure to the oxidising agent (Figure 4.1A). The release of the cNLS-GFP from the nucleus upon H_2O_2 stress is most likely simply due to increased size of the yeast NPC permeability barrier (Mason et al., 2005). Alternatively, Nma111p might be "anchored" inside the nucleus due to an interaction with a thus far unknown binding partner.

4.4.2 Kap95p is an importin for Nma111p

Nuclear localisation of a protein typically requires the presence of a nuclear localisation signal. Nma111p is an exclusively nuclear protein and we have mapped a bipartite basic NLS within the first 35 amino acids of the protein. Such basic NLS sequences are most often recognized by the Kap60p/Kap95p import receptor complex and consequently the expression of Nma111$^{1\text{-}83}$-GFP in a *kap95-3* mutant leads to cytoplasmic redistribution of the fusion protein (Figure 4.2C). Surprisingly, full-length Nma111p is still in the nucleus in this *kap95* mutant. Similarly, ProtA-Nma111p is nuclear, whereas ProtA-Nma111-N is

cytoplasmic in the *kap95-3* mutant (Figure 4.4 and Figure 4.6). These data suggest that Nma111p gets trapped inside the nucleus due to an interaction with a thus far unknown binding partner and that the binding to this partner protein is mediated by the C-terminal HtrA-repeat. This binding partner is unlikely to be Bir1p, since Bir1p interacts with the N-terminal HtrA-repeat of Nma111p (Walter et al., 2006). Alternatively, more than one Kap mediates the nuclear import of the full-length protein, as it known for histones or ribosomal proteins (Mosammaparast et al., 2002; Mosammaparast et al., 2001; Rout et al., 1997). Post-translational modifications can also affect protein localisation and, for example, mask nuclear localisation signals (Poon et al., 2005). The human homologue of Nma111p, Omi/HtrA2, is regulated by phosphorylation (Plun-Favreau et al., 2007) and Nma111p is phosphorylated at serine989 in the C-terminal HtrA-repeat (*Saccharomyces cerevisiae* database). It will be interesting to see if this phoshorylation site in fact is implicated in the regulation of Nma111p nuclear import/retention.

4.4.3 Nuclear localisation is critical for Nma111p function in yeast apoptosis

Our previous studies have revealed that Nma111p and its only known substrate, the inhibitor-of-apoptosis protein Bir1p, are both nuclear proteins (Fahrenkrog et al., 2004; Walter et al., 2006), which indicated that the nucleus appears to play a significant role in yeast apoptosis. This is further supported by our data presented here, which revealed that Nma111p is not shuttling between the nucleus and the cytoplasm under normal and oxidative stress conditions (Figure 4.1). Moreover, disruption of any of the two basic stretches that act as NLS for Nma111p affects its ability to promote cell death in response to oxidative stress and during chronological ageing (Figure 4.5, Figure 4.6 and Figure 4.7). Therefore, Nma111p function in yeast apoptosis appears not linked to mitochondria, which is in clear contrast to its metazoan homologue Omi/HtrA2. Omi/HtrA2 is predominantly localised to mitochondria and is released into the cytosol under apoptotic conditions, which, in turn, allows its interaction with and the degradation of the inhibitor-of-apoptosis protein XIAP due to which executioner caspases get activated (Challa et al., 2007; Hegde et al., 2002; Khan et al., 2008; Suzuki et al., 2001a; Suzuki et al., 2004). It still remains to be seen which proteins are the downstream targets of Nma111p that lead to the execution of apoptosis. Bir1p is one such target and degradation of Bir1p by

Nma111p induces apoptosis, but Bir1p does not directly inhibit the yeast caspase Yca1p (Walter et al., 2006). Therefore the bridging factor to the caspase or an unknown executioner of apoptosis in yeast remains to be elucidated, but it is most likely a nuclear protein.

4.4.4 Serine235 versus serine236 as active catalytic site

Nma111p consists of two tandem HtrA-repeats with the N-terminal HtrA-repeat harbouring a complete catalytic triade characteristic for serine proteases, while the second repeat is lacking two of the three active site residues (Pallen and Ponting, 1997). Consequently, we show here that the N-terminal HtrA-repeat of Nma111p is sufficient and required to induce apoptosis in response to oxidative stress and during chronological ageing (Figure 4.9, Figure 4.10 and Figure 4.11). The active catalytic serine of trypsin-like serine proteases is typically embedded in a sequence motif GNSGG as consensus (Clausen et al., 2002; Pallen and Ponting, 1997), which in Nma111p is ^{234}GSSGS^{238} accordingly. We have previously shown that a mutation in serine235 inhibits Nma111p's ability to promote cell death (Fahrenkrog et al., 2004), while others have recently shown that mutation of serine236 impairs its chaperone activity (Padmanabhan et al., 2009). Consistent with our previous data, we found here that cells that express ProtA-Nma111p-N with a cysteine mutation in serine235 are less sensitive to oxidative stress and ageing induced apoptosis than cells expressing a wild-type ProtA-Nma111p-N (Figure 4.9, Figure 4.10 and Figure 4.11). Therefore, serine235 is unambiguously important for the pro-apoptotic activity of Nma111p. The controversy between our data and Padmanabhan et al. (2009) most likely arises from the different strain background that has been used. While in our background, i.e.BMA41/BMA64/W303 (Fahrenkrog et al., 2004), and in BY4741 wild-type cells (Zuo et al., 2005) deletion of *nma111* causes no growth and morphological defects, it does in the YB332 background used by Padmanabhan et al. (Tong et al., 2006), indicating some strain specific features of the YB332 derivatives. Alternatively, serine235 and serine236 both are critical for the catalytic activity of Nma111p; serine235 primarily for its pro-apoptotic activity and serine236 primarily for its chaperon activity. Future studies are required to address this issue more systematically.

In summary, we have identified and characterized the nuclear localisation signal and nuclear import receptor of Nma111p. Moreover, Nma111p is a non-shuttling protein that remains in the nucleus under steady state as well as apoptotic conditions. This inability of Nma111p to exit the nucleus in essence excludes a role for Nma111p in the mitochondria-dependent apoptotic pathway in yeast. This is further supported by our data that indicate that Nma111p exerts its apoptotic activity in the nucleus and that nuclear localisation is critical for the Nma111p-dependent cell death. Therefore nuclear signalling cascades appear of utmost significance for the execution of apoptosis in yeast.

4.5 Materials and Methods

4.5.1 Yeast strains, media, and plasmids

Enzymes for molecular biology were purchased from New England Biolabs (Beverly, MA) and Sigma-Aldrich (St. Louis, MO) and were used as per manufacturer's instructions. Yeast transformations were performed as described (Woods and Gietz, 2001) as were genetic manipulations, yeast cell culture, and media preparation (Guthrie, 1991). Plasmids pGAL::CCA-GFP and pGAL::H2B-GFP were generous gifts from A. Hopper (Penn State, Hershey, PA). Yeast strains and plasmids used are indicated in Table 1.

Plasmids pKBB282 (Nma111$^{1-35}$-GFP) and pKBB280 (Nma111$^{1-83}$-GFP) were constructed by amplifying the DNA encoding the *NMA111* promoter and the first 35 and 83 amino acids of Nma111p, respectively, and inserting the resulting DNA into pLDB350 (*CEN URA3 GFP*) by homologous recombination. For pKBB282, *NMA111* DNA was amplified using primers KOL 155 (5'- GTA CCG GGC CCC CCC TCG AGG TCG ACG GTA TCG ATA AGC TTG ATA TCG AAT TCCT GCA GGC ATC AGC ATC AGC AAG ATC C-3') and KOL157 (5'- TCC AAC AAG AAT TGG GAC AAC TCC AGT GAA GAG TTC TTC TCC TTT GCT GGC GCT TTC CAA CTG TTT CCT TTT TAC AAG CG–3'). For pKBB280, amplification was performed using KOL155 and KOL156 (5'-ATC TAA TTC AAC AAG AAT TGG GAC AAC TCC AGT GAA GAG TTC TTC TCC TTT GCT CAC TGA TTT AAC AAC GTT GGA GAT GG-3'). pLDB350 was digested with *Eco*RI prior to co-transformation into yeast with the *NMA111* PCR products to stimulate homologous recombination. pKBB428 (Nma111$^{1-83}$*nls1Δ*-GFP) was generated by amplifying *NMA111* by PCR using KOL156 and mutagenic primer KOL207 (5'-CAG TAA AGG TTT TTT AGA TCT ACT AAT GAC CAT ATC GTT GAG CAA TAT AGC TGC TGC TGA CCA TTC TAA AAT TTC CG-3') and inserting the resulting product into pLDB352 as described above. pKBB430 (Nma111$^{1-83}$*nls2Δ*-GFP) was similarly constructed using KOL155 and KOL209 (5'-TCA TGG TCT GTA TAT TCT TCC TCT TGA TCT CCG GTG GCG CTT TCC AAC TGA GCA GCA GCT ACA AGC GAT GAT TCA CCA G-3'). pKBB394 (Nma111$^{1-83}$*nls1Δnls2Δ*-GFP) was generated using KOL209 and KOL207 as primers for

amplification. pKBB460 (Nma111-GFP) was constructed by PCR amplifying the entire *NMA111* gene sequence, including 500 nucleotides of the upstream promoter, using oligonucleotides KOL155 and KOL278 (5'-CAT CAC CAT CTA ATT CAA CAA GAA TTG GGA CAA CTC CAG TGA AGA GTT CTT CTC CTT TGC TAG CTT TTT CAC TTT GGC TGT TGC C-3') and integrating the resulting DNA into pLDB351 using homologous recombination as described above. pKBB465 (Nma111*nls1Δ*-GFP) was generated by site-directed mutagenesis of pKBB460 using U.S.E. mutagenesis (Pharmacia, New York) and mutagenic oligonucleotide KOL288 (5'-CCA TAT CGT TGA GCA ATA TAG CGG CAG CAG ACC ATT CTA AAA TTT CCG ATG G-3') as per manufacturer's instructions. pKBB467 (Nma111*nls2Δ*-GFP) was similarly generated using KOL289 (5'-GGT GAA TCA TCG CTT GTA GCA GCG GCA CAG TTG GAA AGC GCC ACC GG-3') and pKBB466. (Nma111*nls1Δnls2Δ*-GFP) was made by site-directed mutagenesis using both KOL288 and KOL289. All plasmids were rescued from yeast using glass bead lysis (Hoffman and Winston, 1987) and transformed into *E. coli*. pKBB439 (2μ *URA3 GAL::NMA111-GFP*) was constructed by PCR amplifying the entire coding region of *NMA111* using oligonucleotides KOL277 (5'-CAA CAA AAA ATT GTT AAT ATA CC TCT ATA CTT TAA CGT CAA GGA GAA AAA ACT ATA ATG ACC ATA TCG TTG AGC-3') and KOL278 and inserting the resulting DNA into pLDB352 by homologous recombination. Expression of Nma111-GFP in media containing 2% galactose and the lack of expression in the presence of 2% dextrose was confirmed by Western blotting using anti-GFP antibodies (Roche Pharmaceuticals, Basel). The complete nucleotide sequence of all *NMA111* fusions was confirmed using dideoxy nucleotide sequencing on an AbiPrism 310 Genetic Analyser (Applied Biosystems, Foster City, CA).

4.5.2 Shuttling Assay

Nucleocytoplasmic shuttling of Nma111p was performed by the method of Feng and Hopper (2002) with the following modifications. Briefly, pKBB439 (2*μ* URA3 GAL::Nma111-GFP), pGAL::Cca1-GFP, and pGAL::H2B-GFP were transformed into BY4741. Transformants were grown overnight in SD –Ura + 2% raffinose to A_{600} of 0.05

– 0.2, then supplemented with galactose to 2% and grown for 2.5 h at 30°C. Cells were harvested by centrifugation and resuspended in SD –Ura containing 2% glucose and incubated for 2 h at 30°C. Cells were then mixed with an equivalent number of MS739 (*kar1-1*) cells, centrifuged, and resuspended in 50 ml SD –Ura. A slurry of cells was spotted on a YPD plate and incubated at 30°C. Samples collected 3 h and 8 h after mating were fixed in 70% EtOH at 4°C and DAPI stained for DNA visualisation.

4.5.3 Direct fluorescence microscopy

To examine Nma111p protein localisation, plasmids pKBB282 (Nma111$^{1-35}$-GFP), pKBB280 (Nma111$^{1-83}$-GFP), pKBB428 (Nma111$^{1-83}$*nls1Δ*-GFP), pKBB430 (Nma111$^{1-83}$*nls2Δ*-GFP), pKBB394 (Nma111$^{1-83}$*nls1Δnls2Δ*-GFP), pKBB460 (Nma111-GFP), pKBB465 (Nma111*nls1Δ*-GFP), pKBB467 (Nma111*nls2Δ*-GFP), and pKBB466 (Nma111*nls1Δnls2Δ*-GFP) were transformed into strains W303 and BY4741, grown in SD –Ura to A_{600} 0.1 – 0.4, and observed by direct fluorescence microscopy using a Nikon E600 epifluorescence microscope. Localisation in karyopherin mutants was performed by transforming plasmids into PSY1102 (*kap95^{ts}*), EY10609 (*msn5Δ*), and LDY1008 (*crm1^{ts}*). Cells were grown overnight at 25ºC in SD –Ura to early log phase, then shifted to 37°C for 2 – 4 h and observed by direct immunofluorescence microscopy. Images were captured using SPOT cameras and software (Diagnostic Instruments, Inc., Sterling Heights, MI) and final images were produced in Adobe Photoshop CS (Adobe Systems Inc., San Jose CA).

4.5.4 Indirect immunofluorescence microscopy

Indirect immunofluorescence microscopy was performed as described (Fahrenkrog et al., 2004). Primary antibodies were anti-protein A (Sigma, St. Louis, MO) diluted 1:1000. Secondary antibodies: Alexa 488-labelled anti-rabbit-IgG antibody (Molecular Probes, Eugene, OR) diluted 1:1000. Images were recorded using a confocal laser scanning microscope (Leica TCS NT/SP1, Leica, Vienna, Austria) and were analyzed using NIH Image and Adobe Photoshop CS (Adobe Systems Inc., San Jose CA).

4.5.5 Test for apoptotic markers and chronological ageing

For dihydroethidium staining, $1x10^7$ cells were harvested by centrifugation, resuspended in 1 ml of 2.5 μg/ml DHE in PBS and incubated for 15 min in the dark. Cells were washed with 1 ml PBS and analyzed by fluorescence microscopy. DNA was stained using Mowiol containing 1μglml DAPI as mounting medium. TUNEL assay and survival platings/clonogenicity assays were performed as described (Fahrenkrog et al., 2004; Walter et al., 2006). Chronological ageing assays were performed as described elsewhere (Walter et al., 2006).

4.5.6 Annexin V staining

Exposed phosphatidylserine was detected by reaction with FITC-coupled annexin V (Annexin V-FITC Apoptosis detection Kit I, BD Boscience). Yeast cells were washed in sorbitol buffer (1.2 M sorbitol, 0.1mM KPP pH7.4) and digested with zymoylase in sorbitol buffer for 30 min at 30°C. Next cells were washed in incubation buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, 0.6 mM sorbitol), resuspended in 30 μl incubation buffer containing 6 μl propidium iodide (50 μg/ml) and 3 μl Annexin V-FITC and incubated for 20 min at room temperature. The cells were harvested, resuspended in sorbitol buffer, applied to a microscopic slide and imaged by confocal microscopy.

4.6 Acknowledgements

The authors would like to acknowledge Anita Hopper, Lucy Pemberton, Mark Rose, and Pam Silver for generously sharing plasmids and yeast strains. This research was supported by Colgate University and NSF-FIBR grant EF-04245749 to K.D.B., a Swiss National Science Foundation research grant to B.F. as well as by the Kanton Basel Stadt and the M.E. Mueller Foundation.

5

General Discussion

5.1 A histone Crosstalk regulates budding yeast life and death

In this thesis, I have identified and characterized distinct interrelated histone modifications on histone H2B and histone H3 as regulators of apoptosis in yeast (Figure 5.1). Histone H2B K123 ubiquitination is a prerequisite for the methylation of histone H3 at lysine K4 and K79 by the two methyltransferases Set1p and Dot1p, respectively (Briggs et al., 2002; Sun and Allis, 2002). Histone H2B K123 ubiquitination and its downstream effects on histone H3 methylation is involved in a variety of cellular processes including transcriptional regulation and DNA damage response (DDR) (Briggs et al., 2001b; Dover et al., 2002; Henry et al., 2003; Kao et al., 2004; Mutiu et al., 2007; Sun and Allis, 2002; Wyce et al., 2007; Xiao et al., 2005). We found that disruption of *DOT1*, which causes a lack of histone H3 K79 methylation confers apoptosis resistance and that *DOT1* disrupted cells fail to undergo Yca1p-dependent apoptosis (Figure 5.1 and Figure 3.2). In contrast, disruption of the E3 ligase *BRE1* or the methyltransferase *SET1*, which leads to a lack of H2B K123 ubiquitination and H3 K4 methylation, respectively, causes metacaspase Yca1p dependent apoptosis (Figure 5.1, Figure 2.5 and Figure 3.1). Moreover, we found that Dot1p is required for Yca1p-dependent cell death of *Δset1* cells (Figure 5.1 and Figure 3.3). Together, this study highlights the requirement of Dot1p mediated histone H3 K79 methylation for Yca1p-dependent cell death and points to a novel role of the conserved histone H2B/H3 crosstalk in apoptosis regulation.

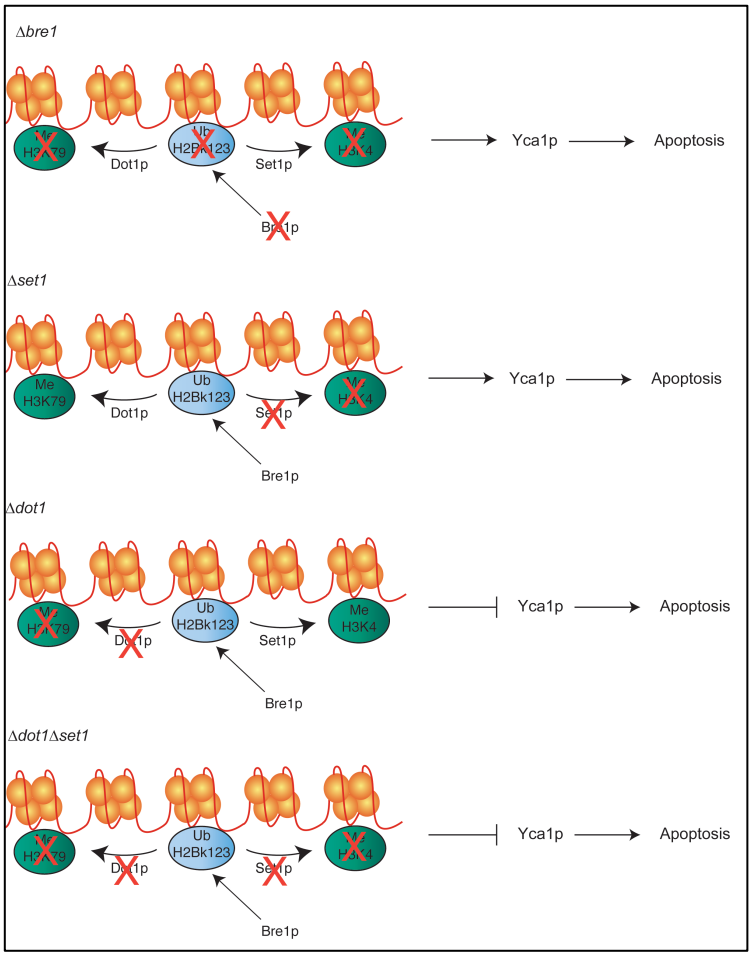

Figure 5.1: A histone Crosstalk regulates budding yeast life and death.
Lack of distinct interrelated histone modifications confers either apoptosis sensitivity or resistance. Disruption of *BRE1* as well as *SET1* causes Yca1p-dependent cell death whereas *DOT1* disruption confers apoptosis resistance. *Δset1Δdot1* cells fail to undergo Yca1p-dependent cell death similar to *Δdot1* cells.

5.1.1 A role of histone H2B ubiquitination in apoptosis regulation

We have uncovered a link between histone H2B ubiquitination and metacaspase Yca1p dependent apoptosis in yeast (Chapter 2, Walter et al., manuscript in revision for Journal of Cell Science). We found that enhanced levels of Bre1p, the E3 ligase that ubiquitinates H2B K123, protect from apoptosis (Figure 2.1). In contrast, cells lacking *BRE1* are prone to metacaspase Yca1p dependent apoptosis (Figure 2.5). Consistent with the idea that Bre1p acts through H2B ubiquitination, cells containing a histone H2B variant with a lysine-to-arginine substitution at amino acid 123 and therefore fail to be ubiquitinated at

H2B, mimic the apoptotic phenotypes similar to a *BRE1* deletion strain (Figure 2.4, Walter et al., manuscript in revision for Journal of Cell Science).

Bettiga and colleagues first uncovered a potential link between histone H2B ubiquitination and apoptosis. They found that yeast cells that exhibit enhanced levels of ubiquitinated histone H2B due 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). These data indicate that high levels of histone H2B ubiquitination can predispose yeast to apoptotic stimuli similar to the lack of histone H2B ubiquitination. However, this study did not address whether or not *Δubp10* cells show increased caspase activity is due to high ubiquitination levels of histone H2B. It is therefore possible that Ubp10p has other targets than ubiquitinated H2B and failure in deubiquitination of these targets may cause apoptosis sensitivity in these cells. To rule out this possibility, we analyzed the apoptosis sensitivity of *Δbre1Δubp10*. We expected that this double mutant would exhibit increased apoptosis sensitivity as compared to *Δbre1* cells, if Ubp10p would act in a histone H2B-independent manner. However, *Δbre1* and *Δbre1Δubp10* cells showed similar apoptosis sensitivity during chronological ageing. After two days in culture, *BRE1* lacking cells showed survival rates of 23% ± 4% as compared to 24% ± 2% of *Δbre1Δubp10* cells (Figure 5.2A). When, after two days in culture, these yeast cells were tested for ROS accumulation, which is causally linked to apoptosis in yeast, 73% of *Δbre1* and 68% of *Δbre1Δubp10* cells accumulated ROS as determined by DHE staining (Figure 5.2B). These data suggest that *UBP10* disruption causes apoptosis sensitivity due to failures in histone H2B deubiquitination. Therefore, the lack of histone H2B ubiquitination as well as high levels of histone H2B ubiquitination appear to predisposes yeast to apoptotic stimuli, indicating that H2B monoubiquitination needs to be tightly regulated to assure cell survival.

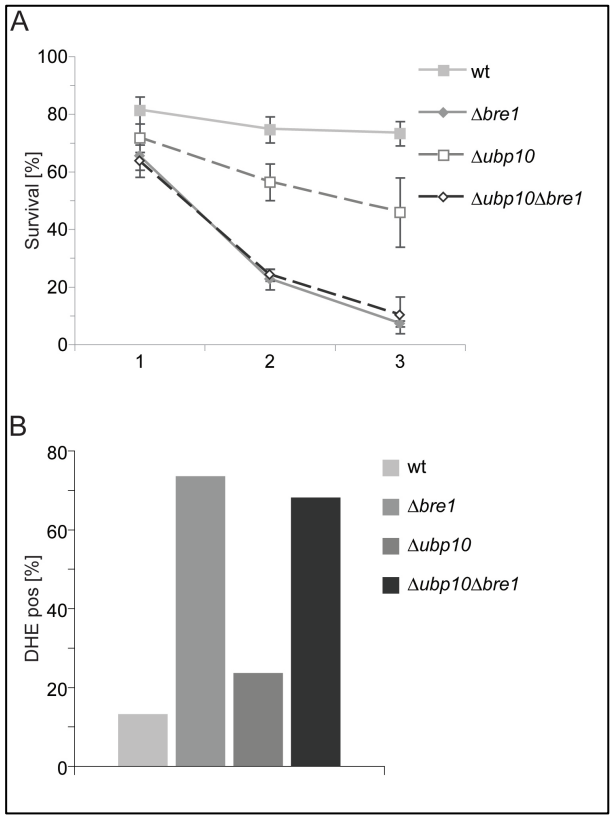

Figure 5.2: Disruption of *UBP10* causes no additional apoptosis sensitivity in *Δbre1* cells
(A) Survival of wild type (wt), *Δbre1*, *Δubp10* and *Δubp10Δbre1* cells determined by clonogenicity during chronological ageing. **(B)** DHE-positive cells were quantified after 2 days in culture using flow cytometry. In each experiment, 10.000 cells were evaluated.

5.1.2 A role of histone H3 methylation in apoptosis regulation

To test whether apoptosis in cells lacking ubiquitinated histone H2B is caused by defects in histone H3 methylation, we analyzed the apoptosis sensitivity of *Δset1* and *Δdot1* cells, which lack methylated histone H3 K4 and histone H3 K79, respectively. We found that *Δset1* cells are prone to Yca1p-dependent apoptosis similar to *Δbre1* cells (Figure 3.1), whereas *DOT1* disruption confers apoptosis resistance. Moreover, we found that *Δdot1* cells fail to undergo Yca1p dependent apoptosis (Figure 3.2). Consistently, Dot1p is required for Yca1p-dependent cell death of *Δset1* cells (Figure 3.3). Together, these studies highlight the requirement of Dot1p mediated histone H3 K79 methylation for an Yca1p-dependent cell death scenario. However, the molecular mechanism as to how

Dot1p mediated histone H3 K79 methylation triggers apoptosis is subject off our current research and will be discussed in the following section.

5.1.3 The DNA damage response is implicated in Dot1p mediated cell death

How does disruption of Dot1p confer apoptosis resistance? Yeast cells that fail to methylate histone H3 due to *DOT1* disruption exhibit defects in the DNA damage response (Giannattasio et al., 2005; Wysocki et al., 2005). Particularly, Dot1p-mediated H3 K79 methylation is required for Rad9p-dependent checkpoint activation after DNA damage (Wysocki et al., 2005). The DNA-damage response is an evolutionarily conserved signaling cascade crucial for sensing DNA damage and activating cellular responses such as cell-cycle arrest, DNA repair and apoptosis. Accordingly, DNA damage response genes have also been implicated in yeast apoptosis. Notably, deletion of the budding yeast *RAD9* gene can partially suppress the lethal effects of the apoptotic *orc2-1* mutation in the origin recognition complex (Watanabe et al., 2002) suggesting that *RAD9*-dependent checkpoint function is required for apoptosis induction in *orc2-1* cells. As Dot1p is required for Rad9p-dependent checkpoint activation, we consider it likely that *Δdot1* and *Δset1Δdot1* cells fail to activate apoptosis as a result of a defective checkpoint function. To test this hypothesis, we analyzed the apoptosis sensitivity of *Δset1Δrad9*. We expected that this double mutant would exhibit decreased apoptosis sensitivity as compared to *Δset1* cells similar to *Δset1Δdot1* cells, if Dot1p would trigger apoptosis in a Rad9p-dependent manner. Strikingly, our recent results show that *Δset1Δrad9* cells exhibit decreased apoptosis sensitivity as compared to *Δset1* cells (Figure 5.3A). After two days of chronological ageing *Δset1Δrad9* cells exhibit survival rates of 59% ± 5% as compared to 35% ± 5% of *Δset1* cells (Figure 5.3A). When, after two days in culture, these yeast cells were tested for ROS accumulation, 44% of *Δset1Δrad9* and 72% of *Δset1* cells accumulated ROS as determined by DHE staining (Figure 5.3B). These data indicate that Dot1p mediates apoptosis in *Δset1* cells trough its function in checkpoint activation and suggest that the DNA damage response is implicated in Yca1p-dependent cell death of *Δset1* cells during chronological ageing. Our findings therefore imply that DNA damage causes apoptosis in *Δset1* cells during chronological ageing. Thus, it will be interesting to see whether *Δset1* cells are also prone

to apoptosis induced by exogenously induced DNA damage and whether additional disruption of *DOT1*, *YCA1* and *RAD9*, respectively, would decrease the DNA damage sensitivity of Δ*set1* cells.

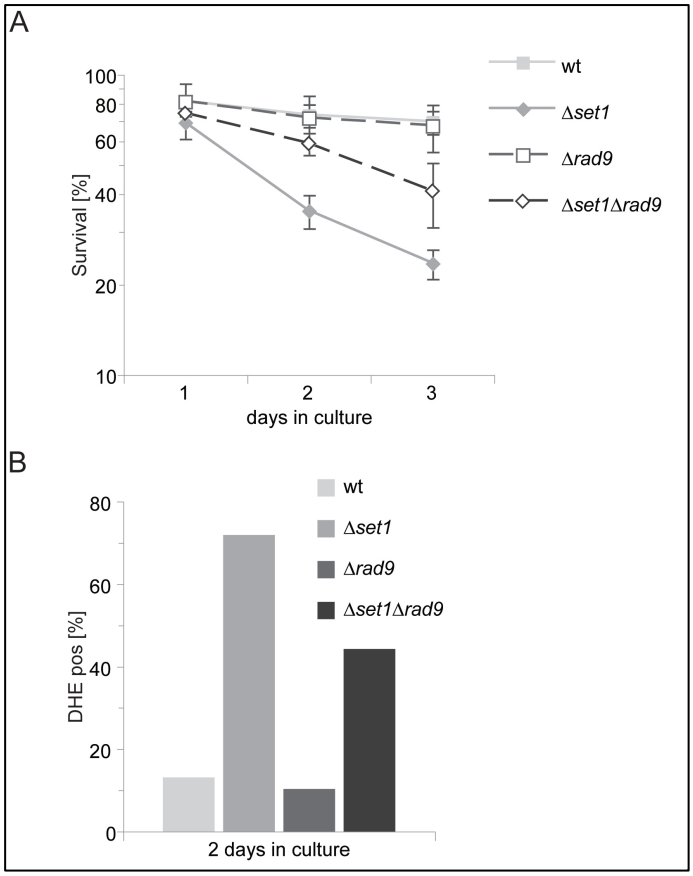

Figure 5.3: Rad9p is required for cell death of *Δset1* cells.
(A) Survival of wild type (wt), *Δset1, Δrad9* and *Δset1Δrad9* cells determined by clonogenicity during chronological ageing. **(B)** DHE-positive cells were quantified after 2 days in culture using flow cytometry. In each experiment, 10.000 cells were evaluated.

5.1.4 Perspective

Finally, my thesis work has led to the identification of a novel regulatory function of a histone cross-talk in yeast apoptosis that is linked to the DNA damage response. Since this cross-talk and the enzyme system responsible for this process are conserved between higher and lower eukaryotes, the mammalian apoptotic cascade may mirror the identified

regulatory yeast apoptotic mechanism. In higher eukaryotes the DNA damage response is implicated in apoptosis mainly through the tumor suppressor protein p53, which assimilates input signals including DNA damage to initiate appropriate outputs such as apoptosis. Yeast does not encode a p53 homologue and less is known about how DNA damage can lead to apoptosis when p53 is inactivated. However, both p53-dependent and -independent apoptotic pathways in higher eukaryotes are regulated by a number of DNA damage response proteins such as the Rad9p homologue BRCA1 (Harkin et al., 1999; Holt et al., 1996). As Rad9p is required for apoptosis of *Δset1* cells (Figure 5.3), these findings support the notion that the described regulatory mechanism may be conserved in higher eukaryotes. However, future research will hopefully answer to this question and will provide more insights into the role of histone modifications and DNA damage response in apoptosis in higher eukaryotes, which could be of great relevance for potential medical applications

5.2 Nma111p needs the nucleus to induce apoptosis

The HtrA-like serine protease Nma111p is a nuclear protein that is able to promote apoptosis in yeast in a serine protease-dependent manner (Fahrenkrog et al., 2004; Walter et al., 2006). We have shown that Nma111p harbours a classical bipartite NLS within the first 35 N-terminal amino acids and identified Kap95p as its nuclear import receptor (Belanger et al., 2009). Also, we have demonstrated that Nma111p is a non-shuttling protein that even under oxidative stress conditions remains nuclear (Belanger et al., 2009). The pro-apoptotic activity of Nma111p requires its nuclear localisation, as mutations of any critical residue in the bipartite NLS reduce Nma111p's ability to mediate apoptosis (Belanger et al., 2009). Moreover, Nma111p's death-promoting activity is restricted to its N-terminal HtrA-repeat, which harbours the NLSs and the active catalytic site of the protein (Belanger et al., 2009).

Our previous studies have revealed that Nma111p and its only known substrate, the inhibitor-of-apoptosis protein Bir1p, are both nuclear proteins (Fahrenkrog et al., 2004; Walter et al., 2006), which indicated that the nucleus appears to play a significant role in yeast apoptosis. This is further supported by the data, which revealed that Nma111p is not shuttling between the nucleus and the cytoplasm under normal and oxidative stress conditions. Moreover, disruption of any of the two basic stretches that act as NLS for Nma111p affects its ability to promote cell death. Therefore, Nma111p function in yeast apoptosis appears not linked to mitochondria, which is in clear contrast to its metazoan homologue Omi/HtrA2. Omi/HtrA2 is predominantly localised to mitochondria and is released into the cytosol under apoptotic conditions, which, in turn, allows its interaction with and the degradation of the inhibitor-of-apoptosis protein XIAP due to which executioner caspases get activated (Khan et al., 2008; Martins et al., 2002; Suzuki et al., 2004). It still remains to be seen which proteins are the downstream targets of Nma111p that lead to the execution of apoptosis. Bir1p is one such target and degradation of Bir1p by Nma111p induces apoptosis, but Bir1p does not directly inhibit the yeast caspase Yca1p (Walter et al., 2006). Therefore the bridging factor to the caspase or an unknown executioner of apoptosis in yeast remains to be elucidated, but it is most likely a nuclear protein.

6 References

Ahn, S.-H., R.L. Diaz, M. Grunstein, and C.D. Allis. 2006. Histone H2B Deacetylation at Lysine 11 Is Required for Yeast Apoptosis Induced by Phosphorylation of H2B at Serine 10. *Molecular Cell*. 24:211-220.

Ahn, S.H., W.L. Cheung, J.Y. Hsu, R.L. Diaz, M.M. Smith, and C.D. Allis. 2005. Sterile 20 kinase phosphorylates histone H2B at serine 10 during hydrogen peroxide-induced apoptosis in S. cerevisiae. *Cell*. 120:25-36.

Ajiro, K. 2000. Histone H2B phosphorylation in mammalian apoptotic cells. An association with DNA fragmentation. *J Biol Chem*. 275:439-43.

Allfrey, V.G., and A.E. Mirsky. 1964. Structural Modifications of Histones and their Possible Role in the Regulation of RNA Synthesis. *Science*. 144:559.

Almeida, B., S. Buttner, S. Ohlmeier, A. Silva, A. Mesquita, B. Sampaio-Marques, N.S. Osorio, A. Kollau, B. Mayer, C. Leao, J. Laranjinha, F. Rodrigues, F. Madeo, and P. Ludovico. 2007. NO-mediated apoptosis in yeast. *J Cell Sci*. 120:3279-88.

Almeida, T., M. Marques, D. Mojzita, M.A. Amorim, R.D. Silva, B. Almeida, P. Rodrigues, P. Ludovico, S. Hohmann, P. Moradas-Ferreira, M. Corte-Real, and V. Costa. 2008. Isc1p Plays a Key Role in Hydrogen Peroxide Resistance and Chronological Lifespan through Modulation of Iron Levels and Apoptosis. *Mol. Biol. Cell*. 19:865-876.

Ambrosini, G., C. Adida, and D.C. Altieri. 1997. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat. Med*. 3:917-921.

Ashrafi, K., D. Sinclair, J.I. Gordon, and L. Guarente. 1999. Passage through stationary phase advances replicative aging in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*. 96:9100-5.

Ballal, N.R., D.A. Goldberg, and H. Busch. 1975. Dissociation and reconstitution of chromatin without appreciable degradation of the proteins. *Biochem Biophys Res Commun*. 62:972-82

Bartke, T., C. Pohl, G. Pyrowolakis, and S. Jentsch. 2004. Dual Role of BRUCE as an Antiapoptotic IAP and a Chimeric E2/E3 Ubiquitin Ligase. *Mol. Cell*. 14:801-811.

Belanger, K.D., D. Walter, T.A. Henderson, A.L. Yelton, T.G. O'Brien, K.G. Belanger, S.J. Geier, and B. Fahrenkrog. 2009. Nuclear localisation is crucial for the proapoptotic activity of the HtrA-like serine protease Nma111p. *J Cell Sci*. 122:3931-41.

Berger, S.L. 2002. Histone modifications in transcriptional regulation. *Curr Opin Genet Dev*. 12:142-8.

Bernstein, B.E., E.L. Humphrey, R.L. Erlich, R. Schneider, P. Bouman, J.S. Liu, T. Kouzarides, and S.L. Schreiber. 2002. Methylation of histone H3 Lys 4 in coding regions of active genes. *Proc Natl Acad Sci U S A*. 99:8695-700.

Bettiga, M., L. Calzari, I. Orlandi, L. Alberghina, and M. Vai. 2004. Involvement of the yeast metacaspase Yca1 in ubpΔ-programmed cell death. *FEMS Yeast Res*. 5:141-7.

Bleazard, W., J.M. McCaffery, E.J. King, S. Bale, A. Mozdy, Q. Tieu, J. Nunnari, and J.M. Shaw. 1999. The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. *Nat. Cell Biol*. 1:298-304.

Bocharova, N.A., S.S. Sokolov, D.A. Knorre, V.P. Skulachev, and F.F. Severin. 2008. Unexpected link between anaphase promoting complex and the toxicity of expanded polyglutamines expressed in yeast. *Cell Cycle*. 7:3943-6.

Bouck, D.C., and K.S. Bloom. 2005. The kinetochore protein Ndc10p is required for spindle stability and cytokinesis in yeast. *Proc. Natl. Acad. Sci. U S A*. 102:5408-13.

Bounhar, Y., K.K. Mann, X. Roucou, and A.C. LeBlanc. 2006. Prion protein prevents Bax-mediated cell death in the absence of other Bcl-2 family members in Saccharomyces cerevisiae. *FEMS Yeast Res*. 6:1204-12.

Bounhar, Y., Y. Zhang, C.G. Goodyer, and A. LeBlanc. 2001. Prion protein protects human neurons against Bax-mediated apoptosis. *J. Biol. Chem*. 276:39145-9.

Brace, J.L., D.J. Vanderweele, and C.M. Rudin. 2005. Svf1 inhibits reactive oxygen species generation and promotes survival under conditions of oxidative stress in Saccharomyces cerevisiae. *Yeast*. 22:641-52.

Braun, R.J., H. Zischka, F. Madeo, T. Eisenberg, S. Wissing, S. Buttner, S.M. Engelhardt, D. Buringer, and M. Ueffing. 2006. Crucial mitochondrial impairment upon CDC48 mutation in apoptotic yeast. *J. Biol. Chem*. 281:25757-67.

Bray, S., H. Musisi, and M. Bienz. 2005. Bre1 is required for Notch signaling and histone modification. *Dev Cell*. 8:279-86.

Briggs, S.D., M. Bryk, B.D. Strahl, W.L. Cheung, J.K. Davie, S.Y. Dent, F. Winston, and C.D. Allis. 2001a. Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in Saccharomyces cerevisiae. *Genes Dev*. 15:3286-95.

Briggs, S.D., M. Bryk, B.D. Strahl, W.L. Cheung, J.K. Davie, S.Y.R. Dent, F. Winston, and C.D. Allis. 2001b. Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in Saccharomyces cerevisiae. *Genes Dev*. 15:3286-3295.

Briggs, S.D., T. Xiao, Z.W. Sun, J.A. Caldwell, J. Shabanowitz, D.F. Hunt, C.D. Allis, and B.D. Strahl. 2002. Gene silencing: trans-histone regulatory pathway in chromatin. *Nature*. 418:498.

Bryk, M., S.D. Briggs, B.D. Strahl, M.J. Curcio, C.D. Allis, and F. Winston. 2002. Evidence that Set1, a factor required for methylation of histone H3, regulates rDNA silencing in S. cerevisiae by a Sir2-independent mechanism. *Curr Biol*. 12:165-70.

Burhans, W.C., M. Weinberger, M.A. Marchetti, L. Ramachandran, G. D'Urso, and J.A. Huberman. 2003. Apoptosis-like yeast cell death in response to DNA damage and replication defects. *Mutat Res*. 532:227-43.

Buttner, S., T. Eisenberg, D. Carmona-Gutierrez, D. Ruli, H. Knauer, C. Ruckenstuhl, C. Sigrist, S. Wissing, M. Kollroser, K.U. Frohlich, S. Sigrist, and F. Madeo. 2007. Endonuclease G regulates budding yeast life and death. *Mol. Cell*. 25:233-46.

Challa, M., S. Malladi, B.J. Pellock, D. Dresnek, S. Varadarajan, Y.W. Yin, K. White, and S.B. Bratton. 2007. Drosophila Omi, a mitochondrial-localized IAP antagonist and proapoptotic serine protease. *Embo J*. 26:3144-56.

Cheeseman, I.M., S. Anderson, M. Jwa, E.M. Green, J. Kang, J.R. Yates, 3rd, C.S. Chan, D.G. Drubin, and G. Barnes. 2002. Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p. *Cell*. 111:163-72.

Chen, D., H. Ma, H. Hong, S.S. Koh, S.M. Huang, B.T. Schurter, D.W. Aswad, and M.R. Stallcup. 1999. Regulation of transcription by a protein methyltransferase. *Science*. 284:2174-7.

Chen, S.-R., D.D. Dunigan, and M.B. Dickman. 2003. Bcl-2 family members inhibit oxidative stress-induced programmed cell death in Saccharomyces cerevisiae. *Free Radic. Biol. Med*. 34:1315-1325.

Cheung, P., C.D. Allis, and P. Sassone-Corsi. 2000. Signaling to chromatin through histone modifications. *Cell*. 103:263-71.

Cheung, W.L., K. Ajiro, K. Samejima, M. Kloc, P. Cheung, C.A. Mizzen, A. Beeser, L.D. Etkin, J. Chernoff, W.C. Earnshaw, and C.D. Allis. 2003. Apoptotic phosphorylation of histone H2B is mediated by mammalian sterile twenty kinase. *Cell*. 113:507-17.

Clark-Adams, C.D., D. Norris, M.A. Osley, J.S. Fassler, and F. Winston. 1988. Changes in histone gene dosage alter transcription in yeast. *Genes Dev*. 2:150-9.

Clausen, T., C. Southan, and M. Ehrmann. 2002. The HtrA family of proteases: implications for protein composition and cell fate. *Mol Cell*. 10:443-55.

Cokol, M., R. Nair, and B. Rost. 2000. Finding nuclear localization signals. *EMBO Rep*. 1:411-5.

Conde, J., and G.R. Fink. 1976. A mutant of Saccharomyces cerevisiae defective for nuclear fusion. *Proc Natl Acad Sci U S A*. 73:3651-5.

Cory, S., and J.M. Adams. 2002. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat. Rev. Cancer*. 2:647-56.

Crook, N.E., R.J. Clem, and L.K. Miller. 1993. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J. Virol*. 67:2168-2174.

Danial, N.N., and S.J. Korsmeyer. 2004. Cell Death: Critical Control Points. *Cell*. 116:205-219.

de la Barre, A.E., D. Angelov, A. Molla, and S. Dimitrov. 2001. The N-terminus of histone H2B, but not that of histone H3 or its phosphorylation, is essential for chromosome condensation. *Embo J*. 20:6383-93.

Defossez, P.A., R. Prusty, M. Kaeberlein, S.J. Lin, P. Ferrigno, P.A. Silver, R.L. Keil, and L. Guarente. 1999. Elimination of replication block protein Fob1 extends the life span of yeast mother cells. *Mol Cell*. 3:447-55.

Dehe, P.M., M. Pamblanco, P. Luciano, R. Lebrun, D. Moinier, R. Sendra, A. Verreault, V. Tordera, and V. Geli. 2005. Histone H3 lysine 4 mono-methylation does not require ubiquitination of histone H2B. *J Mol Biol*. 353:477-84.

DeLotto, R., Y. DeLotto, R. Steward, and J. Lippincott-Schwartz. 2007. Nucleocytoplasmic shuttling mediates the dynamic maintenance of nuclear Dorsal levels during Drosophila embryogenesis. *Development*. 134:4233-41.

Desagher, S., and J.-C. Martinou. 2000. Mitochondria as the central control point of apoptosis. *Trends Cell Biol*. 10:369-377.

Deshaies, R.J., and C.A. Joazeiro. 2009. RING domain E3 ubiquitin ligases. *Annu Rev Biochem*. 78:399-434.

Deveraux, Q.L., and J.C. Reed. 1999. IAP family proteins---suppressors of apoptosis. *Genes Dev*. 13:239-252.

Dover, J., J. Schneider, M.A. Tawiah-Boateng, A. Wood, K. Dean, M. Johnston, and A. Shilatifard. 2002. Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6. *J Biol Chem*. 277:28368-71.

Drakulic, T., M.D. Temple, R. Guido, S. Jarolim, M. Breitenbach, P.V. Attfield, and I.W. Dawes. 2005. Involvement of oxidative stress response genes in redox homeostasis, the level of reactive oxygen species, and ageing in Saccharomyces cerevisiae. *FEMS Yeast Res*. 5:1215-28.

Earnshaw, W.C. 1999. Apoptosis. A cellular poison cupboard. *Nature*. 397:387, 389.

Emre, N.C., K. Ingvarsdottir, A. Wyce, A. Wood, N.J. Krogan, K.W. Henry, K. Li, R. Marmorstein, J.F. Greenblatt, A. Shilatifard, and S.L. Berger. 2005. Maintenance of low histone ubiquitylation by Ubp10 correlates with telomere-proximal Sir2 association and gene silencing. *Mol Cell*. 17:585-94.

Fabrizio, P., L. Battistella, R. Vardavas, C. Gattazzo, L.L. Liou, A. Diaspro, J.W. Dossen, E.B. Gralla, and V.D. Longo. 2004. Superoxide is a mediator of an altruistic aging program in Saccharomyces cerevisiae. *J. Cell Biol*. 166:1055-67.

Fabrizio, P., C. Gattazzo, L. Battistella, M. Wei, C. Cheng, K. McGrew, and V.D. Longo. 2005. Sir2 blocks extreme life-span extension. *Cell*. 123:655-67.

Fabrizio, P., and V.D. Longo. 2003. The chronological life span of Saccharomyces cerevisiae. *Aging Cell*. 2:73-81.

Fabrizio, P., and V.D. Longo. 2008. Chronological aging-induced apoptosis in yeast. *Biochim Biophys Acta*. 1783:1280-5.

Fabrizio, P., F. Pozza, S.D. Pletcher, C.M. Gendron, and V.D. Longo. 2001. Regulation of longevity and stress resistance by Sch9 in yeast. *Science*. 292:288-90.

Fadeel, B., and S. Orrenius. 2005. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *J Intern Med*. 258:479-517.

Fahrenkrog, B., U. Sauder, and U. Aebi. 2004. The S. cerevisiae HtrA-like protein Nma111p is a nuclear serine protease that mediates yeast apoptosis. *J. Cell Sci*. 117:115-26.

Falcon, A.A., and J.P. Aris. 2003. Plasmid accumulation reduces life span in Saccharomyces cerevisiae. *J Biol Chem*. 278:41607-17.

Fannjiang, Y., W.-C. Cheng, S.J. Lee, B. Qi, J. Pevsner, J.M. McCaffery, R.B. Hill, G. Basanez, and J.M. Hardwick. 2004. Mitochondrial fission proteins regulate programmed cell death in yeast. *Genes Dev*. 18:2785-2797.

Feng, C., H.L. Wilson, J.K. Hurley, J.T. Hazzard, G. Tollin, K.V. Rajagopalan, and J.H. Enemark. 2003. Role of conserved tyrosine 343 in intramolecular electron transfer in human sulfite oxidase. *J Biol Chem*. 278:2913-20.

Feng, W., and A.K. Hopper. 2002. A Los1p-independent pathway for nuclear export of intronless tRNAs in Saccharomycescerevisiae. *Proc Natl Acad Sci U S A*. 99:5412-7.

Fink, G.a. 1991. Guide to yeast genetics and molecular biology. *Methods Enzymol*. 194:1-863.

Fischle, W., Y. Wang, and C.D. Allis. 2003a. Binary switches and modification cassettes in histone biology and beyond. *Nature*. 425:475-9.

Fischle, W., Y. Wang, and C.D. Allis. 2003b. Histone and chromatin cross-talk. *Curr Opin Cell Biol*. 15:172-83.

Fleury, D., K. Himanen, G. Cnops, H. Nelissen, T.M. Boccardi, S. Maere, G.T. Beemster, P. Neyt, S. Anami, P. Robles, J.L. Micol, D. Inze, and M. Van Lijsebettens. 2007. The Arabidopsis thaliana homolog of yeast BRE1 has a function in cell cycle regulation during early leaf and root growth. *Plant Cell*. 19:417-32.

Frank, S., B. Gaume, E.S. Bergmann-Leitner, W.W. Leitner, E.G. Robert, F. Catez, C.L. Smith, and R.J. Youle. 2001. The Role of Dynamin-Related Protein 1, a Mediator of Mitochondrial Fission, in Apoptosis. *Dev. Cell*. 1:515-525.

Fraser, A.G., C. James, G.I. Evan, and M.O. Hengartner. 1999. Caenorhabditis elegans inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytokinesis. *Curr. Biol*. 9:292-301.

Frohlich, K.-U., H. Fussi, and C. Ruckenstuhl. 2007. Yeast apoptosis--From genes to pathways. *Semin. Cancer. Biol*. 17:112-121.

Game, J.C., M.S. Williamson, T. Spicakova, and M. Brown. 2006. The RAD6/BRE1 histone modification pathway in Saccharomyces confers radiation resistance through a RAD51-dependent process that is independent of RAD18. *Genetics*:genetics.106.057794.

Gardner, R.G., Z.W. Nelson, and D.E. Gottschling. 2005. Ubp10/Dot4p regulates the persistence of ubiquitinated histone H2B: distinct roles in telomeric silencing and general chromatin. *Mol Cell Biol*. 25:6123-39.

Gavrieli, Y., Y. Sherman, and S.A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol*. 119:493-501.

Giannattasio, M., F. Lazzaro, P. Plevani, and M. Muzi-Falconi. 2005. The DNA damage checkpoint response requires histone H2B ubiquitination by Rad6-Bre1 and H3 methylation by Dot1. *J Biol Chem*. 280:9879-86.

Gietz, D., A. St Jean, R.A. Woods, and R.H. Schiestl. 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res*. 20:1425.

Gillis, A.N., S. Thomas, S.D. Hansen, and K.B. Kaplan. 2005. A novel role for the CBF3 kinetochore-scaffold complex in regulating septin dynamics and cytokinesis. *J. Cell Biol*. 171:773-84.

Glickman, M.H., and A. Ciechanover. 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev*. 82:373-428.

Goldknopf, I.L., and H. Busch. 1975. Remarkable similarities of peptide fingerprints of histone 2A and nonhistone chromosomal protein A24. *Biochem Biophys Res Commun*. 65:951-60.

Gonzalez-Garcia, M., R. Perez-Ballestero, L. Ding, L. Duan, L.H. Boise, C.B. Thompson, and G. Nunez. 1994. bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. *Development*. 120:3033-3042.

Gorczyca, W., J. Gong, and Z. Darzynkiewicz. 1993. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res*. 53:1945-51.

Gorner, W., E. Durchschlag, M.T. Martinez-Pastor, F. Estruch, G. Ammerer, B. Hamilton, H. Ruis, and C. Schuller. 1998. Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev*. 12:586-97.

Gorner, W., E. Durchschlag, J. Wolf, E.L. Brown, G. Ammerer, H. Ruis, and C. Schuller. 2002. Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. *Embo J*. 21:135-44.

Gottlieb, S., and R.E. Esposito. 1989. A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA. *Cell*. 56:771-6.

Gourlay, C.W., and K.R. Ayscough. 2005. The actin cytoskeleton: a key regulator of apoptosis and ageing? *Nat. Rev. Mol. Cell. Biol*. 6:583-9.

Gourlay, C.W., and K.R. Ayscough. 2006. Actin-induced hyperactivation of the Ras signaling pathway leads to apoptosis in Saccharomyces cerevisiae. *Mol Cell Biol*. 26:6487-501.

Gourlay, C.W., L.N. Carpp, P. Timpson, S.J. Winder, and K.R. Ayscough. 2004. A role for the actin cytoskeleton in cell death and aging in yeast. *J. Cell Biol*. 164:803-9.

Gu, X., D. Jiang, Y. Wang, A. Bachmair, and Y. He. 2009. Repression of the floral transition via histone H2B monoubiquitination. *Plant J*. 57:522-33.

Guaragnella, N., L. Antonacci, S. Giannattasio, E. Marra, and S. Passarella. 2008. Catalase T and Cu,Zn-superoxide dismutase in the acetic acid-induced programmed cell death in Saccharomyces cerevisiae. *FEBS Lett*. 582:210-4.

Guarente, L. 2000. Sir2 links chromatin silencing, metabolism, and aging. *Genes Dev*. 14:1021-6.

Gueldener, U., J. Heinisch, G.J. Koehler, D. Voss, and J.H. Hegemann. 2002. A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res*. 30:e23.

Guenther, M.G., L.N. Lawton, T. Rozovskaia, G.M. Frampton, S.S. Levine, T.L. Volkert, C.M. Croce, T. Nakamura, E. Canaani, and R.A. Young. 2008. Aberrant chromatin at genes encoding stem cell regulators in human mixed-lineage leukemia. *Genes Dev*. 22:3403-8.

Guthrie, C. 1991. Messenger RNA splicing in yeast: clues to why the spliceosome is a ribonucleoprotein. *Science*. 253:157-63.

Han, M., and M. Grunstein. 1988. Nucleosome loss activates yeast downstream promoters in vivo. *Cell*. 55:1137-45.

Hanada, M., C. Aime-Sempe, T. Sato, and J.C. Reed. 1995. Structure-function analysis of Bcl-2 protein. Identification of conserved domains important for homodimerization with Bcl-2 and heterodimerization with Bax. *J. Biol. Chem*. 270:11962-9.

Hao, Y., K. Sekine, A. Kawabata, H. Nakamura, T. Ishioka, H. Ohata, R. Katayama, C. Hashimoto, X. Zhang, T. Noda, T. Tsuruo, and M. Naito. 2004. Apollon ubiquitinates SMAC and caspase-9, and has an essential cytoprotection function. *Nat. Cell Biol*. 6:849-860.

Harkin, D.P., J.M. Bean, D. Miklos, Y.H. Song, V.B. Truong, C. Englert, F.C. Christians, L.W. Ellisen, S. Maheswaran, J.D. Oliner, and D.A. Haber. 1999. Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1. *Cell*. 97:575-86.

Hauptmann, P., and L. Lehle. 2008. Kex1 protease is involved in yeast cell death induced by defective N-glycosylation, acetic acid, and chronological aging. *J Biol Chem*. 283:19151-63.

Hauptmann, P., C. Riel, L.A. Kunz-Schughart, K.U. Frohlich, F. Madeo, and L. Lehle. 2006. Defects in N-glycosylation induce apoptosis in yeast. *Mol Microbiol*. 59:765-78.

Hegde, R., S.M. Srinivasula, Z. Zhang, R. Wassell, R. Mukattash, L. Cilenti, G. DuBois, Y. Lazebnik, A.S. Zervos, T. Fernandes-Alnemri, and E.S. Alnemri. 2002. Identification of

Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J Biol Chem*. 277:432-8.

Hellmuth, K., D.M. Lau, F.R. Bischoff, M. Kunzler, E. Hurt, and G. Simos. 1998. Yeast Los1p has properties of an exportin-like nucleocytoplasmic transport factor for tRNA. *Mol Cell Biol*. 18:6374-86.

Henry, K.W., A. Wyce, W.S. Lo, L.J. Duggan, N.C. Emre, C.F. Kao, L. Pillus, A. Shilatifard, M.A. Osley, and S.L. Berger. 2003. Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. *Genes Dev*. 17:2648-63.

Herker, E., H. Jungwirth, K.A. Lehmann, C. Maldener, K.U. Frohlich, S. Wissing, S. Buttner, M. Fehr, S. Sigrist, and F. Madeo. 2004. Chronological aging leads to apoptosis in yeast. *J. Cell Biol*. 164:501-7.

Holt, J.T., M.E. Thompson, C. Szabo, C. Robinson-Benion, C.L. Arteaga, M.C. King, and R.A. Jensen. 1996. Growth retardation and tumour inhibition by BRCA1. *Nat Genet*. 12:298-302.

Hu, S., and X. Yang. 2003. Cellular Inhibitor of Apoptosis 1 and 2 Are Ubiquitin Ligases for the Apoptosis Inducer Smac/DIABLO. *J. Biol. Chem*. 278:10055-10060.

Huang, H.-k., C.A.P. Joazeiro, E. Bonfoco, S. Kamada, J.D. Leverson, and T. Hunter. 2000. The Inhibitor of Apoptosis, cIAP2, Functions as a Ubiquitin-Protein Ligase and Promotes in Vitro Monoubiquitination of Caspases 3 and 7. *J. Biol. Chem*. 275:26661-26664.

Huang, Y. 2002. Transcriptional silencing in Saccharomyces cerevisiae and Schizosaccharomyces pombe. *Nucleic Acids Res*. 30:1465-82.

Hwang, W.W., S. Venkatasubrahmanyam, A.G. Ianculescu, A. Tong, C. Boone, and H.D. Madhani. 2003. A conserved RING finger protein required for histone H2B monoubiquitination and cell size control. *Mol Cell*. 11:261-6.

Igaki, T., Y. Suzuki, N. Tokushige, H. Aonuma, R. Takahashi, and M. Miura. 2007. Evolution of mitochondrial cell death pathway: Proapoptotic role of HtrA2/Omi in Drosophila. *Biochem Biophys Res Commun*. 356:993-7.

Igney, F.H., and P.H. Krammer. 2002. Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer*. 2:277-88.

Imai, S., C.M. Armstrong, M. Kaeberlein, and L. Guarente. 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature*. 403:795-800.

Jagasia, R., P. Grote, B. Westermann, and B. Conradt. 2005. DRP-1-mediated mitochondrial fragmentation during EGL-1-induced cell death in C. elegans. *Nature*. 433:754-760.

Jones, G., D. Jones, L. Zhou, H. Steller, and Y. Chu. 2000. Deterin, a New Inhibitor of Apoptosis from Drosophila melanogaster. *J. Biol. Chem*. 275:22157-22165.

Jurgensmeier, J.M., S. Krajewski, R.C. Armstrong, G.M. Wilson, T. Oltersdorf, L.C. Fritz, J.C. Reed, and S. Ottilie. 1997. Bax- and Bak-induced cell death in the fission yeast Schizosaccharomyces pombe. *Mol. Biol. Cell*. 8:325-39.

Kaeberlein, M., C.R. Burtner, and B.K. Kennedy. 2007. Recent developments in yeast aging. *PLoS Genet*. 3:e84.

Kaeberlein, M., M. McVey, and L. Guarente. 1999. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. *Genes Dev*. 13:2570-80.

Kaeberlein, M., R.W. Powers, 3rd, K.K. Steffen, E.A. Westman, D. Hu, N. Dang, E.O. Kerr, K.T. Kirkland, S. Fields, and B.K. Kennedy. 2005. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science*. 310:1193-6.

Kao, C.F., C. Hillyer, T. Tsukuda, K. Henry, S. Berger, and M.A. Osley. 2004. Rad6 plays a role in transcriptional activation through ubiquitylation of histone H2B. *Genes Dev*. 18:184-95.

Kaufmann, T., S. Schlipf, J. Sanz, K. Neubert, R. Stein, and C. Borner. 2003. Characterization of the signal that directs Bcl-x(L), but not Bcl-2, to the mitochondrial outer membrane. *J. Cell Biol*. 160:53-64.

Kennedy, B.K., N.R. Austriaco, Jr., J. Zhang, and L. Guarente. 1995. Mutation in the silencing gene SIR4 can delay aging in S. cerevisiae. *Cell*. 80:485-96.

Kerr, J.F., A.H. Wyllie, and A.R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 26:239-57.

Khan, F.S., M. Fujioka, P. Datta, T. Fernandes-Alnemri, J.B. Jaynes, and E.S. Alnemri. 2008. The interaction of DIAP1 with dOmi/HtrA2 regulates cell death in Drosophila. *Cell Death Differ*. 15:1073-83.

Kim, J., S.B. Hake, and R.G. Roeder. 2005. The human homolog of yeast BRE1 functions as a transcriptional coactivator through direct activator interactions. *Mol Cell*. 20:759-70.

Kinnally, K.W., and B. Antonsson. 2007. A tale of two mitochondrial channels, MAC and PTP, in apoptosis. *Apoptosis*. 12:857-68.

Kissova, I., L.T. Plamondon, L. Brisson, M. Priault, V. Renouf, J. Schaeffer, N. Camougrand, and S. Manon. 2006. Evaluation of the roles of apoptosis, autophagy, and mitophagy in the loss of plating efficiency induced by Bax expression in yeast. *J. Biol. Chem*. 281:36187-97.

Kornberg, R.D. 1977. Structure of chromatin. *Annu Rev Biochem*. 46:931-54.

Kouzarides, T. 2007. Chromatin modifications and their function. *Cell*. 128:693-705.

Krogan, N.J., J. Dover, S. Khorrami, J.F. Greenblatt, J. Schneider, M. Johnston, and A. Shilatifard. 2002. COMPASS, a histone H3 (Lysine 4) methyltransferase required for telomeric silencing of gene expression. *J Biol Chem*. 277:10753-5.

Lacoste, N., R.T. Utley, J.M. Hunter, G.G. Poirier, and J. Cote. 2002. Disruptor of telomeric silencing-1 is a chromatin-specific histone H3 methyltransferase. *J Biol Chem*. 277:30421-4.

Landry, J., A. Sutton, S.T. Tafrov, R.C. Heller, J. Stebbins, L. Pillus, and R. Sternglanz. 2000. The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proc Natl Acad Sci U S A*. 97:5807-11.

Lange, A., R.E. Mills, C.J. Lange, M. Stewart, S.E. Devine, and A.H. Corbett. 2007. Classical nuclear localization signals: definition, function, and interaction with importin alpha. *J Biol Chem*. 282:5101-5.

Laun, P., A. Pichova, F. Madeo, J. Fuchs, A. Ellinger, S. Kohlwein, I. Dawes, K.U. Frohlich, and M. Breitenbach. 2001. Aged mother cells of Saccharomyces cerevisiae show markers of oxidative stress and apoptosis. *Mol Microbiol*. 39:1166-73.

Lawen, A. 2007. Another piece of the puzzle of apoptotic cytochrome c release. *Mol. Microbiol*.

Lee, J.C., and M.E. Peter. 2003. Regulation of apoptosis by ubiquitination. *Immunol Rev*. 193:39-47.

Lee, J.C., O. Schickling, A.H. Stegh, R.G. Oshima, D. Dinsdale, G.M. Cohen, and M.E. Peter. 2002. DEDD regulates degradation of intermediate filaments during apoptosis. *J. Cell Biol*. 158:1051-1066.

Lens, S.M., R.M. Wolthuis, R. Klompmaker, J. Kauw, R. Agami, T. Brummelkamp, G. Kops, and R.H. Medema. 2003. Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension. *EMBO J*. 22:2934-47.

Lettre, G., E.A. Kritikou, M. Jaeggi, A. Calixto, A.G. Fraser, R.S. Kamath, J. Ahringer, and M.O. Hengartner. 2004. Genome-wide RNAi identifies p53-dependent and -independent regulators of germ cell apoptosis in C. elegans. *Cell Death Differ*. 11:1198-203.

Li, A., and D.A. Harris. 2005. Mammalian prion protein suppresses Bax-induced cell death in yeast. *J. Biol. Chem*. 280:17430-4.

Li, F., P.L. Flanary, D.C. Altieri, and H.G. Dohlman. 2000. Cell Division Regulation by BIR1, a Member of the Inhibitor of Apoptosis Family in Yeast. *J. Biol. Chem*. 275:6707-6711.

Li, L.Y., X. Luo, and X. Wang. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature*. 412:95-99.

Liang, Q., and B. Zhou. 2007. Copper and Manganese Induce Yeast Apoptosis via Different Pathways. *Mol. Biol. Cell*. 18:4741-4749.

Ligr, M., F. Madeo, E. Frohlich, W. Hilt, K.U. Frohlich, and D.H. Wolf. 1998. Mammalian Bax triggers apoptotic changes in yeast. *FEBS Lett*. 438:61-5.

Lin, S.J., P.A. Defossez, and L. Guarente. 2000. Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. *Science*. 289:2126-8.

Lithgow, T., R. van Driel, J.F. Bertram, and A. Strasser. 1994. The protein product of the oncogene bcl-2 is a component of the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane. *Cell Growth Differ*. 5:411-7.

Liu, C.L., T. Kaplan, M. Kim, S. Buratowski, S.L. Schreiber, N. Friedman, and O.J. Rando. 2005. Single-nucleosome mapping of histone modifications in S. cerevisiae. *PLoS Biol*. 3:e328.

Longo, V.D., L.M. Ellerby, D.E. Bredesen, J.S. Valentine, and E.B. Gralla. 1997. Human Bcl-2 Reverses Survival Defects in Yeast Lacking Superoxide Dismutase and Delays Death of Wild-Type Yeast. *J. Cell Biol*. 137:1581-1588.

Longo, V.D., E.B. Gralla, and J.S. Valentine. 1996. Superoxide dismutase activity is essential for stationary phase survival in Saccharomyces cerevisiae. Mitochondrial production of toxic oxygen species in vivo. *J. Biol. Chem*. 271:12275-80.

Longtine, M.S., A. McKenzie, 3rd, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philippsen, and J.R. Pringle. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast*. 14:953-61.

Ludovico, P., F. Rodrigues, A. Almeida, M.T. Silva, A. Barrientos, and M. Corte-Real. 2002. Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in Saccharomyces cerevisiae. *Mol. Biol. Cell*. 13:2598-606.

Ludovico, P., M.J. Sousa, M.T. Silva, C. Leao, and M. Corte-Real. 2001. Saccharomyces cerevisiae commits to a programmed cell death process in response to acetic acid. *Microbiology*. 147:2409-15.

Ly, J.D., D.R. Grubb, and A. Lawen. 2003. The mitochondrial membrane potential (deltapsi(m)) in apoptosis; an update. *Apoptosis*. 8:115-28.

Madeo, F., D. Carmona-Gutierrez, J. Ring, S. Buttner, T. Eisenberg, and G. Kroemer. 2009. Caspase-dependent and caspase-independent cell death pathways in yeast. *Biochem Biophys Res Commun*. 382:227-31.

Madeo, F., E. Frohlich, and K.-U. Frohlich. 1997. A Yeast Mutant Showing Diagnostic Markers of Early and Late Apoptosis. *J. Cell Biol*. 139:729-734.

Madeo, F., E. Frohlich, M. Ligr, M. Grey, S.J. Sigrist, D.H. Wolf, and K.-U. Frohlich. 1999a. Oxygen Stress: A Regulator of Apoptosis in Yeast. *J. Cell Biol*. 145:757-767.

Madeo, F., E. Frohlich, M. Ligr, M. Grey, S.J. Sigrist, D.H. Wolf, and K.U. Frohlich. 1999b. Oxygen stress: a regulator of apoptosis in yeast. *J Cell Biol*. 145:757-67.

Madeo, F., E. Herker, C. Maldener, S. Wissing, S. Lachelt, M. Herlan, M. Fehr, K. Lauber, S.J. Sigrist, S. Wesselborg, and K.U. Frohlich. 2002. A caspase-related protease regulates apoptosis in yeast. *Mol Cell*. 9:911-7.

Mancini, M., B.O. Anderson, E. Caldwell, M. Sedghinasab, P.B. Paty, and D.M. Hockenbery. 1997. Mitochondrial Proliferation and Paradoxical Membrane Depolarization during Terminal Differentiation and Apoptosis in a Human Colon Carcinoma Cell Line. *J. Cell Biol*. 138:449-469.

Manon, S., B. Chaudhuri, and M. Guerin. 1997. Release of cytochrome c and decrease of cytochrome c oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-xL. *FEBS Lett*. 415:29-32.

Manon, S., X. Roucou, M. Guerin, M. Rigoulet, and B. Guerin. 1998. Characterization of the yeast mitochondria unselective channel: a counterpart to the mammalian permeability transition pore? *J. Bioenerg. Biomembr*. 30:419-29.

Maris, A.F., A.L. Assumpcao, D. Bonatto, M. Brendel, and J.A. Henriques. 2001. Diauxic shift-induced stress resistance against hydroperoxides in Saccharomyces cerevisiae is not an adaptive stress response and does not depend on functional mitochondria. *Curr. Genet.* 39:137-49.

Martins, L.M., I. Iaccarino, T. Tenev, S. Gschmeissner, N.F. Totty, N.R. Lemoine, J. Savopoulos, C.W. Gray, C.L. Creasy, C. Dingwall, and J. Downward. 2002. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. *J Biol Chem.* 277:439-44.

Mason, D.A., N. Shulga, S. Undavai, E. Ferrando-May, M.F. Rexach, and D.S. Goldfarb. 2005. Increased nuclear envelope permeability and Pep4p-dependent degradation of nucleoporins during hydrogen peroxide-induced cell death. *FEMS Yeast Res.* 5:1237-51.

Mazzoni, C., E. Herker, V. Palermo, H. Jungwirth, T. Eisenberg, F. Madeo, and C. Falcone. 2005. Yeast caspase 1 links messenger RNA stability to apoptosis in yeast. *EMBO Rep.* 6:1076-81.

Mazzoni, C., P. Mancini, L. Verdone, F. Madeo, A. Serafini, E. Herker, and C. Falcone. 2003. A truncated form of KlLsm4p and the absence of factors involved in mRNA decapping trigger apoptosis in yeast. *Mol Biol Cell.* 14:721-9.

McDonnell, T.J., N. Deane, F.M. Platt, G. Nunez, U. Jaeger, J.P. McKearn, and S.J. Korsmeyer. 1989. bcl-2-Immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell.* 57:79-88.

McGhee, J.D., and G. Felsenfeld. 1980. Nucleosome structure. *Annu Rev Biochem.* 49:1115-56.

Miller, T., N.J. Krogan, J. Dover, H. Erdjument-Bromage, P. Tempst, M. Johnston, J.F. Greenblatt, and A. Shilatifard. 2001. COMPASS: a complex of proteins associated with a trithorax-related SET domain protein. *Proc Natl Acad Sci U S A.* 98:12902-7.

Mimnaugh, E.G., G. Kayastha, N.B. McGovern, S.G. Hwang, M.G. Marcu, J. Trepel, S.Y. Cai, V.T. Marchesi, and L. Neckers. 2001. Caspase-dependent deubiquitination of monoubiquitinated nucleosomal histone H2A induced by diverse apoptogenic stimuli. *Cell Death Differ.* 8:1182-96.

Mitsui, K., D. Nakagawa, M. Nakamura, T. Okamoto, and K. Tsurugi. 2005. Valproic acid induces apoptosis dependent of Yca1p at concentrations that mildly affect the proliferation of yeast. *FEBS Lett.* 579:723-7.

Moazed, D. 2001a. Common themes in mechanisms of gene silencing. *Mol Cell.* 8:489-98.

Moazed, D. 2001b. Enzymatic activities of Sir2 and chromatin silencing. *Curr Opin Cell Biol.* 13:232-8.

Modjtahedi, N., F. Giordanetto, F. Madeo, and G. Kroemer. 2006. Apoptosis-inducing factor: vital and lethal. *Trends Cell Biol.* 16:264-72.

Montpetit, B., T.R. Hazbun, S. Fields, and P. Hieter. 2006. Sumoylation of the budding yeast kinetochore protein Ndc10 is required for Ndc10 spindle localization and regulation of anaphase spindle elongation. *J. Cell Biol.* 174:653-63.

Mosammaparast, N., Y. Guo, J. Shabanowitz, D.F. Hunt, and L.F. Pemberton. 2002. Pathways mediating the nuclear import of histones H3 and H4 in yeast. *J Biol Chem.* 277:862-8.

Mosammaparast, N., K.R. Jackson, Y. Guo, C.J. Brame, J. Shabanowitz, D.F. Hunt, and L.F. Pemberton. 2001. Nuclear import of histone H2A and H2B is mediated by a network of karyopherins. *J Cell Biol.* 153:251-62.

Moye-Rowley, W.S., K.D. Harshman, and C.S. Parker. 1989. Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins. *Genes Dev.* 3:283-92.

Mozdy, A.D., J.M. McCaffery, and J.M. Shaw. 2000. Dnm1p GTPase-mediated Mitochondrial Fission Is a Multi-step Process Requiring the Novel Integral Membrane Component Fis1p. *J. Cell Biol.* 151:367-380.

Mueller, J.E., M. Canze, and M. Bryk. 2006. The requirements for COMPASS and Paf1 in transcriptional silencing and methylation of histone H3 in Saccharomyces cerevisiae. *Genetics.* 173:557-67.

Murray, A.W., and J.W. Szostak. 1983. Pedigree analysis of plasmid segregation in yeast. *Cell*. 34:961-70.

Murray, K. 1964. The Occurrence of Epsilon-N-Methyl Lysine in Histones. *Biochemistry (Mosc)*. 3:10-5.

Mutiu, A.I., S.M. Hoke, J. Genereaux, G. Liang, and C.J. Brandl. 2007. The role of histone ubiquitylation and deubiquitylation in gene expression as determined by the analysis of an HTB1(K123R) Saccharomyces cerevisiae strain. *Mol Genet Genomics*. 277:491-506.

Nagy, P.L., J. Griesenbeck, R.D. Kornberg, and M.L. Cleary. 2002. A trithorax-group complex purified from Saccharomyces cerevisiae is required for methylation of histone H3. *Proc Natl Acad Sci U S A*. 99:90-4.

Nakai, K., and P. Horton. 1999. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem Sci*. 24:34-6.

Narasimhan, M.L., B. Damsz, M.A. Coca, J.I. Ibeas, D.-J. Yun, J.M. Pardo, P.M. Hasegawa, and R.A. Bressan. 2001. A Plant Defense Response Effector Induces Microbial Apoptosis. *Mol. Cell*. 8:921-930.

Ng, H.H., D.N. Ciccone, K.B. Morshead, M.A. Oettinger, and K. Struhl. 2003a. Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: a potential mechanism for position-effect variegation. *Proc Natl Acad Sci U S A*. 100:1820-5.

Ng, H.H., F. Robert, R.A. Young, and K. Struhl. 2003b. Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. *Mol Cell*. 11:709-19.

Nguyen, M., D.G. Millar, V.W. Yong, S.J. Korsmeyer, and G.C. Shore. 1993. Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. *J. Biol. Chem*. 268:25265-8.

Nislow, C., E. Ray, and L. Pillus. 1997. SET1, a yeast member of the trithorax family, functions in transcriptional silencing and diverse cellular processes. *Mol Biol Cell*. 8:2421-36.

Okada, Y., Q. Feng, Y. Lin, Q. Jiang, Y. Li, V.M. Coffield, L. Su, G. Xu, and Y. Zhang. 2005. hDOT1L links histone methylation to leukemogenesis. *Cell*. 121:167-78.

Okamoto, K., and J.M. Shaw. 2005. Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. *Ann. Rev. Gen*. 39:503-536.

Oki, M., and R.T. Kamakaka. 2002. Blockers and barriers to transcription: competing activities? *Curr Opin Cell Biol*. 14:299-304.

Orlandi, I., M. Bettiga, L. Alberghina, and M. Vai. 2004. Transcriptional profiling of ubp10 null mutant reveals altered subtelomeric gene expression and insurgence of oxidative stress response. *J Biol Chem*. 279:6414-25.

Otsuga, D., B.R. Keegan, E. Brisch, J.W. Thatcher, G.J. Hermann, W. Bleazard, and J.M. Shaw. 1998. The dynamin-related GTPase, Dnm1p, controls mitochondrial morphology in yeast. *J. Cell Biol*. 143:333-49.

Padmanabhan, N., L. Fichtner, A. Dickmanns, R. Ficner, J.B. Schulz, and G.H. Braus. 2009. The yeast HtrA orthologue Ynm3 is a protease with chaperone activity that aids survival under heat stress. *Mol Biol Cell*. 20:68-77.

Pallen, M.J., and C.P. Ponting. 1997. PDZ domains in bacterial proteins. *Mol Microbiol*. 26:411-3.

Parrish, J., L. Li, K. Klotz, D. Ledwich, X. Wang, and D. Xue. 2001. Mitochondrial endonuclease G is important for apoptosis in C. elegans. *Nature*. 412:90-94.

Pereira, C., N. Camougrand, S. Manon, M.J. Sousa, and M. Corte-Real. 2007. ADP/ATP carrier is required for mitochondrial outer membrane permeabilization and cytochrome c release in yeast apoptosis. *Mol. Microbiol*. 66:571-582.

Peter, M.E., and P.H. Krammer. The CD95(APO-1//Fas) DISC and beyond. *Cell Death Differ*. 10:26-35.

Piper, P.W. 2006. Long-lived yeast as a model for ageing research. *Yeast*. 23:215-26.

Plun-Favreau, H., K. Klupsch, N. Moisoi, S. Gandhi, S. Kjaer, D. Frith, K. Harvey, E. Deas, R.J. Harvey, N. McDonald, N.W. Wood, L.M. Martins, and J. Downward. 2007. The

mitochondrial protease HtrA2 is regulated by Parkinson's disease-associated kinase PINK1. *Nat Cell Biol*. 9:1243-52.

Poliakova, D., B. Sokolikova, J. Kolarov, and L. Sabova. 2002. The antiapoptotic protein Bcl-x(L) prevents the cytotoxic effect of Bax, but not Bax-induced formation of reactive oxygen species, in Kluyveromyces lactis. *Microbiology*. 148:2789-95.

Ponting, C.P. 1997. Evidence for PDZ domains in bacteria, yeast, and plants. *Protein Sci*. 6:464-8.

Poon, B., J.B. Jowett, S.A. Stewart, R.W. Armstrong, G.M. Rishton, and I.S. Chen. 1997. Human immunodeficiency virus type 1 vpr gene induces phenotypic effects similar to those of the DNA alkylating agent, nitrogen mustard. *J. Virol*. 71:3961-71.

Poon, I.K., C. Oro, M.M. Dias, J.P. Zhang, and D.A. Jans. 2005. A tumor cell-specific nuclear targeting signal within chicken anemia virus VP3/apoptin. *J Virol*. 79:1339-41.

Powers, R.W., 3rd, M. Kaeberlein, S.D. Caldwell, B.K. Kennedy, and S. Fields. 2006. Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes Dev*. 20:174-84.

Reiter, J., E. Herker, F. Madeo, and M.J. Schmitt. 2005. Viral killer toxins induce caspase-mediated apoptosis in yeast. *J Cell Biol*. 168:353-8.

Reverter-Branchat, G., E. Cabiscol, J. Tamarit, and J. Ros. 2004. Oxidative damage to specific proteins in replicative and chronological-aged Saccharomyces cerevisiae: common targets and prevention by calorie restriction. *J Biol Chem*. 279:31983-9.

Rhoads, R.E. 1999. Signal Transduction Pathways That Regulate Eukaryotic Protein Synthesis. *J. Biol. Chem*. 274:30337-30340.

Riedl, S.J., and Y. Shi. 2004. Molecular mechanisms of caspase regulation during apoptosis. *Nat. Rev. Mol. Cell. Biol*. 5:897-907.

Robzyk, K., J. Recht, and M.A. Osley. 2000. Rad6-dependent ubiquitination of histone H2B in yeast. *Science*. 287:501-4.

Rockenfeller, P., and F. Madeo. 2008. Apoptotic death of ageing yeast. *Exp Gerontol*. 43:876-81.

Roguev, A., D. Schaft, A. Shevchenko, W.W. Pijnappel, M. Wilm, R. Aasland, and A.F. Stewart. 2001. The Saccharomyces cerevisiae Set1 complex includes an Ash2 homologue and methylates histone 3 lysine 4. *Embo J*. 20:7137-48.

Roucou, X., P.N. Giannopoulos, Y. Zhang, J. Jodoin, C.G. Goodyer, and A. LeBlanc. 2005. Cellular prion protein inhibits proapoptotic Bax conformational change in human neurons and in breast carcinoma MCF-7 cells. *Cell Death Differ*. 12:783-95.

Roucou, X., Q. Guo, Y. Zhang, C.G. Goodyer, and A.C. LeBlanc. 2003. Cytosolic prion protein is not toxic and protects against Bax-mediated cell death in human primary neurons. *J. Biol. Chem*. 278:40877-81.

Rout, M.P., G. Blobel, and J.D. Aitchison. 1997. A distinct nuclear import pathway used by ribosomal proteins. *Cell*. 89:715-25.

San-Segundo, P.A., and G.S. Roeder. 2000. Role for the silencing protein Dot1 in meiotic checkpoint control. *Mol Biol Cell*. 11:3601-15.

Sandall, S., F. Severin, I.X. McLeod, J.R. Yates, 3rd, K. Oegema, A. Hyman, and A. Desai. 2006. A Bir1-Sli15 complex connects centromeres to microtubules and is required to sense kinetochore tension. *Cell*. 127:1179-91.

Santos-Rosa, H., R. Schneider, A.J. Bannister, J. Sherriff, B.E. Bernstein, N.C. Emre, S.L. Schreiber, J. Mellor, and T. Kouzarides. 2002. Active genes are tri-methylated at K4 of histone H3. *Nature*. 419:407-11.

Sato, T., M. Hanada, S. Bodrug, S. Irie, N. Iwama, L.H. Boise, C.B. Thompson, E. Golemis, L. Fong, H.G. Wang, and et al. 1994. Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. *Proc. Natl. Acad. Sci. U S A*. 91:9238-42.

Schneider, J., A. Wood, J.S. Lee, R. Schuster, J. Dueker, C. Maguire, S.K. Swanson, L. Florens, M.P. Washburn, and A. Shilatifard. 2005. Molecular regulation of histone H3 trimethylation by COMPASS and the regulation of gene expression. *Mol Cell*. 19:849-56.

Selitrennik, M., L. Duek, R. Lotan, and M. Choder. 2006. Nucleocytoplasmic shuttling of the Rpb4p and Rpb7p subunits of Saccharomyces cerevisiae RNA polymerase II by two pathways. *Eukaryot Cell*. 5:2092-103.

Severin, F.F., and A.A. Hyman. 2002. Pheromone Induces Programmed Cell Death in S. cerevisiae. *Curr. Biol*. 12:R233-R235.

Shahbazian, M.D., K. Zhang, and M. Grunstein. 2005. Histone H2B ubiquitylation controls processive methylation but not monomethylation by Dot1 and Set1. *Mol Cell*. 19:271-7.

Sharikabad, M.N., K.M. Ostbye, T. Lyberg, and O. Brors. 2001. Effect of extracellular Mg(2+) on ROS and Ca(2+) accumulation during reoxygenation of rat cardiomyocytes. *Am J Physiol Heart Circ Physiol*. 280:H344-53.

Shilatifard, A. 2006. Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. *Annu Rev Biochem*. 75:243-69.

Silke, J., and D.L. Vaux. 2001. Two kinds of BIR-containing protein - inhibitors of apoptosis, or required for mitosis. *J. Cell. Sci*. 114:1821-7.

Silva, R.D., R. Sotoca, B. Johansson, P. Ludovico, F. Sansonetty, M.T. Silva, J.M. Peinado, and M. Corte-Real. 2005. Hyperosmotic stress induces metacaspase- and mitochondria-dependent apoptosis in Saccharomyces cerevisiae. *Mol Microbiol*. 58:824-34.

Sinclair, D.A., and L. Guarente. 1997. Extrachromosomal rDNA circles--a cause of aging in yeast. *Cell*. 91:1033-42.

Singer, M.S., A. Kahana, A.J. Wolf, L.L. Meisinger, S.E. Peterson, C. Goggin, M. Mahowald, and D.E. Gottschling. 1998. Identification of high-copy disruptors of telomeric silencing in Saccharomyces cerevisiae. *Genetics*. 150:613-32.

Skoufias, D.A., C. Mollinari, F.B. Lacroix, and R.L. Margolis. 2000. Human Survivin Is a Kinetochore-associated Passenger Protein. *J. Cell Biol*. 151:1575-1582.

Smith, J.S., C.B. Brachmann, L. Pillus, and J.D. Boeke. 1998. Distribution of a limited Sir2 protein pool regulates the strength of yeast rDNA silencing and is modulated by Sir4p. *Genetics*. 149:1205-19.

Sokolov, S., A. Pozniakovsky, N. Bocharova, D. Knorre, and F. Severin. Expression of an expanded polyglutamine domain in yeast causes death with apoptotic markers. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. 1757:660-666.

Somasundaran, M., M. Sharkey, B. Brichacek, K. Luzuriaga, M. Emerman, J.L. Sullivan, and M. Stevenson. 2002. Evidence for a cytopathogenicity determinant in HIV-1 Vpr. *Proc. Natl. Acad. Sci. USA*. 99:9503-8.

Sterner, D.E., and S.L. Berger. 2000. Acetylation of histones and transcription-related factors. *Microbiol Mol Biol Rev*. 64:435-59.

Strahl, B.D., and C.D. Allis. 2000. The language of covalent histone modifications. *Nature*. 403:41-5.

Strahl, B.D., R. Ohba, R.G. Cook, and C.D. Allis. 1999. Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in Tetrahymena. *Proc Natl Acad Sci U S A*. 96:14967-72.

Straight, A.F., W. Shou, G.J. Dowd, C.W. Turck, R.J. Deshaies, A.D. Johnson, and D. Moazed. 1999. Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. *Cell*. 97:245-56.

Sun, L., and Z.J. Chen. 2004. The novel functions of ubiquitination in signaling. *Curr Opin Cell Biol*. 16:119-26.

Sun, Z.W., and C.D. Allis. 2002. Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature*. 418:104-8.

Suzuki, Y., Y. Nakabayashi, K. Nakata, J.C. Reed, and R. Takahashi. 2001a. X-linked inhibitor of apoptosis protein (XIAP) inhibits caspase-3 and -7 in distinct modes. *J Biol Chem*. 276:27058-63.

Suzuki, Y., Y. Nakabayashi, and R. Takahashi. 2001b. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and

enhances its anti-apoptotic effect in Fas-induced cell death. *Proc. Natl. Acad. Sci. U S A*. 98:8662-7.

Suzuki, Y., K. Takahashi-Niki, T. Akagi, T. Hashikawa, and R. Takahashi. 2004. Mitochondrial protease Omi/HtrA2 enhances caspase activation through multiple pathways. *Cell Death Differ*. 11:208-16.

Temme, A., M. Rieger, F. Reber, D. Lindemann, B. Weigle, P. Diestelkoetter-Bachert, G. Ehninger, M. Tatsuka, Y. Terada, and E.P. Rieber. 2003. Localization, Dynamics, and Function of Survivin Revealed by Expression of Functional SurvivinDsRed Fusion Proteins in the Living Cell. *Mol. Biol. Cell*. 14:78-92.

Temple, M.D., G.G. Perrone, and I.W. Dawes. 2005. Complex cellular responses to reactive oxygen species. *Trends Cell Biol*. 15:319-26.

Thomas, S., and K.B. Kaplan. 2007. A Bir1p Sli15p Kinetochore Passenger Complex Regulates Septin Organization during Anaphase. *Mol. Biol. Cell*. 18:3820-34.

Tompa, R., and H.D. Madhani. 2007. Histone H3 lysine 36 methylation antagonizes silencing in Saccharomyces cerevisiae independently of the Rpd3S histone deacetylase complex. *Genetics*. 175:585-93.

Tong, W., O.I. Kulaeva, D.J. Clark, and L.C. Lutter. 2006. Topological analysis of plasmid chromatin from yeast and mammalian cells. *J Mol Biol*. 361:813-22.

Trancikova, A., P. Weisova, I. Kissova, I. Zeman, and J. Kolarov. 2004. Production of reactive oxygen species and loss of viability in yeast mitochondrial mutants: protective effect of Bcl-xL. *FEMS Yeast Res*. 5:149-56.

Uren, A.G., T. Beilharz, M.J. O'Connell, S.J. Bugg, R. van Driel, D.L. Vaux, and T. Lithgow. 1999. Role for yeast inhibitor of apoptosis (IAP)-like proteins in cell division. *Proc. Natl. Acad. Sci. U S A*. 96:10170-10175.

Uren, A.G., E.J. Coulson, and D.L. Vaux. 1998. Conservation of baculovirus inhibitor of apoptosis repeat proteins (BIRPs) in viruses, nematodes, vertebrates and yeasts. *Trends Biochem. Sci*. 23:159-62.

Uren, A.G., K. O'Rourke, L.A. Aravind, M.T. Pisabarro, S. Seshagiri, E.V. Koonin, and V.M. Dixit. 2000. Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Mol Cell*. 6:961-7.

Vachova, L., F. Devaux, H. Kucerova, M. Ricicova, C. Jacq, and Z. Palkova. 2004. Sok2p transcription factor is involved in adaptive program relevant for long term survival of Saccharomyces cerevisiae colonies. *J Biol Chem*. 279:37973-81.

Vachova, L., and Z. Palkova. 2005. Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia. *J. Cell Biol*. 169:711-717.

van Leeuwen, F., P.R. Gafken, and D.E. Gottschling. 2002. Dot1p modulates silencing in yeast by methylation of the nucleosome core. *Cell*. 109:745-56.

Vande Walle, L., M. Lamkanfi, and P. Vandenabeele. 2008. The mitochondrial serine protease HtrA2/Omi: an overview. *Cell Death Differ*. 15:453-60.

Vander Heiden, M.G., J.S. Choy, D.J. VanderWeele, J.L. Brace, M.H. Harris, D.E. Bauer, B. Prange, S.J. Kron, C.B. Thompson, and C.M. Rudin. 2002. Bcl-x(L) complements Saccharomyces cerevisiae genes that facilitate the switch from glycolytic to oxidative metabolism. *J. Biol. Chem*. 277:44870-6.

Vaux, D.L., S. Cory, and J.M. Adams. 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*. 335:440-442.

Vaux, D.L., and J. Silke. 2005. IAPs, RINGs and ubiquitylation. *Nat. Rev. Mol. Cell. Biol*. 6:287-297.

Venkatasubrahmanyam, S., W.W. Hwang, M.D. Meneghini, A.H. Tong, and H.D. Madhani. 2007. Genome-wide, as opposed to local, antisilencing is mediated redundantly by the euchromatic factors Set1 and H2A.Z. *Proc Natl Acad Sci U S A*. 104:16609-14.

Verhagen, A.M., E.J. Coulson, and D.L. Vaux. 2001. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol*. 2:R3009.

Verhagen, A.M., J. Silke, P.G. Ekert, M. Pakusch, H. Kaufmann, L.M. Connolly, C.L. Day, A. Tikoo, R. Burke, C. Wrobel, R.L. Moritz, R.J. Simpson, and D.L. Vaux. 2002. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J Biol Chem*. 277:445-54.

Vernooy, S.Y., V. Chow, J. Su, K. Verbrugghe, J. Yang, S. Cole, M.R. Olson, and B.A. Hay. 2002. Drosophila Bruce Can Potently Suppress Rpr- and Grim-Dependent but Not Hid-Dependent Cell Death. *Curr. Biol*. 12:1164-1168.

Wadskog, I., C. Maldener, A. Proksch, F. Madeo, and L. Adler. 2004. Yeast lacking the SRO7/SOP1 encoded tumor suppressor homologue shows increased susceptibility to apoptosis-like cell death on exposure to NaCl stress. *Mol. Biol. Cell*:E03-02-0114.

Walter, D., S. Wissing, F. Madeo, and B. Fahrenkrog. 2006. The inhibitor-of-apoptosis protein Bir1p protects against apoptosis in S. cerevisiae and is a substrate for the yeast homologue of Omi/HtrA2. *J. Cell Sci*. 119:1843-51.

Watanabe, K., J. Morishita, K. Umezu, K. Shirahige, and H. Maki. 2002. Involvement of RAD9-dependent damage checkpoint control in arrest of cell cycle, induction of cell death, and chromosome instability caused by defects in origin recognition complex in Saccharomyces cerevisiae. *Eukaryot Cell*. 1:200-12.

Wei, M., P. Fabrizio, J. Hu, H. Ge, C. Cheng, L. Li, and V.D. Longo. 2008. Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. *PLoS Genet*. 4:e13.

Weinberger, M., L. Feng, A. Paul, D.L. Smith, Jr., R.D. Hontz, J.S. Smith, M. Vujcic, K.K. Singh, J.A. Huberman, and W.C. Burhans. 2007. DNA replication stress is a determinant of chronological lifespan in budding yeast. *PLoS ONE*. 2:e748.

Weinberger, M., L. Ramachandran, L. Feng, K. Sharma, X. Sun, M. Marchetti, J.A. Huberman, and W.C. Burhans. 2005. Apoptosis in budding yeast caused by defects in initiation of DNA replication. *J Cell Sci*. 118:3543-3553.

Widlund, P.O., J.S. Lyssand, S. Anderson, S. Niessen, J.R. Yates, 3rd, and T.N. Davis. 2006. Phosphorylation of the chromosomal passenger protein Bir1 is required for localization of Ndc10 to the spindle during anaphase and full spindle elongation. *Mol. Biol. Cell*. 17:1065-74.

Wilson, R., L. Goyal, M. Ditzel, A. Zachariou, D.A. Baker, J. Agapite, H. Steller, and P. Meier. 2002. The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. *Nat. Cell Biol*. 4:445-50.

Wissing, S., P. Ludovico, E. Herker, S. Buttner, S.M. Engelhardt, T. Decker, A. Link, A. Proksch, F. Rodrigues, M. Corte-Real, K.U. Frohlich, J. Manns, C. Cande, S.J. Sigrist, G. Kroemer, and F. Madeo. 2004. An AIF orthologue regulates apoptosis in yeast. *J. Cell Biol*. 166:969-74.

Wohlschlegel, J.A., E.S. Johnson, S.I. Reed, and J.R. Yates, 3rd. 2004. Global analysis of protein sumoylation in Saccharomyces cerevisiae. *J. Biol. Chem*. 279:45662-8.

Wood, A., N.J. Krogan, J. Dover, J. Schneider, J. Heidt, M.A. Boateng, K. Dean, A. Golshani, Y. Zhang, J.F. Greenblatt, M. Johnston, and A. Shilatifard. 2003a. Bre1, an E3 Ubiquitin Ligase Required for Recruitment and Substrate Selection of Rad6 at a Promoter. *Molecular Cell*. 11:267-274.

Wood, A., J. Schneider, J. Dover, M. Johnston, and A. Shilatifard. 2003b. The Paf1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p. *J Biol Chem*. 278:34739-42.

Woods, R.A., and R.D. Gietz. 2001. High-efficiency transformation of plasmid DNA into yeast. *Methods Mol Biol*. 177:85-97.

Wyce, A., T. Xiao, K.A. Whelan, C. Kosman, W. Walter, D. Eick, T.R. Hughes, N.J. Krogan, B.D. Strahl, and S.L. Berger. 2007. H2B Ubiquitylation Acts as a Barrier to Ctk1

Nucleosomal Recruitment Prior to Removal by Ubp8 within a SAGA-Related Complex. 27:275-288.

Wysocki, R., A. Javaheri, S. Allard, F. Sha, J. Cote, and S.J. Kron. 2005. Role of Dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9. *Mol Cell Biol*. 25:8430-43.

Wysocki, R., and S.J. Kron. 2004. Yeast cell death during DNA damage arrest is independent of caspase or reactive oxygen species. *J Cell Biol*. 166:311-6.

Xiao, T., C.F. Kao, N.J. Krogan, Z.W. Sun, J.F. Greenblatt, M.A. Osley, and B.D. Strahl. 2005. Histone H2B ubiquitylation is associated with elongating RNA polymerase II. *Mol Cell Biol*. 25:637-51.

Xu, Q., and J.C. Reed. 1998. Bax Inhibitor-1, a Mammalian Apoptosis Suppressor Identified by Functional Screening in Yeast. *Mol. Cell*. 1:337-346.

Yamaki, M., T. Umehara, T. Chimura, and M. Horikoshi. 2001. Cell death with predominant apoptotic features in Saccharomyces cerevisiae mediated by deletion of the histone chaperone ASF1/CIA1. *Genes to Cells*. 6:1043-1054.

Yoon, H.-J., and J. Carbon. 1999. Participation of Bir1p, a member of the inhibitor of apoptosis family, in yeast chromosome segregation events. *Proc. Natl. Acad. Sci. U S A*. 96:13208-13213.

Zelivianski, S., D. Liang, M. Chen, B.L. Mirkin, and R.Y. Zhao. 2006. Suppressive effect of elongation factor 2 on apoptosis induced by HIV-1 viral protein R. *Apoptosis*. 11:377-88.

Zhang, L., K. Onda, R. Imai, R. Fukuda, H. Horiuchi, and A. Ohta. 2003. Growth temperature downshift induces antioxidant response in Saccharomyces cerevisiae. *Biochem. Biophys. Res. Commun*. 307:308-14.

Zhang, Y., and D. Reinberg. 2001. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev*. 15:2343-60.

Zhao, Y., J. Cao, M.R. O'Gorman, M. Yu, and R. Yogev. 1996. Effect of human immunodeficiency virus type 1 protein R (vpr) gene expression on basic cellular function of fission yeast Schizosaccharomyces pombe. *J. Virol*. 70:5821-6.

Zhao, Y., M. Yu, M. Chen, R.T. Elder, A. Yamamoto, and J. Cao. 1998. Pleiotropic effects of HIV-1 protein R (Vpr) on morphogenesis and cell survival in fission yeast and antagonism by pentoxifylline. *Virology*. 246:266-76.

Zhou, W., J.J. Ryan, and H. Zhou. 2004. Global analyses of sumoylated proteins in Saccharomyces cerevisiae. Induction of protein sumoylation by cellular stresses. *J. Biol. Chem*. 279:32262-8.

Zhu, B., Y. Zheng, A.D. Pham, S.S. Mandal, H. Erdjument-Bromage, P. Tempst, and D. Reinberg. 2005. Monoubiquitination of human histone H2B: the factors involved and their roles in HOX gene regulation. *Mol Cell*. 20:601-11.

Zoratti, M., I. Szabo, and U. De Marchi. 2005. Mitochondrial permeability transitions: how many doors to the house? *Biochim. Biophys. Acta*. 1706:40-52.

Zuckerman, V., K. Wolyniec, R.V. Sionov, S. Haupt, and Y. Haupt. 2009. Tumour suppression by p53: the importance of apoptosis and cellular senescence. *J Pathol*. 219:3-15.

Zuo, S., S. Zou, J. Luo, W. Guo, L. Xu, J. Dong, and M. Liu. 2005. Effect of antisense MBD1 gene eukaryotic expression plasmid on expression of MBD1 gene in human biliary tract carcinoma cells. *J Huazhong Univ Sci Technolog Med Sci*. 25:658-61.

7

Appendix

7.1 List of publications

Research article

Walter, D., Wissing, S., Madeo, F. and Fahrenkrog, B. (2006).
The inhibitor-of-apoptosis protein Bir1p protects against apoptosis in *S. cerevisiae* and is a substrate for the yeast homologue of Omi/HtrA2.
J Cell Sci 119, 1843-51.

Belanger, K. D., Walter, D., Henderson, T. A., Yelton, A. L., O'Brien, T. G., Belanger, K. G., Geier, S. J. and Fahrenkrog, B. (2009).
Nuclear localisation is crucial for the proapoptotic activity of the HtrA-like serine protease Nma111p.
J Cell Sci 122, 3931-41.

Walter, D., Matter, A. and Fahrenkrog, B. (2010).
Bre1p mediated histone H2B ubiquitination regulates apoptosis in *S. cerevisiae*
Manuscript in Revision for *J Cell Sci*

Walter, D., Matter, A. and Fahrenkrog, B. (2010).
The histone H3 methyltransferase Dot1p is required for apoptosis in budding yeast
Manuscript in preparation

Review

Walter, D., Owsianowski, E. and Fahrenkrog, B. (2008).
Negative regulation of apoptosis in yeast.
Biochim Biophys Acta 1783, 1303-10.

7.2 Curriculum Vitae

Name	David Walter
Date of Birth:	19.03.1979
Place of Birth	Basel, Switzerland
Current Address	Klingelbergstrasse 70 CH-4056 Basel, Switzerland
E-Mail	david.walter@unibas.ch
Telefon	+41 61 267 2255

EDUCATION

04/2006	**Start of PhD thesis** in the lab of Prof. Dr. Birthe Fahrenkrog M.E.Müller Institute for Structural Biology, Biozentrum, University of Basel, Switzerland Advisor: Prof. Dr. Birthe Fahrenkrog Thesis topic: Histone modifications and apoptosis in yeast
10/2004 - 11/2005	**Master in Molecular Biology**, Major in Biochemistry M.E.Müller Institute for structural Biology, Biozentrum, University of Basel, Switzerland Prof. Dr. Ueli Aebi Advisor: Prof. Dr. Birthe Fahrenkrog Thesis topic: Characterization of the functional role of Bir1p in yeast apoptosis
07/2004 - 09/2004	**Internship** in the lab of Prof. Dr. Renata Freitas University of Ouro Preto, Brasil Topic: Differential regulation of plasma cholesterol by Apolipoprotein E isoforms

07/2004	**Bachelor in Molecular Biology** Major Molecular Biology, elective subject: Medical Biology University of Basel, Switzerland
10/2001	Matriculation at the University of Basel, Switzerland
1993 - 2000	High school; Matura Typus B; main subjects: Latin Kantonsschule Solothurn, Switzerland
1986 – 1993	Primary school at Grenchen, Switzerland

TALKS AND POSTERS PRESENTED AT MEETINGS AND ATTENDED COURSES

Talks	2009, Graz, Austria 7th international meeting on yeast apoptosis A histone crosstalk regulates budding yeast life and death
	2009, Engelberg, Switzerland PhD retreat of the Biozentrum, University of Basel The E3 ubiquitin ligase Bre1p protects against apoptosis in *S. cerevisiae*
	2008, Leuven, Belgium 6th international meeting on yeast apoptosis Bre1p protects against apoptosis in *S. cerevisiae* through its E3 ubiquitin ligase activity
Posters	2009, Jerusalem, Israel 17th School in Life Sciences "Nuclear Organization and Dynamics" A histone crosstalk regulates budding yeast life and death
	2008, San Francisco, US ASCB 48th Annual Meeting The E3 ubiquitin ligase Bre1p protects against DNA replication stress and apoptosis in *S. cerevisiae*
	2008, Leuven, Belgium 6th international meeting on yeast apoptosis Identification of substrates for the ubiquitin-ligase Bre1p
	2007, Dresden, Germany ELSO Annual Meeting

	Bre1p protects against apoptosis in *S. cerevisiae* and is a direct binding partner of the inhibitor-of-apoptosis protein Bir1p
	2006, Kutná Hora, Czech Republic 5th international meeting on yeast apoptosis The inhibitor of apoptosis protein Bir1p protects against apoptosis in *S. cerevisiae* and is a substrate for the yeast homologue of Omi/HtrA2
Courses	One week course at "The Onassis Foundation Science Lecture Series" Heraklion, Crete Topic: Programmed Cell Death and Cell Signaling in Development and Disease

SUPERVISION

2008 - 2009	Anja Matter Master Student, Biozentrum, University of Basel, Switzerland
2007 - 2008	Therese Tschon Master Student, Biozentrum, University of Basel, Switzerland

TEACHING

2006 - Present	Immunofluorescence microscopy course Part of the Blockkurs "Biophysics and Structural Biology" Biozentrum, University of Basel

PROFFESIONAL EXPERIENCE

2001 - 2003	Working as a Werkstudent in a lab of the Pharmacy company Polyform AG in Allschwil (Switzerland)
2000 - 2001	Web designer at buysite.ch AG in Basel

7.3 Acknowledgement

First, I would like to thank my thesis advisor Prof. Dr. Birthe Fahrenkrog for all her guidance and support during my years in her lab. She has always had an open door and ear for any problems I was having and never failed to be enthusiastic in helping me solve these issues. I am also grateful to Prof. Dr Ueli Aebi for his support throughout my whole time at the M.E. Müller Institute for Structural Biology.

I am thankful to Prof. Dr. Joris Winderickx and Prof. Dr. Peter Philippsen who readily agreed to act as co-referee and as chairman in my PhD exam.

Many thanks to thank PD Dr. Cora-Ann Schoenenberger and Dr. Christian Mittelholzer for inspiring discussions about my project and helpful suggestions.

I've had the luck of supervising two excellent Master students, Anja Matter and Therese Tschon. It was a privilege to work together with them on my project.

I would like to thank Dr. Teba Al-Haboubi for being a good friend throughout my whole time at the M.E. Müller Institute for Structural Biology.

I am grateful to the former members of the lab Dr. Sara Paullilo, Dr. Joachim Köser, Dr. Esther Owsianowski, Dr. Kyrill Schwarz Herion, Dr. Ulrich Schröder, Prof. Dr. Laurent Kreplak, Dr Bohumil Maco, Ilona Hügi, Josephine Jüttener, Kai Schleicher for being always very helpful.

I am grateful for the excellent technical and administrative support form Robert Wyss, Beat Schuhmacher, Liselotte Walti, Margrit Jenni, Roland Buerki and Barbara Merz.

I would like to thank Prof. Dr. Roderick Lim, all the members of the Lim lab, Marija Plodinec, Dr. Marko Loparic and Unai Silvan for all their help and for the good time we had outside the lab.

I am grateful to the M.E. Müller Institute for Structural Biology and the Swiss National Foundation for financial support of my project.

Finally, Yvonne Lussi has been the most important person in my life for the past years. We have had many adventures during the time of our PhD thesis and I can't wait to see what comes next.

Last but not least I would like to thank my family for all their support throughout my PhD thesis, it would not have been possible without all of you.