Apoptosis in the Yeast *Saccharomyces cerevisiae*:

A Novel Cell Death Process Regulated by the Ubiquitin–Proteasome System

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Gerade Tatsachen gibt es nicht,
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1 Zusammenfassung

In der hier vorgelegten Arbeit wurde die Hefe *Saccharomyces cerevisiae* als ein Modellsystem zur Untersuchung der Apoptose – einer Form des programmierten Zelltods – verwendet. Die auch als Bäckerhefe bekannte *S. cerevisiae* ist Methoden von der Biochemie bis zur molekularen Genetik zugänglich und daher ein ideales Modellsystem für eine Eukaryontenzelle. In dieser Arbeit wurde *S. cerevisiae* insbesondere dazu benutzt, die Funktionen des Ubiquitin-Proteasomen-Systems in der Apoptose zu studieren.

Vielzellige Organismen müssen während der Entwicklung die Anordnung ihrer Zellen kontrollieren. Im Erwachsenen-Stadium muss die Zahl der Zellen konstant gehalten werden. In beiden Prozessen / Zuständen spielt die Kontrolle der Zellen durch programmierten Zelltod eine wichtige Rolle: So müssen überflüssige, gealterte, geschädigte aber auch "gefährliche" Zellen entfernt und durch neu erzeugte Zellen ersetzt werden. Der Verlust der Balance zwischen dem Zellwachstum und dem programmierten Zelltod führt kann daher während der Entwicklung zum Auftreten schwerer Missbildungen führen, im erwachsenen Organismus aber die Ursache für fatale Erkrankungen wie zum Beispiel Krebs, neurodegenerative oder Autoimmunerkrankungen sein.

Apoptose, als besondere Form des programmierten Zelltods, hat eine wichtige Rolle in der Entwicklung und Homöostase von Metazoen. Der Apoptoseprozess ist morphologisch durch das Auftreten bestimmter Veränderungen charakterisiert: So findet man ein Aufhebung der asymmetrischen Verteilung der Phospholipide der Plasmamembran, die Kondensation und Anlagerung des Chromatins an der Kernhülle sowie schließlich die Fragmentierung der Zellen in so genannte "apoptotische Körper". Die so entstandenen Zellfragment werden anschließend durch Makrophagen aufgenommen und verdaut. Apoptose verläuft nach einem definierten Programm und in einer geordneten Art und Weise, während Nekrose eine

nicht organisierte Form des Zelltods darstellt, die zu einem Austritt des Zellinhalts in den extrazellulären Raum sowie zur Induktion eine entzündlichen Prozesses führt.

Das Apoptoseprogramm wird unter anderem reguliert durch eine Reihe von Bcl-2 verwandten Proteinen. Diese gehören zu einer konservierten Proteinfamilie und umfassen sowohl Apoptose-induzierende Proteine wie Bax, Bak, Bcl-XS als auch Antagonisten wie Bcl-2 und Bcl-XL. Die Entscheidung den Apoptose-Prozess auszulösen wird durch das direkte Zusammenspiel dieser Komponenten getroffen. Dieser Prozess beeinflusst sehr wahrscheinlich die Ausbildung von Kanälen in intrazellulären Membranen sowie die Interaktion mit anderen Protein. Der ultimative Zweck dieser Induktion ist die Aktivierung einer besonderen Gruppe von Cysteinproteasen, den Caspasen.

Caspasen sind zentralen Effektoren der Apoptose; sie spalten eine Reihe bestimmter Zielproteine. Die pro-apoptotischen Komponenten der Bcl-2 Familie lösen den Zelltod durch Induktion der Freisetzung von Cytochrom C (und anderen apoptotischen Effektoren) aus den Mitochondrien aus. Freigesetztes Cytochrom C bindet an das Apaf-1 Protein, das schließlich als Adaptor einen Komplex mit Caspase-9 eingeht und so das so genannte Apoptosom ausbildet. Hierbei wird angenommen, dass die Antagonisten der Apoptose auf zwei Ebenen der Induktion des Zelltods entgegenwirken können: Sie sind in der Lage einerseits die Freisetzung von Cytochrom C aus den Mitochondrien zu verhindern, können andererseits auch die Aktivierung der Casapase Kaskade durch Bindung und Inhibition des Apaf-1 Proteins blockieren.

Auch auf Zellebene muss die Konzentration der unterschiedlichen Komponenten und Zellbestandteile genau reguliert werden. Proteolyse spielt hierbei eine wichtige Rolle in der Kontrolle der Effektoren der Zelle, der Proteine. So müssen konstant Proteine abgebaut werden, die durch Synthesefehler oder bestimmte Umgebungseinflüsse geschädigt wurden.

Andererseits müssen Proteine – zum Beispiel bestimmte metabolische Enzyme - entfernt werden, deren Funktion momentan nicht gebraucht wird. Proteolytische Prozesse spielen darüber hinaus eine lebenswichtige Rolle für die Steuerung unterschiedlichster Zellprogramme, wie zum Beispiel des Programms der Zellteilung.

Um die Schädigung weiter benötigter Proteine zu garantieren muss intrazelluläre Proteolyse streng kontrolliert und hoch-spezifisch ablaufen. Die dafür notwendige Selektivität wird durch Kompartimentierung erreicht. Proteolyse findet daher hauptsächlich im membranumschlossenen Raum des Lysosoms oder im Mikrokompartiment hochmolekularer, aus einer Vielzahl von Untereinheiten aufgebauter Proteasekomplexe, den Proteasomen statt.

Proteasomen sind die zentralen Funktionseinheiten eines besonderen Abbauweges, des Ubiquitin-Proteasomen-Systems. Proteine, die durch das Proteasom erkannt und degradiert werden, müssen durch Modifizierung mit Polyubiquitinketten markiert sein. Die Ubiquitinierung wird durch eine komplexes Enzymsystem vermittelt: Ubiquitin – ein kleines Protein aus 76 Aminosäuren - wird dabei zuerst durch Bindung seines C-terminalen Glycineinheit an ein internes Cystein und Bildung eines Thioesterbindung mit einem Ubiquitin-aktivierenden Enzym (E1) aktiviert. Dieses überträgt anschließend die Ubiquitineinheit auf das aktive Cystein eines Ubiquitin-konjugierenden Enzym (E2). Dieses modifiziert schließlich – in den meisten Fällen unter Mithilfe eine Substraterkennungsproteins (E3) –das jeweilige Zielsubstrat durch Transfer des Ubiquitins auf die ε-Aminosäure eines Lysinrests des Substrats.

Ubiquitinierte Substrate werden durch das 26S Proteasom erkannt und abgebaut. Dieses besteht aus einen proteolytisch aktiven 20S Kernkomplex (20S Proteasom) sowie zwei zusätzliche 19S Regulatorkomplexen. Das 20S Proteasom ist ein Hohlzylinderförmiger Komplex, der aus vier Lagen von Ringen mit je sieben Unterein-

heiten aufgebaut wird. Die mittleren Ringe bestehen aus jeweils sieben β-Untereinheiten, während die äußeren Ringe aus je sieben α-Untereinheiten aufgebaut sind. Die aktiven Zentren des Komplexes liegen auf β-Typ-Untereinheiten und sind in die zentrale Reaktionskammer des 20S Proteasoms gerichtet. Proteine, die durch das Proteasom abgebaut werden, müssen daher entfaltet und in den zentralen Kanal des 20S Proteasoms transferiert werden. Diese Funktion wird neben der Substraterkennung durch die an beide Enden des 20S Proteasoms assoziierten 19S regulatorischen Komplexe vermittelt. Diese Komplexe binden über bestimmte Untereinheiten ubiquitinierte Substratproteine, entfalten diese und transportieren die Peptidkette zu den aktiven Zentren des Kernmoduls. Dabei spielen ATPase Untereinheiten, die zur Familie der AAA Proteine gehören, ein Rolle. Während Ubiquitin im Verlauf des Abbauprozesses durch Ubiquitinasen abgespalten und für weitere Ubiquitinierungen wiederverwertet wird, spaltet das Proteasom das Substratprotein in kurzkettige Oligo-Peptide.

In einer vorausgehenden Arbeit war gezeigt worden, dass eine Mutation im *CDC48* Gen, das für eine ATPase der AAA-Familie codiert, in Hefezellen einen Zelltod auslösen kann, der mit den in Säugetierzellen Apoptose-typischen morphologischen Veränderungen einhergeht. Dabei konnte bislang für Cdc48 jedoch keine Funktion in der Apoptose aufgezeigt werden. Es wurde aber klar, dass die Expression des aus Säugetierzellen stammenden pro-apoptotischen Regulators Bax in Hefe einen Zelltod auslöst. Dieser Prozess konnte darüber hinaus durch gleichzeitige Expression des ebenfalls aus Säugetierzellen stammenden Apoptoseinhibitors Bcl-XL verhindert werden. Diese Daten legten nahe, dass Bax und Bcl-XL in Hefezellen ähnliche wenn nicht identische Funktionen ausüben können. Es war aber nicht bekannt ob der durch Bax in *S. cerevisiae* induzierte Zelltod tatsächlich Apoptose-typisch verläuft.

Um die Art des durch Bax in Hefezellen ausgelösten Zelltods näher zu charakterisieren wurde versucht, Tests für die in Säugerzellen auftretenden Apoptosemerkmale in Hefezellen zu etablieren. Um den Zustand der Plasmamembran im Verlauf des Bax induzierten Zelltods in Hefe zu messen wurde an GFP (green fluorescent protein) gekoppeltes Annexin V eingesetzt. Annexin V bindet in Gegenwart von Calcium Ionen an Phosphatidylserin. Diese befindet sich in lebenden Eukaryontenzellen fast vollständig (95%) in der inneren Lage des Membran-Lipidschichten. Wird GFP-Annexin V auf intakte nicht apoptotische Zellen angewandt, tritt keine Bindung der Zelloberfläche auf. Im Fluoreszenzmikroskop ist keine Markierung der Zelloberfläche mit GFP-Annexin feststellbar. Die Phosphatitylserinasymetrie wird aber früh im Verlauf der Apoptose aufgehoben. An der Oberfläche der Zelle kann dann eine GFP-Annexinmarkierung beobachtet werden. Zum Nachweis von Änderungen der Kernmorphologie wurden Zellen, bei denen das Chromatin durch den einen DNA-interkalierenden Fluoreszenzfarbstoff angefärbt wurde, im Elektronenmikroskop untersucht (elektronenmikroskopische Untersuchungen wurden in Kooperation mit Dr. Eleanore Fröhlich durchgeführt). Um den Intaktheitsgrad der DNA zu messen wurde der so genannte TUNEL Test (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) angewandt. Bei diesem Test werden freie 3'-Enden der DNA mit Hilfe eine farbmarkierten Nukleotids modifiziert. Der bei Apoptose-induzierter DNA-Fragmentierung (Auftreten einer Vielzahl von 3'-Enden) mögliche Einbau von markiertem Nukleotid kann entweder durch direkte Fluoreszenzmikroskopie oder indirekte Immunofluoreszenzmikroskopie nachgewiesen werden.

Mit Hilfe dieser Methoden durchgeführte Studien zeigten, dass das Bax Protein bei Expression in Hefezellen einen Zelltod induziert, der die typischen Anzeichen der Apoptose in Säugerzelle aufweist: So wurde ein Verlust der asymmetrischen Verteilung von Phosphatidylserin in der Plasmamembran sowie das Auftreten von Vesikeln an der Plasmamembran

(membrane blebbing) beobachtet. Darüber hinaus war ein Kondensieren des Chromatins sowie eine anschließende Anlagerung an die Kernhülle feststellbar. Außerdem konnte mit Hilfe des TUNEL Tests eindeutig eine Fragmentierung der DNA nachgewiesen werden. Die gleichzeitige Überexpression des Apoptose-Inhibitors Bcl-XL konnte nicht nur den Tod der Hefezellen, sondern auch das Auftreten dieser typischen morphologischen Veränderung verhindern. Diese Untersuchungen - unter Einsatz typischer für den Nachweis von Apoptose in Säugerzellen verwendeter Tests - zeigten im Gegensatz zu früheren Studien klar, dass der durch Bax in *S. cerevisiae* Zellen induzierte Zelltod klar apoptotischer Natur ist. Diese Ergebnisse führten zu der Frage über welchen Mechanismus Bax in Hefe den Zelltod auslöst. In Säugerzellen induziert Bax die Apoptose indem es die Aktivierung der Caspasen bewirkt. Diese Proteasen fehlen in Hefezellen vollständig. Es war deshalb interessant herauszufinden in welcher Weise Bax in Hefezellen wirkt.

Sauerstoffradikale sind wichtige Mediatoren der Apoptose in Metazoen. Aus der Literatur war zumindest ein Fall bekannt, bei dem Bax den apoptotischen Zelltod nicht durch Aktivierung der Caspasen sondern durch Erhöhung der Produktion reaktiven Sauerstoff bewirkte. Die vermehrte Bildung aktiven Sauerstoffs kann mit hoher Wahrscheinlichkeit auf die Freisetzung von Cytochrom C aus dem mitochondriellen Intermembranraum und damit einer Störung der Atmungskette zurückgeführt werden.

Um die Rolle von Sauerstoffradikalen im durch Bax induzierten Zelltod der Hefezelle zu untersuchen, wurden Zellen in denen Bax überexprimiert wurde, mit Dihydro-Rhodamin-123 versetzt. Dieses farblose Reagens kann in Zellen eindringen und dort durch Oxidation mit reaktivem Sauerstoff in eine fluoreszierende Form überführt werden. Durch Auftreten dieser Fluoreszenz lies sich daher im Mikroskop das Vorhandensein von reaktivem Sauerstoff nachweisen. Dies war für Bax exprimierende Zellen

tatsächlich der Fall. Bei Verminderung der Radikale durch Einsatz von Radikalfängern oder bei Anziehen der Zellen in sauerstofffreiem Medium konnte der durch Bax induzierte Zelltod verhindert werden. Ähnliche Befunde wurden für den durch mutiertes Cdc48 ausgelösten Zelltod der Hefe erhalten. Diese Daten legen nahe, dass reaktive Radikale nicht als Nebenprodukte der Apoptose der Hefezelle auftreten, sondern als Apoptose auslösende Komponenten wirken.

Es konnte außerdem gezeigt werden, dass bei Anwendung niedrige Konzentrationen von H_2O_2 durch Einwirkung von Cycloheximid (Inhibition der Proteinneusynthese) die Überlebensrate von Hefezellen erhöht wurde. Durch elektronenmikroskopische Untersuchungen sowie mit Hilfe des TUNEL Tests konnte gezeigt werden, dass Cycloheximid sowohl die apoptotische Chromatin-Kondensation und DNA-Fragmentierung verhindert wie auch zu einem erhöhten Auftreten von nekrotischen Zellen führt. Die Verhütung des Zelltods durch Inhibition der Proteinneusynthese ist in Metazoen ein spezifisches Zeichen, zur Unterscheidung zwischen Apoptose und Nekrose. Die hier vorgelegten Daten deuten darauf hin, dass der durch niedrige Konzentration an H_2O_2 ausgelöste Zelltod nicht in einer generellen Schädigung der Zelle begründet ist, sondern eine aktive Beteiligung der Zelle am tödlichen Prozess benötigt. Umfangreiche Schädigungen der Zellstrukturen und des Zellmetabolismus verhindern offensichtlich eine aktive Beteiligung der Zelle am programmierten Zelltod: Wie sich zeigen lies, führt der Einsatz hoher Konzentrationen an H_2O_2 einerseits zu einer Auflösung der intrazellulären Strukturen, verhindert aber andererseits das Auftreten der Apoptose-typischen Zeichen. Stationäre Hefezellen – das heißt Zellen, die an einen Mangel an Nährstoffen im Umgebungsmedium adaptiert sind - zeigen eine deutliche erniedrigte Sensitivität gegen Oxidationsstress. In logarithmisch wachsenden Zellen Apoptose auslösende H_2O_2 Konzentrationen, führen in stationären Zellen zu keinem apoptotischen

Phänotyp. Bei höheren H_2O_2 Konzentrationen konnte in stationären Zellen aber eine starke TUNEL-Färbung induziert werden.

Aus der Literatur ist bekannt, dass unterschiedliche zytotoxische Substanzen, die bei hoher Konzentration eine Zellnekrose verursachen, bei niedriger Konzentration Apoptose auslösen. Daraus kann möglicherweise ein Hinweis auf den Ursprung des apoptotischen Zelltods abgeleitet werden. Sauerstoffradikale sind natürliche Auslöser der Zellalterung, schwerer Zellschäden und des Zelltods. Die Entwicklung des Apoptoseprogramms mag in dieser Eigenschaft begründet sein. Überlebt eine Zelle die durch Radikale verursachte Schädigung, kann sie möglicherweise weiterhin Nährstoffe katabolisieren, aber nicht mehr in der Lage sein gesunde Nachkommen zu produzieren. Für eine klonale Zellpopulation kann es unter diesen Bedingungen durchaus von Vorteil sein, solche geschädigten Zellen durch einen programmierten Zelltod zu eliminieren und so Nährstoff und Energie für die gesunden Zellen der Population aufzusparen. Unter solchen Bedingungen kann die Möglichkeit Apoptose durchzuführen selbst für Kolonien eines einzelligen Lebewesens einen evolutionären Vorteil bedeuten. Ein solcher altruistischer Zelltod wurde tatsächlich für stationäre Hefezellkulturen beschrieben: *S. cerevisiae* Zellen können lange Zeit in reinem Wasser überleben, zeigen aber drastisch reduzierte Überlebensraten in Medien denen bestimmte Nährstoffe entzogen wurden. Dieses Verhalten ermöglicht wahrscheinlich die Bewahrung vorhandener Nährstoffe für die am besten adaptierten Klone. Bcl2 kann tatsächlich das Absterben der Zellen unter solchen Bedingungen verzögern.

Obwohl keines der in Säugerzellen etablierten Apoptoseproteine in Hefe vorgefunden wird, können diese in Hefe ähnliche Funktionen erfüllen. In Säugetierzellen kann Bax Caspase-unabhängig die Produktion reaktiver Sauerstoffspezies induzieren. Die hier vorgelegten Befunde, der Anhäufung von Sauerstoffradikalen bei Expression von Bax in Hefezellen – die darüber hinaus durch Expression von Bcl-XL verhütet werden kann –

spricht für das Vorliegen eines konservierten Mechanismus, der durch die Apoptoseregulatoren aus Säugetieren angesprochen werden kann. Substanzen mit reaktivem Sauerstoff, bzw. Sauerstoffradikale sind daher die ersten gemeinsam regulatorischen Elemente der Apoptose in Säugetier- und Hefezelle.

Aus der Literatur waren experimentelle Ergebnisse bekannt, die annehmen ließen, dass das Ubiquitin-Proteasomen-System in Säugerzellen durch Abbau eines kurzlebigen pro-apoptotischen Proteins in die Kontrolle der Apoptose eingreift. Um herauszufinden ob Ubiquitin-abhängige Proteolyse in *S. cerevisae* eine ähnliche Rolle spielen kann, wurde in einem zweistufigen Ansatz nach potenziell beteiligten Proteinen gesucht. Dazu wurde im ersten Schritt nach Proteinen gesucht, deren Überexpression in Zellen mit einem partiellen Defekt des proteasomalen System zum Zelltod führt. Dabei identifizierte Proteine stellen wahrscheinlich Substrate des Ubiquitin-Proteasomen-Systems dar. Im zweiten Schritt wurden erhaltene Proteine nach Kandidaten durchsucht deren Überexpression in Hefezellen einen Zelltod mit den bekannten morphologischen Veränderungen der Apoptose auslösten. Dabei wurden sechs Proteine mit dieser Eigenschaft identifiziert. Eines dieser Proteine, Stm1 wurde näher untersucht. Stm1 ist proteolytisch instabil in Wildtyp Zellen. In proteasomalen Mutanten tritt eine nahezu vollständige Stabilisierung ein. Damit konnte Stm1 als in vivo Substrat des proteasomalen Systems charakterisiert werden. Lokalisationstudien zeigten, dass Stm1 in der Peripherie des Zellkerns lokalisiert ist. Stm1 wird für das Wachstum unter mutagenen Bedingungen gebraucht. Zellen denen Stm1 fehlt zeigen einen Defekt in der Induktion der Apoptose durch niedrige Konzentrationen von H_2O_2. Die Überlebensrate der Hefezellen unter solchen Bedingungen ist signifikant erhöht. Eine ähnliche Erhöhung der Überlebensrate wird auch durch Inhibition der Proteinsynthese erreicht. Man kann daher hypothetisch annehmen, dass Stm1 ein Aktiva-

tor der Apoptose in Hefe ist. Bei Inhibition seiner Synthese wird Zellen vorhandenes Stm1 Protein durch proteasomale Proteolyse entzogen und so die Rate des Zelltods vermindert. Bei Überexpression in proteasomalen Mutanten kann, durch eine Akkumulation des Stm1 Proteins schon unter normalen Bedingungen der apoptotische Tod induziert werden.

2 Introduction

A car is a complex system. To sustain its functionality, the driver has to fill up the gasoline or replenish water in the cooler from time to time. But this is only a part of the job. Occasionally, the driver also has to replace worn out parts, like tires, oil, or brake shoes. If the driver neglected the maintenance, he would not only decrease the functionality of his car, but most of all he would jeopardize his own safety: old tires, malfunctioning brakes, and icy road are a deadly mix.

Like a car, a multicellular organism is a complex system with its own need for maintenance, or homeostasis.[1] On the most coarse level it is the necessity to modulate the position of cells in a developing organism, and thus to carve its shape. After an organism reaches its adult form, the number of the cells must be kept constant; the process of cell division must be counterbalanced by cell death, the excess, aged, damaged, or dangerous cells must be replaced by new ones. The loss of equilibrium between cell death and cell division will lead to developmental malformations, or to severe diseases and death of the organism. I shall discuss the mechanisms of cell death and its regulation in Section 2.1.

Cells constituting a multicellular body, as well as unicellular life forms, also have to maintain the balance of their constituents. They must constantly eliminate molecules that were worn out during metabolic or regulatory processes, or those that were damaged. The newly synthesized molecules must replace the ones that were removed. The processes a cell has to keep in equilibrium are synthesis and degradation. I shall present a summary of protein degradation, or proteolysis, in Section 2.2.

In my work I set out to establish a single cell eukaryote *Saccharomyces cerevisiae* (also known as the baker's or budding yeast, Section 2.3) as a model organism for study of apoptosis — a form of programmed

[1] Homeostasis is "a relatively stable state of equilibrium or a tendency toward such a state between the different but interdependent elements or groups of elements of an organism, population, or group" [Mish 1997].

cell death. I used this model to examine the role of ubiquitin-dependent proteolysis in this cell death process. In Section 3 I shall summarize the results of my published work (see Appendices) and discuss it in the context of previous findings.

2.1 Programmed Cell Death

Every cell that is living can be killed. The death may be non-physiological (accidental, necrotic), or physiological (programmed). Necrotic cell death occurs when some process that is essential for survival is interrupted, or when a cell succumbs to an injury inflicted from outside. By contrast, programmed cell death is a deliberate reaction of a cell to extracellular or intracellular signals.

2.1.1 Apoptosis

Apoptosis is a prominent form of programmed cell death in Metazoa, and plays an essential role during their development and homeostasis. Developing organisms use this cell death process to sculpt their tissues from excess cells produced during early stages of development and later to maintain the tissue architecture [Jacobson et al. 1997]. In the immune system, apoptosis regulates lymphocyte maturation and receptor repertoire selection [Lam et al. 1997]. A number of disease states, such as cancer, and neurodegenerative and autoimmunity disorders, are associated with breakdown in regulation of apoptosis [Thompson 1995].

The dying cells undergo a characteristic morphological change, during which the cell and its nucleus shrink and frequently fragment. Whereas cells that die by necrosis spill their cytosolic contents into the extracellular space and elicit an inflammatory response, cells that die by apoptosis disappear in a way that is more efficient for the organism: they are so rapidly phagocytosed by macrophages (or other neighboring cells) that there is no leakage of cytosolic components and no inflammatory response.

2.1.1.1 Morphological Definition of Apoptosis

In the late 60' Kerr, Wyllie, and Currie performed series of histological studies on various tissues exposed to various pathological stimuli. The observation they made was striking: despite the heterogeneity of the tissues and treatments, the pattern of cell death was similar. The cells shrunk to half their original volume, lost contact with their neighbors, rearranged and condensed their nuclear chromatin, and, rarely but significantly, were caught in the act of implosion, emerging as condensed, membrane-surrounded apoptotic bodies. Such cells were efficiently phagocytosed and degraded [Kerr et al. 1972].

Another morphological marker of apoptosis is the early exposition of phosphatidylserine, a lipid normally concentrated in the luminal layer of the plasma membrane, on the cell surface [Martin et al. 1995]. Phosphatidylserine is recognized and bound by a receptor on macrophages and other phagocytic cells, and this results in the rapid clearance of the dying cell from the body [Fadok et al. 2000].

Finally, an important diagnostical, although not strictly morphological, marker is cleavage of DNA. DNA is cleaved between nucleosomes [Wyllie 1980] by the caspase-activated nuclease CAD [Enari et al. 1998].

For an example of mammalian cells undergoing apoptosis see Figure 1 (E-H, J).

2.1.1.2 Signals Leading to Apoptosis

A signal that tells the cell to turn on its apoptotic program can have two origins: It can emanate from the extracellular space, and is transmitted to the cell through ligand binding to receptors on the cell surface; or it can originate inside the cell, and is usually integrated and further transmitted by mitochondria.

2.1.1.2.1 Death Receptors

Death receptors, involved in transducing death signals, are a subset of the tumor necrosis factor receptor (TNF-R) family. The prototype receptor of this group, CD95 (Fas/Apo-1), is expressed in various tissues, but its

ligand CD95L is expressed mainly in activated T lymphocytes and natural killer cells [Nagata 1997]. Binding of the CD95L to the receptor induces its trimerization and association with a complex of proteins called death inducing signaling complex (DISC) [Kischkel et al. 1995]. The adaptor protein FADD (Fas-associated death domain) binds to CD95 as a part of DISC [Chinnaiyan et al. 1995]. FADD contains a DED domain in its amino-terminal region and uses it to recruit procaspase-8, which has a similar DED domain in its prodomain [Boldin et al. 1996]. Initiator procaspase-8 is then proteolytically activated and released to the cytosol [Muzio et al. 1996] where it activates downstream efector caspases.

2.1.1.2.2 Mitochondrial Pathway

Mitochondria are not only the centers for energy production in eukaryotic cells, but also play a major role in regulation of apoptosis under certain circumstances. This was first suggested in a study that showed that cytosolic extracts capable to induce apoptotic changes in nuclei isolated from *Xenopus* eggs had to contain mitochondria [Newmeyer et al. 1994]. Later it turned out that after receiving a death signal mitochondria release a number of proteins from their intermembrane space, such as cytochrome c [Liu et al. 1996], AIF (apoptosis inducing factor, a mitochondrial oxidoreductase) [Susin et al. 1999b], or caspases [Susin et al. 1999a]. Cytochrome c participates in caspase activation (see Section 2.1.1.4.1), while AIF appears to induce apoptotic-like changes in a caspase-independent fashion.

While some apoptotic pathways (for example CD95 pathway) require the cooperation of mitochondria only under some circumstances [Scaffidi et al. 1998], others seem to completely depend on apoptotic function of mitochondria. Irradiation or treatment with chemotherapeutics fails to induce apoptosis in cells lacking Apaf-1 or caspase-9. These two proteins form, together with cytochrome c released from mitochondria, a complex with caspase activity [Rodriguez & Lazebnik 1999] and act therefore downstream of mitochondria in this apoptotic pathway.

The release of apoptotic effectors from mitochondria is triggered by translocation of pro-apoptotic members of the Bcl-2 family to the mitochondrial outer membrane. The signal for translocation is for example dephosphorylation of Bad by calcineurin in response to calcium elevations [Wang et al. 1999]. Bid is cleaved following ligand binding to CD95 [Li et al. 1998], Bax dimerizes in response to survival factor deprivation [Wolter et al. 1997]. All these modifications cause the pro-apoptotic Bcl-2–like proteins to move to mitochondria. There they appear to form protein-conducting channels [Susin et al. 1999b], or facilitate opening of pre-existing channels, leading to mitochondrial swelling and bursting [Vander Heiden et al. 1999]. This impact of pro-apoptotic Bcl-2 family members can be neutralized by their interaction with anti-apoptotic family members. The fine balance between these two groups of regulators can therefore contribute to the susceptibility of a cell to apoptosis [Oltvai et al. 1993].

2.1.1.3 Bcl-2 Family of Apoptotic Regulators

Bcl-2, the parent member of the Bcl-2 family of apoptotic regulators, was isolated at the chromosomal breakpoint of t(14;18) present in follicular B cell lymphomas. At this breakpoint the Bcl-2 gene becomes controlled by a strong constitutive immunoglobulin promoter, leading to constant expression and inhibition of apoptosis in the B cell lymphoma cells [Bakhshi et al. 1985].

Bcl-2–related proteins contain at least one of the four conserved regions called Bcl-2 homology domains (BH1–BH4). These motifs are formed by α helices and enable the different members of the family to form either homo- or heterodimers and to regulate each other [Kelekar & Thompson 1998]. The Bcl-2–related proteins display either anti-apoptotic or pro-apoptotic function. The members that inhibit apoptosis, such as Bcl-2 or Bcl-XL, possess at least three BH domains. Among the death promoters, some (Bax, Bak) contain BH1, BH2, and BH3 and closely resemble Bcl-2. Others (Bid, Bad) have only the BH3 domain, which is essential for both their interaction with other family members and their

death-promoting activity [Gross et al. 1999]; these are therefore called "BH3-only proteins". Most Bcl-2 family proteins also contain a carboxy-terminal hydrophobic domain that targets them to intracellular membranes [Goping et al. 1998]. In the absence of a death signal, anti-apoptotic members are integral membrane proteins found in the mitochondria, endoplasmic reticulum, or nuclear membrane [Hockenbery et al. 1990, Krajewski et al. 1993]. In contrast, a substantial fraction of the pro-apoptotic members localize to the cytosol or the cytoskeleton [Hsu et al. 1997, Puthalakath et al. 1999]. Following a death signal, the pro-apoptotic members of the Bcl-2 family undergo a conformational change that enables them to integrate into membranes, especially the outer mitochondrial membrane.

2.1.1.4 Caspases

A core component of the apoptotic program in Metazoa is the proteolytic system of caspases. They are the effectors that in response to apoptotic signals cause the apoptotic morphology.

The term *caspase* is based on two characteristic features of the proteases of the caspase family: First, they are cysteine proteases and use cysteine as the nucleophylic group for substrate cleavage, and second, they cleave the peptide linkage carboxy-terminal to aspartic acid residues [Alnemri et al. 1996].

The first caspase, caspase-1, also known as ICE (interleukin-1β converting enzyme), was identified due to its ability to convert the precursor of interleukin-1β to its mature form, a mediator of inflammation [Cerretti et al. 1992, Thornberry et al. 1992]. Later *ced-3*, a pro-apoptotic gene in *Caenorhabditis elegans*, has been cloned and it turned out that it encodes a protein highly homologous to ICE [Yuan et al. 1993]. Since then, at least 14 mammalian caspases have been characterized. Based on their role in apoptosis, caspases are divided into three functional groups: Initiator caspases, effector caspases, and caspases involved in the inflamatory response.

2.1.1.4.1 Activation of Caspases

Caspases are synthesized as inactive precursors — procaspases. Procaspases are composed of three domains: an amino-terminal domain, and the p20 and p10 domains, which are found in the mature enzyme. In all cases examined so far, the mature enzyme is a heterotetramer containing two p20/p10 heterodimers and two active sites [Earnshaw et al. 1999].

Initiator caspases are activated as a response to an apoptotic signal. For example, when CD95 receptor is aggregated by CD95L (ligand), procaspase-8 (an initiator caspase) is recruited to the death receptor and becomes proteolytically activated via an autocatalytic mechanism [Muzio et al. 1996]. Some T lymphocytes kill their target cells using perforin and granzyme B. They deposit granules containing apoptotic effectors at the T cell – target cell interface, and with the help of perforin, granzyme B finds its way to the target cell where it triggers apoptosis by directly cleaving and activating caspase-8 [Medema et al. 1997]. Caspase-9, another initiator caspase, is activated by association with cytochrome *c* and Apaf-1 [Li et al. 1997] and forms with them a stable proteolytically active complex [Rodriguez & Lazebnik 1999]. Effector procaspases are usually activated by other proteases, either initiator caspases or granzyme B [Medema et al. 1997].

2.1.1.4.2 Proteolytical Targets of Caspases

Once activated, the effector caspases are ready to cleave their substrates. It is in this execution phase of apoptosis, when cells acquire the typical apoptotic morphology as a result of changes caused by the cleavage of caspase targets. The number of identified caspase substrates has increased immensely in the past years, therefore I shall present in this section only a small selection.

During apoptosis, nuclear DNA is condensed and degraded into fragments corresponding to the multiples of the length of nucleosomes [Wyllie 1980]. Endonuclease CAD (caspase-activated DNAse), which is re-

sponsible for the cleavage, is present in healthy cells in cytosol as an inactive complex with ICAD (inhibitor of caspase-activated DNAse) [Enari et al. 1998]. In apoptotic cells, ICAD is cleaved by caspase-3 and caspase-7, releasing CAD to degrade nuclear DNA [Sakahira et al. 1998]. Another caspase-3–activated factor, named acinus, induces chromatin condensation without affecting DNA fragmentation [Sahara et al. 1999].

The cytoskeleton of an apoptotic cell undergoes profound changes as the nucleus fragments, the cell body shrinks, and the cell becomes detached from surrounding cells and the basal membrane. These changes can be explained by cleavage of cytoskeletal proteins by caspases. For example lamins, the intermediate filament scaffold proteins of the nuclear envelope, are cleaved mainly by caspase-6 and inactivated, leading to nuclear fragmentation in the final phases of apoptosis [Lazebnik et al. 1995, Takahashi et al. 1996]. Cleavage of gelsolin (a cytosolic actin-depolymerizing enzyme) by caspase-3 produces a fragment with constitutive activity, facilitating the disassembly of the actin cytoskeleton [Kothakota et al. 1997].

Anti-apoptotic members of the Bcl-2 family of apoptotic regulators (Section 2.1.1.3) are cleaved by caspases as a means of amplifying the apoptotic cascade. The conversion of anti-apoptotic regulators into a pro-apoptotic force constitutes a positive feedback loop in the terminal phase of apoptosis, removing anti-apoptotic brakes and accelerating caspase activation. The anti-apoptotic Bcl-2 and Bcl-XL can each be cleaved by effector caspases to generate fragments that have pro-apoptotic activity [Cheng et al. 1997, Clem et al. 1998]. The amino-terminal BH4 domain, present only in antiapoptotic members of the Bcl-2 family, is removed by caspase cleavage, so that the resulting carboxy-terminal fragment resembles the pro-apoptotic Bcl-2 family members Bax and Bak [Cheng et al. 1997].

Cleavage of Bcl-2 family members can occur also earlier during the initiation of apoptosis. Bid, a Bcl-2–related pro-apoptotic protein containing only the BH3 domain, is cleaved by initiator caspase-8 following death

receptor activation [Li et al. 1998, Luo et al. 1998]. Bid is normally cytosolic and inactive. After proteolytic processing by caspase-8 it translocates to mitochondria and triggers the mitochondrial death pathway (Section 2.1.1.2.2).

The number of known proteolytic targets of caspases reflects the complexity of the terminal apoptotic morphology. However, caution is necessary when assigning a causal relationship between a substrate cleavage and the apoptotic process: the substrate may well be cut as an innocent bystander and its cleavage may be just a consequence, but not a cause, of apoptotic death.

2.1.2 Other Forms of Programmed Cell Death in Metazoa

Besides apoptosis, at least two other morphologically distinct forms of physiological cell death have been described: autophagic degeneration and non-lysosomal disintegration [Schweichel & Merker 1973]. Although morphologically resembling necrosis, they appear to be developmentally programmed and are under genetic control in a broad sense [Zakeri et al. 1995]. However, molecular mechanisms involved in these necrotic-like cell deaths are still obscure.

Interestingly, when caspases are inhibited in some apoptotic systems, cells still die, but with a morphology resembling in some aspects necrotic death. In human leukemic Jurkat cells that were induced to undergo apoptosis by overexpression of the Bcl-2 pro-apoptotic family member Bax inhibition of caspases did not inhibit cell death. Instead, such treatment led to cytoplasmic vacuolization and only partial chromatin condensation. Death of these cells was accompanied by production of reactive oxygen species [Xiang et al. 1996].

Programmed cell death with non-apoptotic morphology has also been observed in the cases where caspase activity was not artificially inhibited. Tumor necrosis factor TNF induces not only apoptosis, but also cell death with a necrotic morphology, depending on the cell type [Schulze-Osthoff et al. 1994]. TNF-induced cell death is an active process

(and not just perturbation of the cellular homeostasis), because it requires synthesis of the TNF receptor and intact downstream signaling pathways. As in the case of Jurkat cells killed by Bax in the absence of caspases, downstream events in the signaling pathway involve production of reactive oxygen species [Schulze-Osthoff et al. 1994]. Importantly, this TNF-induced programmed cell death is caspase-independent [Vercammen et al. 1998].

2.2 Degradation of Proteins

Proteins in living cells are in a state of constant turnover [Vickery et al. 1940, Schoenheimer 1942]. Protein turnover has two components: synthesis of proteins from amino acids on ribosomes and degradation (or disassembly) of proteins into their constituent amino acids by proteases and peptidases. Cells have to restrict proteolysis rigorously to avoid indiscriminate destruction of cellular proteins. Containment is achieved by compartmentalization; proteolysis takes place in most cases either in the microcompartment of the multisubunit enzyme proteasome in cytosol or in membrane bound lysosomal compartments.

2.2.1 The Ubiquitin-Proteasome System

In the ubiquitin-proteasomal pathway, protein substrates to be degraded are first decorated with chains of small polypeptide ubiquitin and are then recognized by the 26S proteasome complex. While the ubiquitin chains are released and recycled into monomeric ubiquitin, the substrate is cleaved into oligopeptides by the proteolytic core of the 26 S complex.

2.2.1.1 Ubiquitin

Ubiquitin was first isolated [Goldstein et al. 1975] and sequenced [Schlesinger et al. 1975] as a lymphocyte differentiation promoting factor. Later, the histone H24A was found to be a covalent complex of histone H2A and a ubiquitin molecule [Goldknopf & Busch 1975, Goldknopf et al. 1975], joined through an isopeptide linkage between the ε-amino group of

histone's lysine residues and the carboxy-terminal glycine of ubiquitin [Goldknopf & Busch 1977]. In another line of work, rabbit reticulocytes were found to contain an ATP-dependent proteolytic system [Etlinger & Goldberg 1977], which required an "ATP-Dependent Proteolysis factor I" [Ciechanover et al. 1978, Hershko et al. 1979]. This factor formed covalent complexes with proteolytic substrates in an ATP-dependent manner [Ciechanover et al. 1980, Hershko et al. 1980] and was subsequently identified as ubiquitin [Wilkinson et al. 1980].

Ubiquitin deserves its name: its genes were found in all eukaryotic cells examined and in several viruses [Dunigan et al. 1988, Guarino 1990, Russell & Rohrmann 1993, Tautz et al. 1993]. Early reports, though, suggesting presence of ubiquitin in eubacteria [Goldstein et al. 1975] have never been confirmed. The finding of ubiquitin in an archaebacterium [Wolf et al. 1993] has been recently nullified by the absence of ubiquitin genes in the completely sequenced genome of the same archaebacterial species [Ruepp et al. 2000].

Ubiquitin genes come in two flavors: polyubiquitin genes and ubiquitin extension genes. Polyubiquitin genes consist of 3 to 52 tandem head-to-tail repeats of 228 base pairs with various carboxy-terminal extensions of 1-3 amino acids [Schlesinger & Bond 1987, Callis & Vierstra 1989]. These extensions may serve to block ligation of polyubiquitin chains to their protein targets before being cleaved into ubiquitin monomers by deubiquitinating endopeptidases [Wilkinson et al. 1989, Tobias & Varshavsky 1991, Baker et al. 1992]. Monoubiquitin genes are in-frame fusions of single monoubiquitin coding units and single open reading frames coding for components of 40S or 60S ribosomal subcomplexes [Finley et al. 1989]. The transient association of ubiquitin and ribosomal proteins (before cleavage of the fusion protein by deubiquitinating enzymes, see also Section 2.2.1.2.5) facilitates the incorporation of the latter into ribosomal subcomplexes [Finley et al. 1989].

Ubiquitin with its 76 amino acids is arguably one of the most conserved proteins known. The invariant mammalian ubiquitin differs only at

one amino acid position from yeast ubiquitin, and at three amino acid positions from the most distant plant ubiquitin [Schlesinger & Bond 1987, Callis & Vierstra 1989]. The carboxy-terminal amino acids Arg-Gly-Gly are absolutely conserved and required for conjugation of ubiquitin to proteins targeted for degradation [Wilkinson & Audhya 1981].

2.2.1.2 Ubiquitin-Conjugating Cascade

Ubiquitin is conjugated to its target in a reaction consisting of three steps: chemical activation of ubiquitin, recognition of the target substrate, and creation of a covalent linkage between ubiquitin and the substrate. This process is called ubiquitination[2].

2.2.1.2.1 Ubiquitin-Activating Enzymes

Ubiquitin activating enzyme (E1) initiates the conjugation cascade [Ciechanover et al. 1981] by adenylating the carboxy-terminal glycine of ubiquitin using ATP. Activated ubiquitin is then attached via a high energy thiol-ester linkage to a cysteine in the catalytic site of the same E1 molecule with a concomitant release of AMP [Haas & Rose 1982, Haas et al. 1982].

2.2.1.2.2 Ubiquitin-Conjugating Enzymes

Ubiquitin conjugating enzymes (E2s) are defined as a family of related proteins able to form a thiol-ester with ubiquitin accepted from the E1 protein [Hershko et al. 1983, Pickart & Rose 1985]. Purified E2 enzymes catalyze formation of isopeptide linkages between ε-amino groups of lysines of proteolytic substrates and the carboxy-terminal glycine of ubiquitin [Haas & Bright 1988] as well as linkages between 48th lysine (Lys^{48}) of one ubiquitin molecule and carboxy-terminal glycine of another ubiquitin molecule [Chen & Pickart 1990, Banerjee et al. 1993].

[2] Ubiquitin covalently linked to other protein is called ubiquityl by some authors (like methane – methyl). The process of linking is then termed ubiquitylation and results in ubiquitylated substrate [Levy et al. 1996].

All E2 proteins contain a conserved catalytic core of approximately 150 amino acids, called UBC domain [Jentsch et al. 1990]. This domain surrounds the active site cysteine required for thiol-ester formation [Sullivan & Vierstra 1991, Sullivan & Vierstra 1993]. Based on the three-dimensional structure of an E2 enzyme [Cook et al. 1992b], a model of interaction between E2 and ubiquitin was proposed [Cook et al. 1992a, Sullivan & Vierstra 1993].

2.2.1.2.3 Ubiquitin Ligases

Although in certain cases the substrate specificity of ubiquitination may be implemented by E2 enzymes, the currently prevailing view is that in most cases ubiquitin ligases (E3s) are required for substrate recognition as well.

The prototype ubiquitin ligase is E6-AP, which, in a complex with viral E6 protein,[3] is responsible for ubiquitination of the tumor suppressor protein p53 in mammalian cells infected with human papilloma virus [Scheffner et al. 1990, Scheffner et al. 1993]. E6-AP forms a thioester with a ubiquitin moiety received from a ubiquitin-E2 thioester complex [Scheffner et al. 1995]. Transesterification takes place on a cysteine within the conserved carboxy-terminal domain of E6-AP [Scheffner et al. 1995]. This E3 is thus not a mere docking protein with recognition function, but it has an enzymatic activity on its own. This enzymatic activity, together with the presence of a HECT (homologous to E6-AP carboxy-terminal) domain [Huibregtse et al. 1993, Huibregtse et al. 1995], defines the best characterized group of ubiquitin ligases.

The HECT domain, however, does not seem to be a common feature of all E3s. For example the yeast E3 enzyme Ubr1 [Bartel et al. 1990] lacks this domain. Instead it has a RING-H2 (rather interesting new gene domain with 2 histidines) domain, which is in this case required for ubiquitination of one class of substrates [Xie & Varshavsky 1999]. In addition to the RING-H2 domain, Ubr1 also contains a BRR (basic residue rich) re-

[3] Viral protein E6 is not a part of ubiquitination cascade as the name might suggest. The nomenclature of viral proteins is independent of E1-E2-E3-E4 nomenclature of enzymes involved in ubiquitination pathway.

gion responsible for the interaction with the ubiquitin conjugating enzyme Ubc2. Although the RING-H2 of Ubr1 is required neither for E2 or substrate binding, evidence is still lacking for its direct role in the formation of the ubiquitin-substrate isopeptide bond.

Even though the RING-H2 domain, like the HECT domain, is not a defining structural feature of ubiquitin ligases — it is also involved in protein sorting pathways [Radisky et al. 1997] and structural organization of the MAPK (mitogen activated protein kinase) pathway [Inouye et al. 1997] — it is tightly linked to the ubiquitination pathway. The RING-H2 domain is functionally essential in all three known modular ubiquitin ligases [Kamura et al. 1999, Seol et al. 1999, Gmachl et al. 2000, Leverson et al. 2000]: SCF (Skp1-cullin-F box receptor), APC (anaphase promoting complex), and VCB-like (VHL-elonginC/B, based on VHL – *v*on *H*ippel *L*indau tumor suppressor protein) complexes. Of these three the yeast SCF complex is best characterized. It contains adapter subunits called F-box proteins, which recognize different substrates through specific protein-protein interaction (WD40) domains. The F-box motif is a docking site for a core consisting of Skp1, the yeast cullin Cdc53, and the RING-H2 protein Hrt1. Hrt1 recruits ubiquitin conjugating enzyme Cdc34 to the complex [Bai et al. 1996, Seol et al. 1999]. Thus the complex brings together the ubiquitin–E2 thioester and the target substrate. The transfer of the ubiquitin is thought to come about without any further thioester intermediate. The E3 function of the SCF complex is therefore limited to substrate recognition and recruitment of activated E2.

2.2.1.2.4 Multiubiquitin Chain Assembly

Most substrates are modified by multiubiquitin chains, where individual ubiquitins are linked by isopeptide bonds [Chau et al. 1989]. The basic principle for establishing these bonds is the same as for isopeptide linkages between a target substrate and ubiquitin, with the exception that here the incoming ubiquitin is linked to a ubiquitin already conjugated to the substrate or that is part of a polyubiquitin chain.

In most proteasomal substrates the carboxy-terminal glycine of the incoming ubiquitin is linked to Lys^{48} of ubiquitin terminating the existing chain. This type of linkage seems to be preferred if the ubiquitinated substrate is going to be degraded by the 26S proteasome [Chau et al. 1989]. There are, however, several ways how ubiquitins can be linked to form a polyubiquitin chain (ubiquitin has six lysine residues on which the rest of a polyubiquitin chain can be appended). For example, linkage through Lys^{63} results in polyubiquitinated substrates that are not degraded by the proteasome. Instead, this type of polyubiquitin chain has a regulatory role in DNA repair [Hofmann & Pickart 1999], targeting of plasma-membrane proteins for degradation in vacuole [Galan & Haguenauer-Tsapis 1997], mitochondrial inheritance [Fisk & Yaffe 1999], and possibly cell cycle-dependent regulation of translation [Spence et al. 2000].

For efficient polyubiquitination an additional cofactor, E4, is needed in at least one case. It cooperates with E1, E2, and E3 in extending existing polyubiquitin chains [Koegl et al. 1999]. Moreover, Cdc48, an ATPase of the AAA family, functions in the proteolytic pathway following the E4. It perhaps takes part in assembly or disassembly of complexes involved in ubiquitination or targeting of substrates to the proteasome [Koegl et al. 1999].

2.2.1.2.5 Deubiquitinating Enzymes

Deubiquitinating enzymes have three important roles in the ubiquitin-proteasome system. First, they free monomeric ubiquitin from protein products of polyubiquitin and ubiquitin-fusion genes (see Section 2.2.1.1). Second, they release ubiquitin during proteasomal degradation of a ubiquitinated protein. In yeast, for example, the deubiquitinating enzyme Doa4 liberates the polyubiquitin chain from its binding site on the 26S proteasome and from the lysine-containing peptide remnant of the degraded substrate [Papa & Hochstrasser 1993]. Another deubiquitinating enzyme, Ubp14, then disassembles the polyubiquitin chain into individual ubiquitins [Amerik et al. 1997], making them available for a next round of ubiquitination. Ubiquitin itself is degraded with slow kinetics [Ligr & Malek

1997] by an unknown mechanism. Lastly, in addition to these basal functions, deubiquitinating enzymes may provide another level of substrate selectivity. A subunit of the mammalian 19S regulatory cap of the 26 proteasome has a deubiquitinating activity. By truncating polyubiquitin chains of substrates bound to the cap it reduces their chance of being degraded. Substrates that are already poorly ubiquitinated can have their polyubiquitin chains shortened enough to be completely rescued – such mechanism may prolong the lifetime of substrates with poor ubiquitin-conjugating signals [Lam et al. 1997].

2.2.1.3 The 26 S Proteasome

26 S proteasome[4] is the protease responsible for degradation of polyubiquitinated proteins. It consists of the 20 S catalytic core barrel and 19 S regulatory caps attached at openings of the barrel. It is thought that the substrate is recognized through its ubiquitin tag, unfolded by the regulatory cap, translocated through a channel from the regulatory cap to the 20 S core, and there cleaved into short polypeptides [Hilt & Wolf 1992, Larsen & Finley 1997].

Cytosolic particles called prosomes were identified in the early 70's [Shelton et al. 1970], but it was not until 1987 that prosomes, identical to 20 S proteasomes, were associated with the ATP-dependent protein degradation pathway as a component of the 26 S proteasome [Hough et al. 1987, Waxman et al. 1987].

2.2.1.3.1 The 20 S proteasome

The 20 S proteasome is ubiquitous in eukaryotes, and in addition was found in archaebacteria [Dahlmann et al. 1989], as well as eubacteria [Lupas et al. 1994, Tamura et al. 1995]. The structure of the yeast 20 S proteasome has been solved crystalographically [Groll et al. 1997]. It is a hollow, barrel-like particle. Its inside is divided into three chambers, two

[4] *26 S* denotes the size of the particle, measured in svedberg units S; svedberg is a unit of time amounting to 10^{-13} s that is used to measure the sedimentation velocity of particles in an ultracentrifuge.

antechambers and one central chamber which harbors catalytic sites. The ends of the yeast 20 S barrel are sealed. On the molecular level, the 20 S proteasome is made of 28 subunits [Heinemeyer et al. 1994], organized in four stacked heptameric rings. The outer rings are built of α subunits, which lack any catalytic activity. The inner rings consist of β subunits. Three out of seven subunits in each of the β rings are catalytically active, and the active sites are facing the central chamber.

Five distinct proteolytic activities have been identified [Orlowski et al. 1993], each associated with a defined β subunit of the 20 S proteasome [Dick et al. 1998]. Three activities, the trypsine-like, the chymotrypsine-like, and the peptidyl-glutamyl-peptide hydrolyzing, cleave peptide bonds on the carboxyl side of basic, hydrophobic, and acidic amino acid residues, respectively. The fourth activity cleaves bonds preferentially on the carboxyl side of amino acids with branched chains, the fifth between small neutral amino acids. In higher vertebrates, the cytokine interferon-γ induces the expression of altered versions of the activity providing 20 S proteasomal subunits, which replace their constitutive counterparts [Groettrup et al. 1996b].

After entering the central chamber, the peptide bonds of a substrate protein are subjected to nucleophylic attacks by amino-terminal threonines of active β subunits [Seemuller et al. 1995], cleaving their substrates into oligopeptides of 3–15 amino acids [Ehring et al. 1996, Kisselev et al. 1998].

2.2.1.3.2 19 S Regulatory Caps

The 20 S core proteasome complex is able to cleave denatured proteins and short artificial substrates, but it lacks any selectivity for ubiquitinated proteins. Such competence is provided by association of 19 S regulatory caps at the ends of the 20 S barrel.

The 19 S regulatory cap is composed of two subassemblies: the eight-subunit base, and the eight-subunit lid [Glickman et al. 1998]. The lid assembly seems to contain subunits necessary for recognition of ubiq-

uitinated proteins. The base consists of ATPases of the AAA family and exhibits chaperone-like activity [Braun et al. 1999]. The base may have two functions: activation of the 20 S core by opening its otherwise closed channels at the ends of the barrel, and unfolding and feeding the substrate through this channel to the lumen of the 20 S catalytic core [Glickman et al. 1998].

Unlike the 19 S cap, an 11 S regulatory cap is found only in vertebrates. The hexa- or heptameric 11 S complex, which associates with the 20 S catalytic core in an ATP-independent manner [Dubiel et al. 1992], is formed by two nonidentical subunits [Mott et al. 1994]. The existence of the 20 S core with attached 11 S complexes was demonstrated visually by electrone microscopy [Gray et al. 1994] and in vivo by immunoprecipitation experiments [Yang et al. 1995]. The 11 S cap appears to be required for immunological adaptation. In vertebrates, the peptides generated as degradation products by the proteasome are delivered to major histocompatibility class I molecules for display on the cell surface [Michalek et al. 1993]. The 11 S cap, together with the interferon-γ–induced catalytic subunits, modulates the cleavage mechanism of the 20 S core and improves the yield of antigenic peptides [Dick et al. 1996, Groettrup et al. 1996a].

2.2.2 Lysosomal Compartments

Lysosomes (including yeast vacuoles) are membrane-surrounded organelles containing a number of acid hydrolytic enzymes, including proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases, and sulfatases. The internal acidic environment of lysosomes is generated by a membrane-bound H^+-ATPase that pumps protons into the lysosomal lumen.

2.2.2.1 Lysosomal proteases

Lysosomes contain over 20 different proteases [Bohley & Seglen 1992]. Most of them are designated as cathepsins and include both endo- and

exopeptidases. One of the most prominent cathepsins is cathepsin D, an aspartic endopeptidase [Knecht et al. 1998]. Its yeast orthologue is protease yscA/PrA, a product of the *PRA1*/*PEP4* gene [Mechler & Wolf 1981]. As a potent protease, yscA may pose danger to the yeast cell if the integrity of the vacuolar membrane is compromised and leakage of vacuolar content to the cytosol occurs. This at least seems to be an explanation for existence of a cytosolic inhibitor of yscA, I_{A3} [Saheki et al. 1974]. A similar inhibitor exists also for protease yscB, the second endopeptidases of the yeast vacuole [Schu et al. 1991], as well as for yscY, an unspecific serine exopeptidase [Lenney 1975].

2.2.2.2 Uptake of Proteins into Lysosomes

There are several ways for cytosolic proteins to gain access to vacuolar proteases:

In mammalian cells, transport of specific cytosolic proteins into lysosomes requires the presence of a consensus peptide motif KFERQ [Chiang & Dice 1988]. A cytosolic chaperone of hsc73 binds to the substrate proteins at the region containing the motif sequence, and this binding of hsc73 stimulates the direct transport of substrate proteins into lysosomes [Chiang et al. 1989]. A second chaperone located within the lysosomal lumen (lysosomal hsc73) is also necessary for the selective uptake of substrate proteins [Agarraberes et al. 1997]. An integral lysosomal membrane protein LGP96 acts as the receptor for the hsc73-mediated lysosomal degradation pathway [Cuervo & Dice 1996].

Another pathway for protein entry into the lysosome is microautophagy. Here the lysosomal membrane invaginates at multiple locations to form intralysosomal vesicles, which then pinch off [Ahlberg et al. 1982]. The vesicles are disintegrated in the lumen of the lysosome and their content can be processed by proteases.

During autophagy, in contrast to microautophagy, autophagic vesicles are formed as invaginations of the endoplasmic reticulum [Dunn 1990a] and engulf large volumes of cytosol or complete organelles. These vesicles are then maturing into autophagosomes by acquiring vacuolar

proteases [Dunn 1990b]. In yeast, the autophagic vesicles fuse with the vacuole [Baba et al. 1994].

Integral membrane proteins of the plasma membrane are delivered to vacuolar proteases by endocytosis. The signal for internalization is in many cases ubiquitination [Hicke 1999]. The substrates are mono- or di-ubiquitinated, or short di-ubiquitin chains are linked through Lys63, and are therefore ignored by the 26 S proteasome (which require much longer ubiquitin chains for substrate recognition).

2.3 Yeast – A Model Organism

Unlike most other eukaryotic organisms, yeast *Saccharomyces cerevisiae* cells can proliferate in both a haploid and a diploid state. Recessive mutations can thus be conveniently isolated and manifested in haploid strains, and complementation tests can be carried out in diploid strains.

The development of DNA transformation has made yeast particularly accessible to gene cloning and genetic engineering techniques. Structural genes corresponding to virtually any genetic trait can be identified by complementation from plasmid libraries. Plasmids can be introduced into yeast cells either as replicating molecules or by integration into the genome.

Integrative recombination of transforming DNA in yeast proceeds preferentially via homologous recombination. Exogenous DNA with at least partial homologous segments can therefore be directed deliberately to specific locations in the genome. Also, homologous recombination, coupled with yeasts' high levels of gene conversion, has led to the development of techniques for the direct replacement of genetically engineered DNA sequences into their normal chromosome locations. Thus, normal wild-type genes, even those having no previously known mutations, can be conveniently replaced with altered and disrupted alleles. The phenotypes arising after disruption of yeast genes has contributed significantly toward understanding of the in vivo function of a number of proteins.

Also unique to yeast, transformation can be carried out directly with synthetic oligonucleotides, permitting the convenient productions of numerous altered forms of proteins. These techniques have been extensively exploited in the analysis of gene regulation, structure-function relationships of proteins, chromosome structure, and other general questions in cell biology.

The yeast *S. cerevisiae* was the first eukaryote whose genome was completely sequenced [Goffeau et al. 1996]. This facilitated a new way of approach to study gene function. Instead of first selecting a mutant with an interesting phenotype, cloning the gene, and analyzing the sequence of the gene, with the "reverse genetics" approach a scientist can select an interesting gene (for example by homology searches in databases) and can then create mutants based on the published yeast genome sequence.

The knowledge of the full yeast genomic sequence also enable highly effective use of proteomics methods, where cellular proteins are separated on two-dimensional gels, protein spots are extracted and identified by combining mass spectroscopic data with theoretical properties of the proteins calculated from published genomic sequence [Rout et al. 2000, Verma et al. 2000].

DNA microarray technology allows printing of DNA spots, each representing a predicted open reading frame from the genomic sequence, on a single glass slide. mRNA samples are then differentially labeled and hybridized onto the slide. The result is then a complete map of transcriptional activity of the yeast under given conditions. Genes with similar transcriptional profiles can then be found and grouped together; this may help to uncover the function(s) for uncharacterized genes or aid in finding common features of the promoters of grouped genes [Spellman et al. 1998].

3 Discussion of Results

In this section, I shall present a brief overview of results of my work and discuss them in the framework of published literature. I shall present evidence that yeast can undergo cell death resembling mammalian apoptosis, that this process is dependent on the production of reactive oxygen species, and that the ubiquitin-proteasome system participates in this process through degradation of specific substrates. I shall also present some hypotheses and speculations to indicate possible direction for further research.

3.1 Bax Triggers Apoptotic-Like Cell Death in Yeast

When using the yeast two hybrid system to study physical interactions between the members of the Bcl-2 family of apoptotic regulators (see Section 2.1.1.3), Reed and colleagues observed that overexpression of Bax, a pro-apoptotic member of the family, leads to cell death [Sato et al. 1994, Hanada et al. 1995]. Furthermore, they noticed that the toxic effect of Bax expression can be neutralized by simultaneous expression of Bcl-2 or Bcl-XL, both anti-apoptotic members of the Bcl-2 family. A similar Bax/Bcl-2 functional relationship was observed in mammalian cells [Oltvai et al. 1993] and therefore the finding made in yeast suggested that these mammalian proteins preserve their functionality even in a monocellular eukaryote.

The observation that Bax (or Bak) can kill yeast cells raised questions about the terminal phenotype: Is the death caused by Bax in yeast apoptotic? Several electron microscopic studies were performed in *S. cerevisiae* and *S. pombe*, but they did not provide a clear answer. In one case the morphology appeared to be apoptotic [Ink et al. 1997], in other cases the cells underwent massive vacuolization and apparently autophagic degradation [Zha et al. 1996, Jürgensmeier et al. 1997].

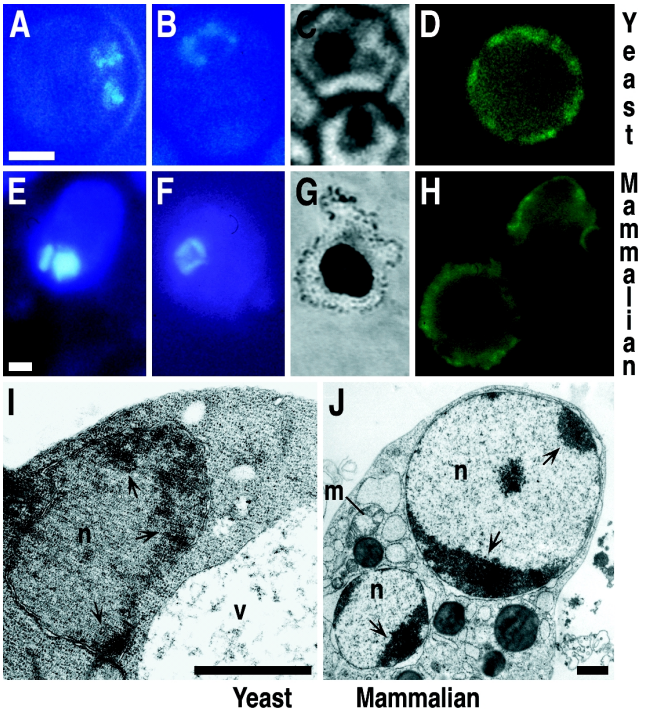

Figure 1: Cytological markers of apoptosis in yeast and mammalian cells.

S. cerevisiae treated with 1 mM H_2O_2 (A, B, C, I) or mutated in *CDC48* (*cdc48-S565G*, D), porcine thyrocytes treated with 30 µM retinol (E, F, H, J), and human WISH cells treated with 1 mM H_2O_2 (G) stained for chromatin with DAPI (A, B) or bisbenzimide (E, F), for DNA fragmentation with the TUNEL assay (C, G), for the exposition of phosphatidylserine with FITC-labelled annexin V (D, H), or investigated by electron microscopy for chromatin condensation (I, J). Bars 10 µm (A-H), 1 µm (I, J). Reprinted from Fröhlich K-U, Madeo F (2000) Apoptosis in yeast — a monocellular organism exhibits altruistic behaviour. *FEBS Lett* 473: 6–9. Copyright © 2000, Federation of European Biochemical Societies.

There was at the time one published observation of cell death in yeast that resembled in many ways the mammalian apoptosis. Yeast carrying a mutation in the AAA protein Cdc48 (*cdc48-S565G*) displayed all the typical hallmarks of apoptosis (see Section 2.1.1.1 and Figure 1) [Madeo et al. 1997]. However, there was no obvious connection between apoptotic cell death program and Cdc48 and therefore it was not clear if the observed phenotypes were relevant manifestation of an apoptosis-like process.

To clarify if yeast cells have the capacity for an apoptosis-like cell death process, I decided to test comprehensively the nature of cell death triggered in yeast by Bax. I introduced into a wild type yeast strain a plasmid carrying the open reading frame of murine Bax under the control

of the strongly inducible *GAL1* promoter. When this strain was grown on galactose medium (hence Bax was overexpressed), cells were dying. To uncover the nature of Bax-induced death I applied a set of tests originally developed for investigation of apoptotic cell death in mammalian cells.

To probe the state of the plasma membrane, I used annexin V linked to the green fluorescent protein GFP. Annexin V binds to phosphatidyl serine in the presence of calcium. If GFP-annexin V is incubated with intact non-apoptotic cells, no binding is observed, since 95% of phosphatidyl serine is located in the inner leaflet of the plasma membrane, facing the cytosol. Early during apoptosis this asymmetry is lost, and binding of GFP-annexin V to the cell surface can be observed by fluorescence microscopy (for an example see Figure 1 D). Changes in the nuclear morphology were observed using diaminophenylindole, a DNA-intercalating fluorescent dye (for an example see Figure 1 A,B), and by electron microscopy (performed by Dr. Eleanore Fröhlich; see Figure 1 I). To probe the physical state of DNA I used terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. This test detects physiological double strand brakes in DNA by attaching a labeled nucleotide to free 3’ ends. The incorporation of the labeled nucleotide can be visualized either by direct fluorescence microscopy or indirect immunofluorescence microscopy, allowing an estimation of the extent of DNA cleavage (for illustration see Figure 1 C).

The dying cells overexpressing Bax displayed a series of changes identical to morphological markers defining apoptosis in mammalian cells. Early during Bax induction the plasma membrane of the cells lost apparently its asymmetry as phosphatidylserine was exposed on the cell surface. Chromatin underwent condensation and margination and nuclear DNA was fragmented. Some degree of plasma membrane blebbing was observed as well. These alterations were absent in the control cells cultivated under identical conditions, as well as in cells where the lethal effect of Bax expression was compensated by co-expression of the murine apoptotic inhibitor Bcl-XL.

My observations confirmed that an established mammalian pro-apoptotic regulator can trigger in yeast changes similar to those it causes in mammalian cells. This demonstrated that the unicellular eukaryote *S. cerevisiae* has a capacity to undergo cell death resembling mammalian apoptosis. The results also provided an indirect evidence that the changes observed in the *cdc48-S565G* mutant were the relevant manifestation of an apoptotic process. Additional evidence was provided later by reports defining Cdc48 as a regulator of the mammalian apoptotic pathway [Shirogane et al. 1999, Wu et al. 1999].

If yeast can indeed set off an active cell death program, what is the biological meaning of such a process? It is accepted that altruistic cell death occurs in Metazoa to facilitate the development and to preserve the integrity of the organism, but in a unicellular eukaryote the need for such a process has not been widely recognized. Some clue to the function of programmed cell death in unicellular organisms came from studies in bacteria. There apparently altruistic programmed cell death has been described in populations faced with nutrient limitation.

During the stringent response of *Escherichia coli* to amino acid deprivation these cells start to produce guanosine-3',5'-bispyrophosphate (ppGpp). The enzyme responsible for ppGpp synthesis is encoded by the *relA* gene; it is activated by uncharged tRNA and thereby by limitation of amino acid availability or by inhibition of amino acylation. The synthesis of ppGpp can be also activated by carbon source limitation and is then *spoT* dependent [Cashel et al. 1996]. The *relA* gene is part of an operon in which a pair of genes *mazE* and *mazF* are located downstream of the *relA* gene [Metzger et al. 1988]. MazF is a toxic, highly stable protein. MazE, on the other hand, is a labile protein (it is degraded by ClpPA ATP-dependent protease), which protects the cells from the toxic effect of MazF. The expression of the mazEF module is inhibited by ppGpp. Under conditions of nutritional starvation the levels of ppGpp increase and the coexpression of *mazE* and *mazF* is inhibited. Because MazE is an unstable protein, its cellular concentration is decreased more rapidly than that of

MazF. MazE is not able to neutralize the toxicity of MazF anymore, and MazF then causes cell death by an unknown mechanism [Aizenman et al. 1996]. This shows that bacteria possess a mechanism to induce a cell death program.

Clonal populations of single-cell organisms may to some extent behave like Metazoan organisms. Let us assume that the ultimate purpose of an organism is the transmission of its genetic information to the progeny. In a multicellular organism cells that are infected, mutated, or damaged sacrifice themselves to preserve the integrity of the whole organism and thus do not compromise the transmission of the genetic information. When a population of *E. coli* is faced with nutrient limitation, some cells start a suicidal program in order to preserve the dwindling resources for the remaining cells.

Yeast populations may use a similar strategy for survival under nutritional stress. The growth cycle of a yeast culture in rich glucose medium follows a distinct pattern. First, there is a phase of logarithmic growth when cell density is relatively low and glucose is fermented. After glucose is used up the cells undergo a shift to the respiratory metabolism and growth slows. Finally, yeast cells enter stationary phase; stationary yeast cell staying in used up medium quickly lose viability, but this can be prevented by shifting them to pure water. In water yeast cells can survive for weeks without any added nutrients. The loss of viability of stationary yeast cells in nutrient-depleted medium is accompanied by changes resembling apoptosis [Madeo and Ligr, manuscript in preparation]. This indicates that in nutrient-depleted medium a part of yeast population undergoes an apoptotic-like cell death, presumably to preserve the remaining resources for the rest of the population, which can then survive. In pure water there are no nutrients to compete for, and it only makes sense that no cell death takes place. This also implies that nutrient sensing and associated signaling pathways may be involved in the control of the apoptotic-like cell death program in yeast.

Yeast is not the first monocellular eukaryote that has been shown to possess the capacity to undergo programmed cell death with apoptotic features. The parasites *Trypanosoma cruzi* [Ameisen et al. 1995], *Trypanosoma brucei rhodensiense* [Welburn et al. 1996], the free-living ciliate *Tetrahymena thermophila* [Christensen et al. 1995], the slime mold *Dictyostelium discoideum* [Cornillon et al. 1994], and the dinoflagellate *Peridinium gatunense* [Vardi et al. 1999] commit in response to environmental stress or extracellular signals cell suicide with changes characteristic of apoptosis.

Until recently the major argument against the existence of an apoptotic program in yeast was the absence of any orthologues of established metazoan apoptotic regulators. Database searches now revealed presence of caspase-related molecules, named metacaspases, in plants, Fungi (including *S. cerevisiae*), and Protozoa [Uren et al. 2000]. It will be interesting to see if these proteins play a role of executioners of apoptosis in yeast.

The occurrence of apoptosis in yeast is supported also by looking at phylogenetic distribution of organisms that were shown to undergo apoptosis or an apoptosis-like cell death process. In Metazoa the apoptosis penetrates the phylogenetic tree all the way down to *Hydra* [Cikala et al. 1999]. Plants undergo a form of programmed cell death know as hypersensitive response after being infected by pathogens. The purpose of this process is to prevent the spread of the pathogen by killing the infected cells and surrounding tissues. Plant cells undergoing hypersensitive response display two morphological markers that define mammalian apoptosis: cleavage of DNA and the loss of the plasma membrane asymmetry, manifested by the exposure of phosphatidylserine at the cell surface [Ryerson & Heath 1996, O'Brien et al. 1997]. Hypersensitive response can be elicited by expression of mammalian Bax [Lacomme & Santa Cruz 1999], and prevented by expression of anti-apoptotic Bcl-2–family members Bcl-XL and Ced-9 [Mitsuhara et al. 1999]. Plants contain caspase activities [del Pozo & Lam 1998, De Jong et al. 2000] and an orthologue of

the mammalian Bax inhibitor BI-1 [Kawai et al. 1999, Sanchez et al. 2000].

Looking at the phylogenetic tree highlighting the species known to be able to undergo apoptosis (Figure 2), it is apparent that the phylogenetic distance between Metazoa and Viridiplantae, two kingdoms where apoptosis has been firmly established, is larger than the phylogenetic distance between *Saccharomyces* (Fungi) and Metazoa. This suggests that apoptosis is an evolutionary old process, and that the ability to undergo apoptosis may well have been preserved during evolution of *Saccharomyces*. This of course does not imply that the apoptotic processes in Metazoa and yeast are identical. It is more likely that in Metazoa the apoptotic cell death and its regulation developed and refined as the organisms became more dependent on it for their development and homeostasis. In yeast, where this process is probably not so extensively used (and probably not essential), the evolutionary original apoptotic pathway may have been preserved. This presents the opportunity to use yeast to get insight into the origins of apoptosis and thus to learn more about the advanced mammalian apoptosis and possibly other death processes (see Section 2.1.2) that occur in mammalian cells.

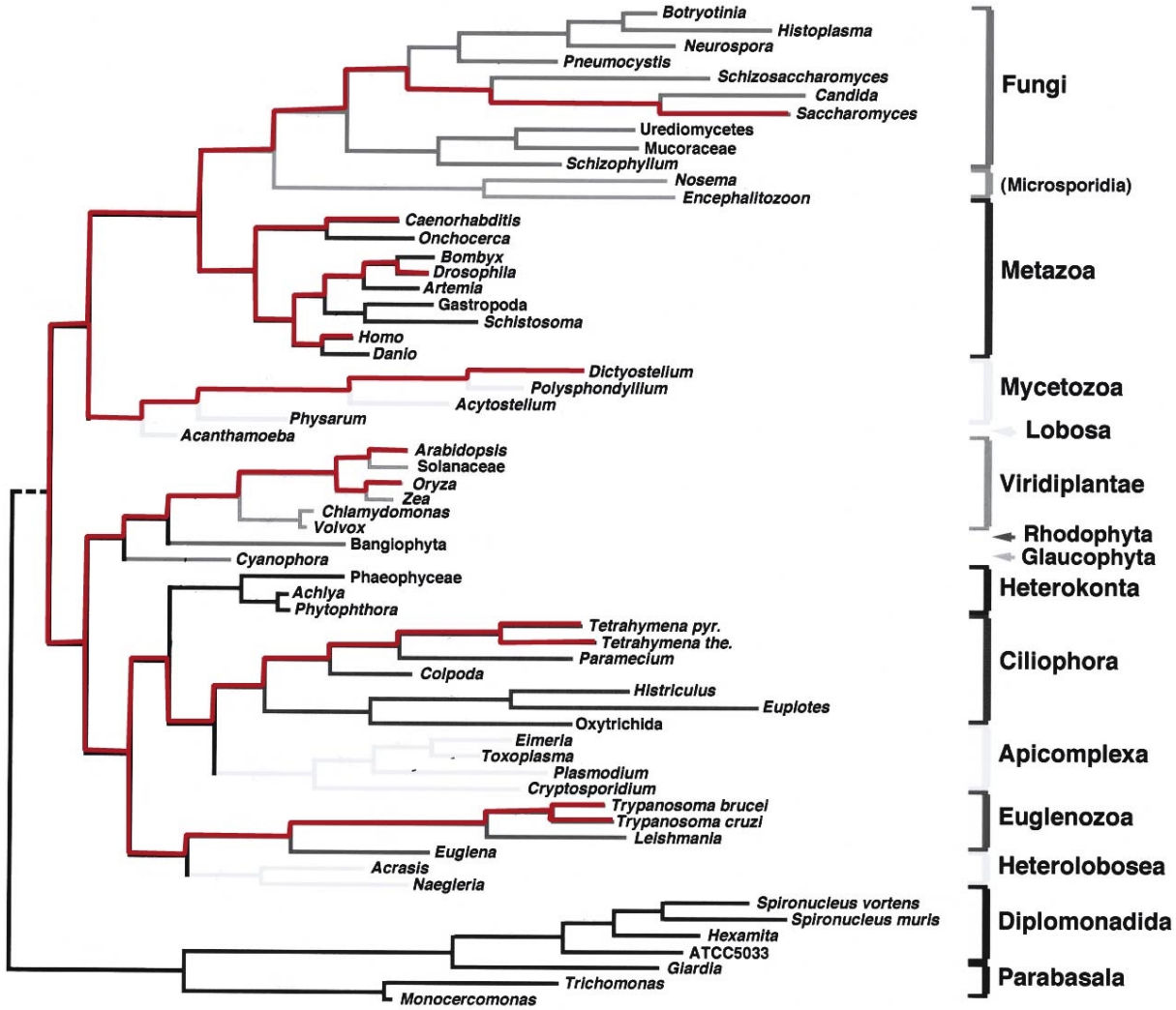

Figure 2: A kingdom-level phylogeny of eukaryotes, with clades containing species capable of apoptosis highlighted in red.

The tree is based on data from Baldauf SL, Roger AJ, Wenk-Seifert I, Doolittle WF (2000) A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* 290: 972–977.

3.2 Oxygen Radicals Regulate Yeast Apoptosis

The observation that yeast can undergo cell death resembling apoptosis led to the next question: What is the mechanism behind this cell death process? There was at least one report in the literature describing a Bax-induced cell death partially resembling apoptosis and taking place when caspases were inhibited. This process was accompanied by production of reactive oxygen species (ROS) [Xiang et al. 1996]. I therefore tested yeast cells overexpressing Bax for production of ROS. For this purpose the cells were incubated with a colorless, cell permeable dye dihydrorhodamine-123. This dye turns fluorescent after contact with ROS, becomes charged and accumulates then mainly in mitochondria. Cells overexpressing Bax exhibited a strong fluorescent signal. Control cells grown under identical conditions in the absence of any transgene, as well as the cells that were rescued from Bax-induced death by co-expression of Bcl-XL, did not show any significant fluorescence.

These observations indicated that ROS play a role in Bax-induced apoptosis in yeast. To test if ROS were just a byproduct of cell death, or if they played an active role in the process, I tried to rescue the cells overexpressing Bax by treatment with free radical spin traps.[5] Such treatment did not only restore viability of the cells, but also led to the disappearance of the apoptotic morphology associated with Bax-induced cell death. A similar effect was achieved when Bax-expressing cells were incubated under anaerobic conditions.

Next, the identical experiments were performed with the *cdc48-S565G* mutant strain, which spontaneously undergoes apoptotic-like cell death. The mutant cells were shown to accumulate oxygen radicals, and again, treatment with free radical spin traps and anaerobiosis led to a significant increase in viability of these cells, as well as the loss of apoptotic morphology.

[5] Spin traps are molecules that are able to react with unstable radicals to produce stable, non-reactive radical species.

To further confirm the causal role of ROS in apoptotic-like cell death, a strain lacking γ-glutamylcysteine synthetase was examined. Due to this defect the cells cannot synthesize glutathione, a peptide that plays a crucial role in protection of yeast cells against oxidative stress. Cells growing without supplemented glutathione displayed the phenotypic markers of apoptosis: DNA breakage, chromatin condensation and margination, and the exposition of phosphatidylserine.

These experiments demonstrated conclusively that ROS are not byproducts generated in dying yeast, but that they are active participants in the process, in every known case of yeast apoptotic-like cell death. Furthermore, the ability of cycloheximide to inhibit apoptotic-like death in yeast after exposure to mild oxidative stress showed that this cell death is an active process, involving de novo protein synthesis and therefore active participation of the cell.

ROS are well established apoptotic inducers in mammalian cells [Jacobson 1996]. Intracellular ROS accumulate in neural cells deprived of nerve growth factor or potassium as a late step of the apoptotic pathway, downstream of the action of Bax and caspases [Schulz et al. 1996, Schulz et al. 1997]. This accumulation is a prerequisite for the ensuing cell death. In addition, exogenous oxygen stress triggers the apoptotic cascade by itself [Hockenbery et al. 1993, Kane et al. 1993, Greenlund et al. 1995].

ROS have been also associated with death of stationary yeast cells in nutritionally poor medium [Longo et al. 1996, Longo et al. 1997, Longo et al. 1999], hypersensitive response in plants [Chen et al. 1993], and with apoptotic death of monocellular dinoflagellate *Peridinium gatunense* [Vardi et al. 1999]. This, together with results of our experiments, suggests that ROS are evolutionary old apoptotic inducers.

The finding that overexpression of Bax leads to accumulation of ROS provides an insight into its function. In mammalian cells, Bax translocates, after receiving an appropriate apoptotic signal, from the cytosol to mitochondrial membrane [Wolter et al. 1997]. After the insertion to the membrane it causes a series of changes in mitochondrial metabolism,

leading to the release of pro-apoptotic factors from the mitochondrial intermembrane space, including cytochrome c [Eskes et al. 1998, Jurgensmeier et al. 1998]. In mammalian cells this results in the activation of the caspase cascade [Li et al. 1997]. The release of cytochrome c was also observed in yeast [Manon et al. 1997], however, yeast cytochrome c is not able to induce apoptotic changes in nuclei isolated from *Xenopus* eggs [Newmeyer et al. 1994]. This makes it less likely that yeast cytochrome c participates in the activation of metacaspases [Uren et al. 2000], in case their role in yeast apoptotic-like cell death is established.

I suggest that the evolutionary original function of cytochrome c release during apoptosis is not activation of caspases, but the production of ROS. When cytochrome c is released from mitochondria, the electron transport chain is perturbed, mitochondrial respiration is inhibited, and superoxide is produced due to a switch from the normal 4-electron reduction of O_2 to a 1-electron reduction [Cai & Jones 1998]. ROS are then the active agents responsible for triggering of the apoptotic program, and cytochrome c release to the cytosol is just a prerequisite for ROS generation. The presence of cytochrome c in the cytosol concomitant to cell death was probably later in the evolution exploited for more efficient activation of the caspase cascade.

What is the target of ROS in the yeast cell that is about to undergo apoptosis-like death? One possible target may be vacuole, the yeast equivalent of the mammalian lysosomal compartment. Damage by mild oxidative stress may cause a limited leakage of vacuolar proteases to the cytosol, where they could for example activate the putative metacaspases, or cleave other “apoptotic”substrates [Fossel et al. 1994].

When lysosomes of human cells are loaded with acridine orange and irradiated, or when the cells are subjected to mild oxidative stress, the lysosomes start to leak their content, even though they appear structurally intact [Brunk & Ericsson 1972]. One of the factors that are released from the lysosome under such conditions is cathepsin D, a highly conserved orthologue of the yeast vacuolar protease yscA. The limited leak-

age of lysosomes leads to cell death with classical apoptotic features [Fossel et al. 1994, Brunk et al. 1995, Brunk et al. 1997, Nilsson et al. 1997]. During apoptosis induced by external oxidative stress, lysosomal release of cathepsin D precedes relocation of cytochrome c to the cytosol, and cell death with accompanying apoptotic features can be prevented by treatment with the cathepsin D inhibitor pepstatin A [Roberg et al. 1999].

In a screen for proteasomal substrates that trigger apoptosis when stabilized (see the next section), I isolated a cDNA coding for a subunit Ppa1 of vacuolar H^+-ATPase. I overexpressed this gene in a strain with defective proteasome and examined the morphology of the vacuole. To visualize possible changes in vacuolar morphology I used a peptide-dye adduct that is cell permeable and non-fluorescent, but after cleavage at the yscA recognition site turns fluorescent and becomes unable to cross cellular membranes. In wild type cells this dye is used to visualize the vacuole, which appears as a disc with sharp edges, with no cytosolic fluorescence. In the cells overexpressing Ppa1 the vacuoles appeared normal under Nomarski optics, but the fluorescence was diffused, with significant signal in the cytosol. Cells treated with low concentration of H_2O_2, cells overexpressing Bax, and *cdc48-S565G* mutants displayed similar vacuolar morphology (unpublished observations). This can be interpreted in two ways: the dye entered the vacuole, got activated by cleavage and leaked to the cytosol; or, the dye was activated in the cytosol by a protease (yscA/cathepsin D) leaking from the vacuole. Although this is just a preliminary experiment, it indicates that vacuolar leakage of proteases may play a role also in the yeast apoptotic-like cell death.

3.3 The Proteasome and Apoptosis

The 26 S proteasome is a protease responsible for the bulk of cytosolic protein degradation, including regulatory proteolysis of a number of short-lived substrates involved in the control of various processes in the cell. It is perhaps not surprising that the interference with proteasomal proteoly-

sis appears to have pro-apoptotic or anti-apoptotic effect, depending on the cell context [Orlowski 1999].

The contradictory effects of proteasome inhibition may be explained by the proliferative state of the cell and by the mechanism of induction of apoptosis. In proliferating cells the inhibition of proteasome may lead to the blockage of the cell cycle and possibly to a signaling conflict between accumulating levels of the cdk inhibitor $p27^{Kip1}$ (leading to antiproliferative signal) and c-myc (leading to proliferative signal) [Drexler 1997], both of which are proteasomal substrates [Pagano et al. 1995, Gregory & Hann 2000]. In contrast, in terminally differentiated cells the same proteasomal inhibitors have the opposite effect and block apoptosis, possibly by preventing proteasome-mediated degradation of a caspase inhibitor [Yang et al. 2000].

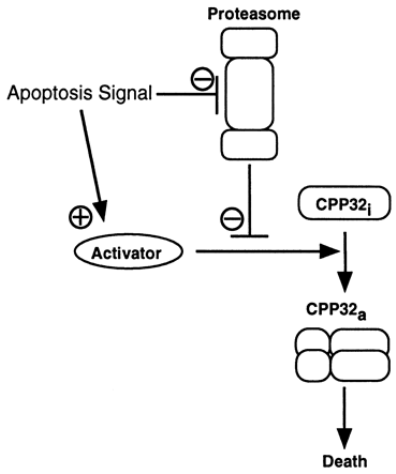

Figure 3: A model of induction of apoptosis in proliferating cells by proteasome inhibition.

An apoptotic signal leads to stabilization and accumulation of a putative activator of apoptosis. This in turn activates a caspase cascade (here represented by caspase CPP32), leading to cell death. Reprinted with permission from DrexlerHC (1997) Activation of the cell death program by inhibition of proteasome function. Proc Natl Acad Sci USA 94: 855–860. © 1997 National Academy of Sciences, U.S.A

The proteasome plays a major role in the degradation of many short-lived proteins, some of which are known to influence apoptosis. Examples include p53 [Scheffner et al. 1990], NF-κB [Palombella et al.

1994], Bax [Li & Dou 2000], Bid [Breitschopf et al. 2000b], and Bcl-2 [Breitschopf et al. 2000a]. Thus, it is likely that application of proteasome inhibitors will lead to different effects depending on which apoptosis-inducing agent is used in a particular cell type.

The ubiquitin-proteasome system has been proposed to control apoptosis in proliferating mammalian cells by degrading a short-lived pro-apoptotic protein [Drexler 1997] (Figure 3). To find out if the ubiquitin system plays a similar role in yeast I performed a two layer screen. In the first step I looked for potential yeast proteasomal substrates that when overexpressed cause cells with defective proteasome to arrest growth. In the second step, I screened these putative substrates for their ability to cause cell death and to elicit diagnostical markers of apoptosis in yeast cells. Six proteins were found whose overexpression in the proteasomal mutant led to exposure of phosphatidylserine on the cell surface, chromatin condensation, DNA breakage, and cell death.

One of them was Stm1, a protein that was known to bind quadruplex DNA [Frantz & Gilbert 1995] and purine-rich triplex DNA [Nelson et al. 2000] in vitro. Quadruplex structures were suggested to be present at chromosome ends [Liu et al. 1993]. However, using a one-hybrid assay for telomere binding proteins [Bourns et al. 1998] I could not detect any expression of the reporter gene that would indicate interaction of Stm1 with telomeric DNA (data not shown). Consistent with its predicted ability to interact with DNA, I found that *stm1-Δ1* cells are sensitive to UV light and treatment with bleomycin, a drug mimicking the effect of ionizing radiation. They are not, however, sensitive to the alkylating agent MMS, suggesting that Stm1 might function in a specific aspect of DNA repair. Stm1 shows weak diffused cytosolic and strong perinuclear staining in fixed cells. Its localization at the periphery of spread nucleoids suggest direct interaction with DNA. These results are consistent with the detection of Stm1 in the highly enriched nuclear envelope fraction [Rout et al. 2000] and with the presence of a putative nuclear localization sequence in the protein.

Stm1 is an in vivo substrate of the proteasome, as evidenced by its rapid turnover in wild type cells and its complete stabilization in mutants with severely impaired proteasome. Because degradation of Stm1 is blocked also under non-lethal conditions (normal expression of Stm1 from its endogenous promoter) it can be excluded that Stm1 stabilization is a consequence of cell death. Therefore the data strongly suggest that the lethal effect of overexpressed Stm1 in *pre1-1 pre4-1* mutants is a result of accumulation of the stabilized protein.

The conspicuous feature of the *pre1-1 pre4-1* cells killed by overexpression of Stm1 is the appearance of phenotypes found in metazoan cells undergoing apoptosis and yeast cells killed for example by exposure to low concentrations of H_2O_2. I tested the sensitivity of *stm1-Δ1* cells to treatment with H_2O_2 using a halo assay and a survival test in liquid culture. In both cases a significant portion of *stm1-Δ1* cells survived exposure to low doses of H_2O_2 that are toxic to wild type cells. In addition, DNA cleavage was correspondingly reduced in *stm1* null mutant cells, indicating that increased survival of these mutants is due to suppression or absence of the apoptosis-like cell death. Cycloheximide treatment — and thereby blocking of protein synthesis — has a protective effect on wild type yeast cells exposed to low levels of H_2O_2 [Collinson & Dawes 1992], but this phenomenon was absent in *stm1-Δ1* mutants. Cycloheximide increases survival of H_2O_2 treated cells by inhibiting a translation-dependent apoptosis-like cell death process (see the previous section). Taken together, these findings led to the idea that protection against H_2O_2 induced cell death is based at least in part on depletion of Stm1 activity due to deletion of the *STM1* gene (*stm1-Δ1* cells) or blocking of its synthesis (application of cycloheximide). Hence, the data suggest that the Stm1 protein is an activator of the cell death process triggered by exposure of cells to low concentrations of H_2O_2. Control of its synthesis and/or degradation may be regulatory steps of H_2O_2 induced apoptosis-like cell death in yeast.

STM1 was originally identified as a multicopy suppressor of *tom1*, *htr1*, and *pop2* mutations, each of them being involved in an aspect of cell

cycle control (for summary see [Nelson et al. 2000]). In a genome-wide two hybrid screen, Stm1 was found to interact with a product of a predicted gene *YJR072C* [Uetz et al. 2000], which has conserved orthologues in *Caenorhabditis elegans* and humans. Stm1 itself has a highly conserved orthologue in *Schizosaccharomyces pombe* and a putative orthologue in *Drosophila melanogaster* [Nelson et al. 2000]. This hints that Stm1 may regulate or participate in an evolutionary conserved process.

Another cDNA detected in the screen encoded the GTP-binding protein Sar1, a component of COPII coated vesicles involved in transport from the endoplasmic reticulum to the Golgi compartment [Kuehn et al. 1998]. Caspase-dependent cleavage of human BAP31 occurring during apoptosis leads to disruption of the endoplasmic reticulum – Golgi transport [Maatta et al. 2000]. Conversely, perturbation of the early secretory pathway by brefeldin A has been shown to trigger p53-independent apoptosis [Shao et al. 1996] in a ceramide signaling pathway dependent manner [Linardic et al. 1996]. Ceramide levels rise in yeast during heat shock [Wells et al. 1998], and preventing ceramide accumulation enhances survival of cells after heat treatment [Mandala et al. 1998]. Lysosomes (see the previous section) appear to integrate the signals from the ceramide pathway as well [Monney et al. 1998, Heinrich et al. 1999]. The ceramide signaling pathway, which is implicated in control of apoptosis in mammals [Hannun & Luberto 2000], may thus turn out to be a player in regulation of apoptotic-like cell death also in yeast.

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5 Appendices

Appendix A: Ligr M, Madeo F, Fröhlich E, Hilt W, Fröhlich K-U, Wolf DH (1998) Mammalian Bax triggers apoptotic changes in yeast. *FEBS Lett* 438: 61–65.

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Mammalian Bax triggers apoptotic changes in yeast

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Abstract Apoptosis is co-regulated by the conserved family of Bcl-2-related proteins, which includes both its agonists (Bax) and antagonists (Bcl-X_L). A mutant strain of the yeast *Saccharomyces cerevisiae* has been shown to express all morphological signs of apoptosis. Overexpression of Bax is lethal in *S. cerevisiae*, whereas simultaneous overexpression of Bcl-X_L rescues the cells. We report that overexpression of mammalian Bax in a *S. cerevisiae* wild type strain triggers morphological changes similar to those of apoptotic metazoan cells: the loss of asymmetric distribution of plasma membrane phosphatidylserine, plasma membrane blebbing, chromatin condensation and margination, and DNA fragmentation. Simultaneous overexpression of Bcl-X_L prevents these changes. We demonstrate that Bax triggers phenotypic alterations in yeast strongly resembling those it causes in metazoan apoptotic cells.

Key words: Apoptosis; Bax; Yeast *Saccharomyces cerevisiae*

1. Introduction

Apoptosis, a form of programmed cell death, is a part of normal development and homeostasis in metazoans. Breakdown of its regulation leads to a variety of diseases such as cancer, and neurodegenerative and autoimmunity disorders [1]. Morphologically apoptosis is defined by the loss of asymmetric distribution of plasma membrane phospholipids [2], condensation and margination of chromatin, DNA fragmentation [3], plasma membrane blebbing, and finally the breakage of the cell into membrane-enclosed apoptotic bodies [4]. This process is co-regulated by the conserved family of Bcl-2-related proteins, which includes both agonists (Bax, Bak, Bad, Bcl-X_S) and antagonists (Bcl-2, Bcl-X_L) of apoptosis [5]. The decision on entry to apoptosis is achieved by direct interaction between these two groups. This interplay may regulate the constitution of channels in intracellular membranes and interaction with additional proteins [6]. The ultimate purpose of these interactions in mammalian cells is to control the activation of caspases. These are a group of cysteine proteases thought to be the actual effectors of apoptosis [7], which cleave specific target substrates [8].

A model has emerged recently, according to which the pro-apoptotic members of the Bcl-2 family trigger cell death by facilitating the release of cytochrome *c* from mitochondria. Cytochrome *c* then binds to CED-4/Apaf-1, an adaptor protein which in turn processes caspase-9/CED-3 to its active form. Negative apoptotic regulators of the Bcl-2 family are suggested to act at two levels. They are able to block release of cytochrome *c*, or prevent the activation of the caspase cascade by binding to CED-4/Apaf-1 [9].

While studying the interaction of Bax with other Bcl-2-like proteins, Reed and colleagues observed that its overexpression inhibited the growth of the yeast *Saccharomyces cerevisiae*, a phenotype which could be suppressed by simultaneous overexpression of antiapoptotic members of the Bcl-2 family [10,11]. This no-growth phenotype was demonstrated to be caused by cell death, and not simply by growth arrest [12]. These observations led to the key question: is the death triggered in yeast by metazoan apoptotic regulators equivalent to apoptosis? The ensuing studies gave equivocal results [13–15]. Recently a mutation in an indigenous *S. cerevisiae* protein Cdc48p has been described, which caused a set of phenotypic changes in yeast normally defining apoptosis in metazoan cells [16]. We therefore asked if overexpression of the mammalian protein Bax leads to a similar apoptotic phenotype in this unicellular eukaryote.

2. Materials and methods

2.1. Yeast strains and plasmids

Plasmids pSD10.a-Bax and pSD10.a-Bcl-X_L [17] contain a cDNA fragment of the respective gene under the control of a hybrid GAL1-10/CYC1 promoter in a pRS316-based vector with URA3 marker [18]. The marker in pSD10.a-Bcl-X_L was changed to LEU2, yielding pL009. Wild type *S. cerevisiae* strain WCG4 (*MATα his3-11,15 leu2-3,112 ura3*) was transformed with plasmids pSD10.a-Bax (CEN6, URA3), pL009 (CEN6, LEU2), and control vectors pRS315 (CEN6, LEU2) and pRS316 (CEN6, URA3) [19]. Strains were pregrown in synthetic complete (SC) medium (0.67% nitrogen base without amino acids, amino acids and nucleotide bases) lacking leucine and uracil, and containing 2% glucose as the carbon source at 30°C to a cell density of about 0.5×10^6/cm^3. To induce the expression of Bax and Bcl-X_L, cells were washed three times and resuspended in SC medium with 2% galactose instead of glucose. Cells with control plasmids and with Bax plus Bcl-X_L plasmids were diluted so that the cell density at the end of treatment was about 0.5×10^6/cm^3. The cell density of the culture of cells expressing Bax did not change within 22 h of further incubation.

To visualize nuclei the cells were incubated with 1 μg/cm^3 DAPI in HEPES buffer (10 mM HEPES/NaOH buffer pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) for 20 min, washed three times with HEPES buffer, and examined under the fluorescence microscope.

2.2. Annexin V staining

Externalization of PS was detected as described previously [16]. Cells were resuspended in digestion buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, 35 mM PO_4^{3-}, pH 6.8) and incubated for 2 h at 30°C with 15 U/cm^3 zymolyase 100T (Seikagaku Corporation, Tokyo, Japan) and

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Abbreviations: DAPI, diaminophenylindole; PI, propidium iodide; PS, phosphatidylserine; SC, synthetic complete growth medium; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

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5.5% glusulase (NEN, Boston, MA). After cell wall digestion cells were washed in binding buffer containing sorbitol (1.2 M sorbitol, 10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). The protoplasts were resuspended in 38 μl of binding buffer and incubated with 2 μl annexin-FITC (Clontech) and 2 μl PI (500 μg/ml) in the dark for 20 min at room temperature. Cells were mounted on a slide and examined under the fluorescence microscope.

2.3. Electron microscopy

Yeast cells were prepared for electron microscopy as described previously [16]. Cells were fixed with phosphate-buffered glutardialdehyde, cell walls were removed, and the cells were postfixed with osmium tetroxide and uranyl acetate and dehydrated as described by Byers and Goetsch [20] for stationary-phase cells. Following the 100% ethanol washes, cells were washed with 100% acetone, infiltrated with 50% acetone/50% Epon for 30 min and with 100% Epon for 20 h. Cells were transferred to fresh 100% Epon and incubated at 56°C for 48 h before cutting thin sections and staining with lead acetate.

2.4. TUNEL staining

A previously described protocol for TUNEL detection of fragmented nuclear DNA in yeast [16] was used with small changes. Cells were fixed in 3.7% formaldehyde for 1 h and the cell walls were removed as described above. The protoplasts were then applied to polylysine-coated slides. In Situ Cell Death Detection Kit POD (Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturer's instructions. After mounting the coverslip with a drop of Kaiser's glycerol gelatin (Merck, Darmstadt, Germany) the cells were examined under the light microscope.

3. Results

3.1. Bax causes the loss of plasma membrane asymmetry in yeast

Lipid composition of the inner and outer leaflets is different in most biological membranes [21], including the yeast plasma membrane [22]. This asymmetry is maintained by an enzymatic activity [23,24] linked in both mammalian and yeast cells to ABC transporters [25,26]. In cells undergoing apoptosis the asymmetry of the plasma membrane is lost and phosphatidylserine (PS) is exposed on the cell surface [2]. In mammalian cells, this process is enhanced by the loss of the aminophospholipid translocase activity and the presence of extracellular calcium [27].

To test if overexpression of Bax in budding yeast leads to the loss of membrane asymmetry and the exposure of PS on the cell exterior, protoplasts were incubated with FITC-labeled annexin V, a PS binding protein. In parallel, the intactness of the protoplasts was assessed by their ability to exclude propidium iodide (PI). After 6 h of galactose induction the binding of annexin V was visible in some cells as punctate structures at the plasma membrane (Fig. 1a), after 15 h about 10% of the cells showed green halos surrounding the cells (Fig. 1c,e).

Exposure of PS on the cell surface accompanying the Bax-induced death of yeast may be due to loss of the putative aminophospholipid translocase activity in a process similar to PS exposure in apoptotic mammalian cells. In mammalian systems the display of PS on the cell surface serves as a recognition signal for removal of apoptotic cells by macrophages [28]. The presence of this phenomenon already in yeast indicates a more fundamental connection to apoptosis.

3.2. Overexpression of Bax leads to chromatin condensation and plasma membrane blebbing

Bax expression induced changes of nuclear morphology of yeast cells were followed by diaminophenylindole (DAPI) staining of DNA. After overexpression of Bax for 15 h about 20% of the cells showed abnormal nuclear morphology: alterations ranged from accumulation of DAPI staining in ring-like structures (Fig. 2e), presence of multiple DAPI-stained regions within the area of the nucleus (Fig. 2c), to the appearance of multiple stained regions within a single cell (Fig. 2a).

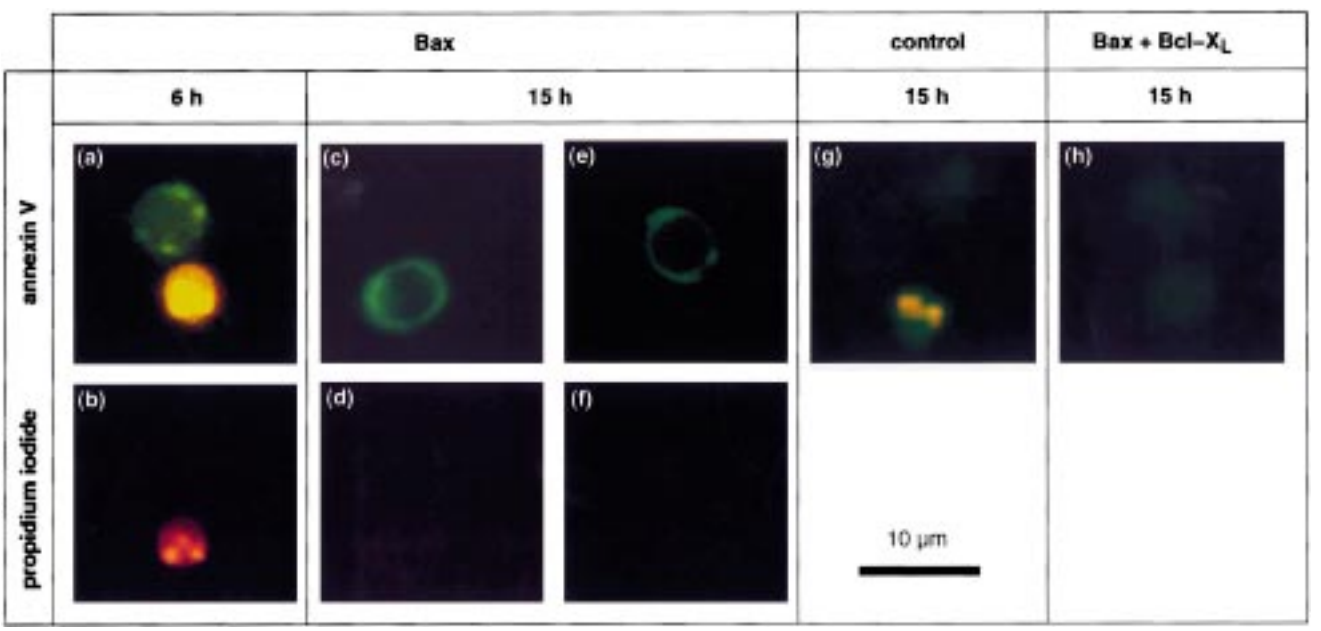

Fig. 1. Overexpression of Bax leads to the exposure of phosphatidylserine on the outer leaflet of the plasma membrane of *S. cerevisiae* cells. Cells transformed with control plasmid pRS315 plus plasmid pSD10.a-Bax carrying Bax (a–f), control plasmids pRS315 and pRS316 (g), or plasmids pSD10.a-Bax and pL009 carrying Bcl-X_L (h). Cells were probed with FITC-annexin V conjugate and propidium iodide after 6 h (a,b) or 15 h (c–h) of induction on galactose medium. Figures (a,b), (c,d), (e,f) show the same cells examined with the FITC (a,c,e,g,h) and PI (b,d,f) filters. Cells binding annexin V exclusively on the outer leaflet of the plasma membrane are recognized by a green halo and no orange staining.

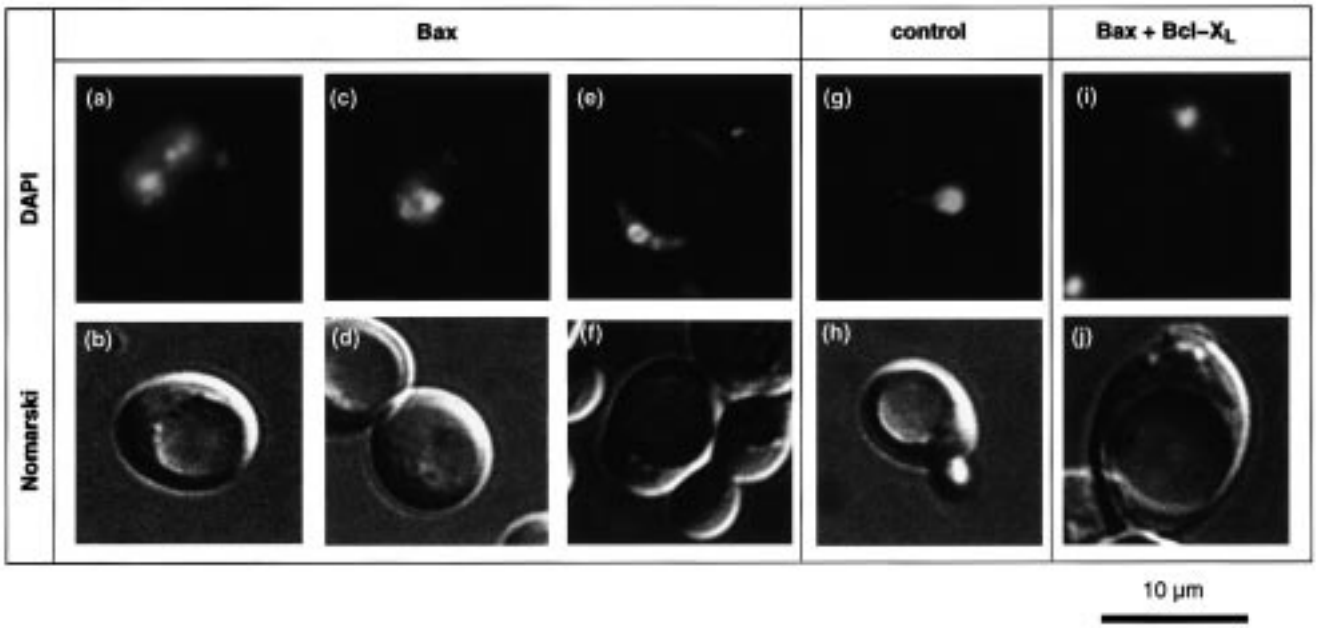

Fig. 2. Nuclei of cells overexpressing Bax show abnormal morphology. Yeast cells containing the control plasmid pRS315 plus plasmid pSD10.a-Bax carrying Bax (a–f), control plasmids pRS315 and pRS316 (g,h), or plasmids pSD10.a-Bax and pL009 carrying Bcl-X_L (i,j) were stained with DAPI after 15 h of induction on galactose. Figures (a,b), (c,d), (e,f), (g,h), (i,j) show the same cells examined with the DAPI filter (a,c,e,g,h,i) and Nomarski optics (b,d,f,h,j).

Electron microscopic examination of same culture as used for DAPI staining revealed the presence of nuclear regions of condensed chromatin, often located at the periphery of the nucleus (Fig. 3c,d). These alterations in the nuclear morphology are reminiscent of the changes observed in apoptotic mammalian cells [29], and very similar to those described in the yeast *cdc48*^{S565G} mutant [16].

Chromatin condensation as a result of expression of Bax or its homologue Bak had been observed in *Schizosaccharomyces pombe* [14,15]. However, changes in nuclear morphology reported by these groups were accompanied by extensive vacuolization, perhaps induced by prolonged expression of pro-apoptotic proteins (for up to 48 h). This may mirror a situation during terminal stages of mammalian apoptosis when the cellular content is being disposed of. On the other hand, when ced-4 was expressed in *S. pombe* for only 12 h, extensive chromatin condensation and disruption of the nucleus took place in the absence of any vacuolization [30]. Under our experimental conditions no vacuolization was observed. Only some degree of plasma membrane blebbing occurred (Fig. 3c), paralleling the situation during the onset of mammalian apoptosis.

3.3. Nuclear DNA is fragmented in yeast overexpressing Bax

To examine if the alterations in nuclear morphology are also accompanied by changes in the physical intactness of DNA, we inspected the Bax expressing cells by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) assay [31]. During this procedure terminal deoxynucleotidyl transferase attaches FITC-conjugated dUTP tails to free 3′ ends of DNA in a template-independent fashion. With the breakage of DNA the number of free 3′ ends increases significantly, giving rise to a stronger TUNEL staining in the affected nuclei.

When performing the TUNEL assay, approximately 30% of the Bax-expressing cells showed staining in their nuclei (Fig. 4a), indicating the presence of damaged DNA. A similar rate of TUNEL staining had been found in the exponentially growing *cdc48*^{S565G} mutant [16]. In mammalian cells the cleavage of DNA as a result of Bax-induced apoptosis is thought to be dependent on the activity of caspases [32] and caspase-activated DNase(s) [8]. In bacteria, on the contrary, the expression of Bax led to DNA damage in the absence of caspases [33], as in our yeast system.

3.4. Bcl-X_L prevents the appearance of Bax-induced apoptotic phenotypes in yeast

Bcl-X_L prevents Bax-induced cell death in both metazoan and yeast cells. We observed that co-expression of Bax and Bcl-X_L not only rescues the yeast cells from Bax-induced cell death, but also prevents the characteristic phenotypes that accompany it. We observed no binding of annexin V to the intact protoplasts (Fig. 1h). The morphology of the nucleus examined by DAPI staining appeared wild type-like (Fig. 2i), as well as the appearance of the nuclei under the electron microscope (Fig. 3b). In addition no significant TUNEL staining was detected in the nuclei of these cells (Fig. 4c). These data show that the phenotypes associated with Bax overexpression in yeast are tightly linked with its killing function, since co-expression of its antagonist, Bcl-X_L, eliminated both the Bax-induced cell death and its associated phenotypes.

4. Discussion

S. cerevisiae cells overexpressing the mammalian apoptotic stimulator Bax display a series of changes, identical to morphological markers defining apoptosis in mammalian cells. Early during Bax induction the plasma membrane of the cells apparently loses its asymmetry as PS is exposed on the surface of the cell. Chromatin undergoes condensation and margin-

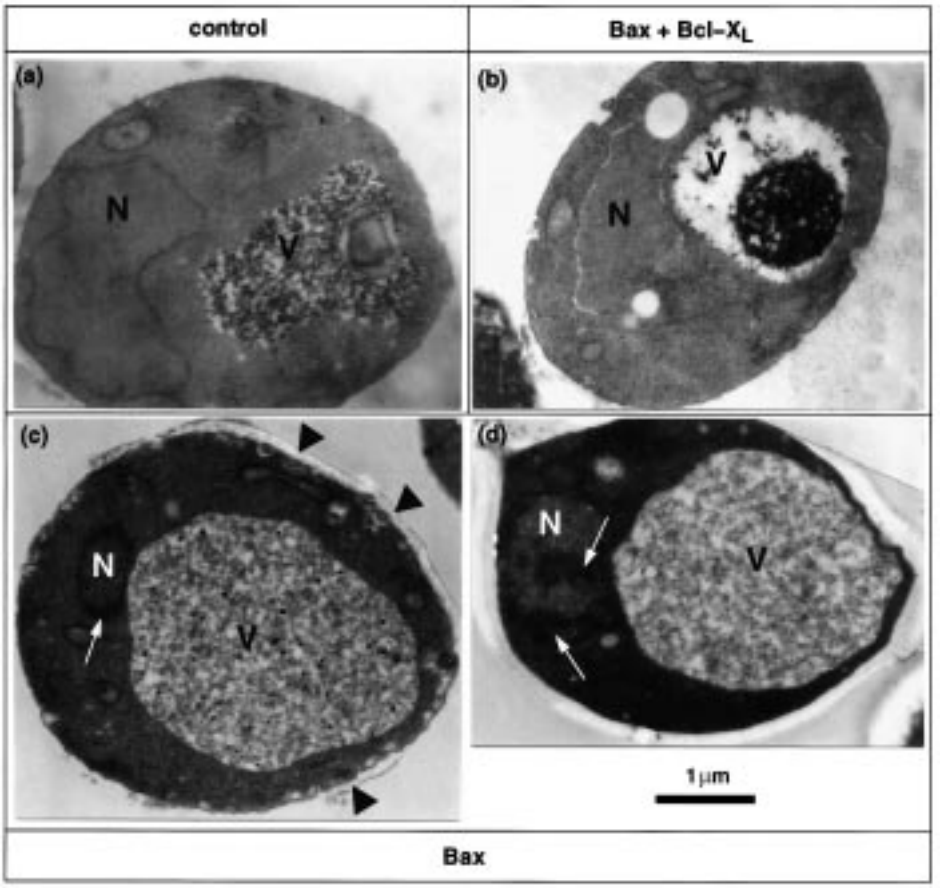

Fig. 3. Overexpression of Bax results in chromatin margination and condensation, as well as blebbing of the yeast plasma membrane. Electron micrographs of yeast cells transformed with control plasmids pRS315 and pRS316 (a), plasmids pSD10.a-Bax and pL009 carrying Bax and Bcl-X_L, respectively (b), or with control plasmid pRS315 plus plasmid pSD10.a-Bax carrying Bax (c,d). Cells were induced on galactose for 15 h. Arrows point to regions of nuclei with condensed and marginalized chromatin, arrowheads mark the blebbing of the plasma membrane. N, nucleus; V, vacuole.

ation, and nuclear DNA is fragmented. Some degree of plasma membrane blebbing was observed as well. These alterations are absent in the control cells cultivated under identical conditions, as well as in the cells where the lethal effect of Bax expression was compensated by co-expression of the mammalian apoptotic inhibitor Bcl-X_L. In a screen for genes involved in the putative apoptotic pathway in yeast only 9% of the clones that could be induced to undergo cell death showed positive annexin V and TUNEL staining (M. Ligr and F. Madeo, unpublished observations). Therefore the observed

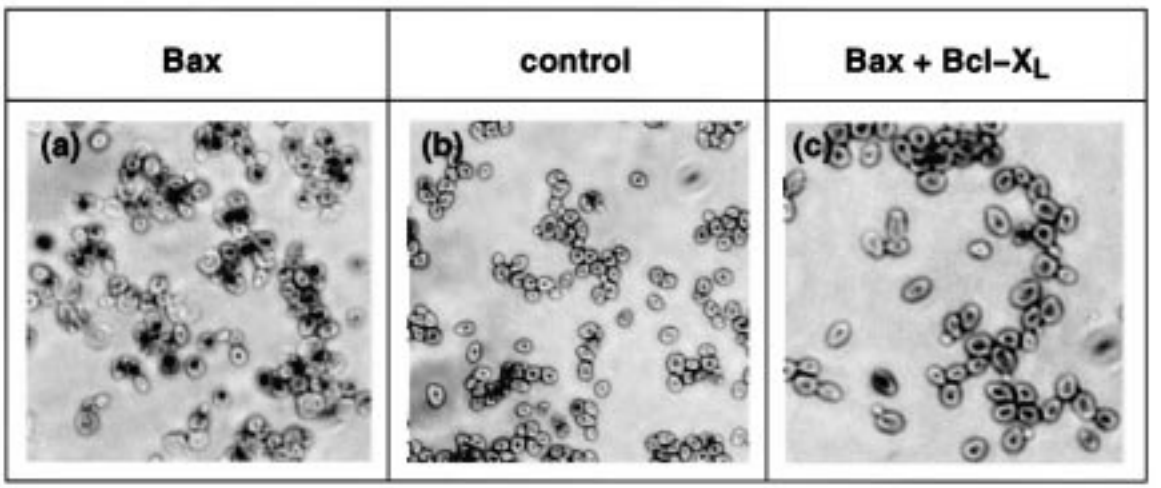

Fig. 4. The nuclei of yeast cells overexpressing Bax contain fragmented DNA. Yeast cells transformed with control plasmid pRS315 plus plasmid pSD10.a-Bax carrying Bax (a), control plasmids pRS315 and pRS316 (b), and plasmids pSD10.a-Bax and pL009 carrying Bax and Bcl-X_L (c) were induced on galactose for 15 h. Fragmentation of nuclear DNA was detected with the TUNEL test (formation of black-stained nuclei).

apoptotic phenotypes are not likely to be just general features of dying yeast. We do not see any connection among the observed phenotypes of Bax-killed yeast cells other than their link to metazoan apoptosis.

The effects of Bax expression in *S. pombe* and *S. cerevisiae* reported previously were based largely on electron microscopy and led to conclusion that Bax-induced cell death in yeast differs from apoptosis [13–15]. In contrast, using a comprehensive set of tests for morphological markers of apoptosis, we show that Bax-induced cell death in budding yeast has an apoptotic character.

The apoptotic program in metazoan cells is thought to depend on the proteolytic action of caspases [7]. The complete sequence of the yeast genome [34] confirmed the absence of homologues of known apoptotic regulators of the Bcl-2 family, as well as of caspases in *S. cerevisiae*. Interestingly, Bax is able to kill mammalian cells in the absence of caspase activity as well [32]. In this case the lethal effect of Bax is accompanied by the production of reactive oxygen species.

These findings point to the presence of a pathway responsible for execution of cell death common in yeast and metazoan cells. This putative pathway could function in parallel with the well studied caspase-dependent pathway in multicellular eukaryotes, or it could be a basic framework upon which the caspase pathway developed.

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Appendix B: Madeo F, Fröhlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH, Fröhlich K-U (1999) Oxygen stress: a regulator of apoptosis in yeast. *J Cell Biol* 145: 757-767.

Oxygen Stress: A Regulator of Apoptosis in Yeast

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Abstract. Oxygen radicals are important components of metazoan apoptosis. We have found that apoptosis can be induced in the yeast *Saccharomyces cerevisiae* by depletion of glutathione or by low external doses of H_2O_2. Cycloheximide prevents apoptotic death revealing active participation of the cell. Yeast can also be triggered into apoptosis by a mutation in *CDC48* or by expression of mammalian *bax*. In both cases, we show oxygen radicals to accumulate in the cell, whereas radical depletion or hypoxia prevents apoptosis. These results suggest that the generation of oxygen radicals is a key event in the ancestral apoptotic pathway and offer an explanation for the mechanism of *bax*-induced apoptosis in the absence of any established apoptotic gene in yeast.

Key words: apoptosis • glutathione • oxygen stress • reactive oxygen species • *Saccharomyces cerevisiae*

APOPTOSIS is an active form of cell death crucial for the development and homeostasis of metazoan organisms. In mammals, apoptosis can be induced by various intra- or extracellular stimuli and via several signaling pathways, depending on, for example, cell type, cellular environment, and differentiation state. Virtually every triggering event or component essential for apoptosis in one system has been shown to be dispensable in some other system, e.g., apoptosis in the presence of caspase inhibitors (Xiang et al., 1996; Monney et al., 1998), or in the absence of bax (Knudson et al., 1995; McCurrach et al., 1997). Apoptosis is characterized by a set of phenotypical alterations such as the exposition of phosphatidylserine on the cell surface (Martin et al., 1995), a characteristic condensation of chromatin to the nuclear envelope ("margination," Clifford et al., 1996), DNA fragmentation, and the formation of membrane-enclosed cell fragments called apoptotic bodies (Kerr et al., 1972).

Its obviously altruistic function seemed to limit apoptosis to multicellular organisms. Consistently, in a blast database search of the *Saccharomyces cerevisiae* genome no obvious homologues of any crucial regulator of metazoan apoptosis (members of the bax/bcl-2 family, caspases, Apaf-1/CED-4, p53) were detected. However, it has been noted that yeast cells, both *S. cerevisiae* and *Schizosaccharomyces pombe*, can be killed by the expression of a number of proapoptotic mammalian genes, for example, bax (Sato et al., 1994; Hanada et al., 1995; Greenhalf et al., 1996) or p53 (Bischoff et al., 1992; Nigro et al., 1992).

Our recent observations that a certain *S. cerevisiae cdc48* mutant as well as cells overexpressing *bax* coordinately show the phenotypic markers of apoptosis, chromatin condensation and fragmentation, DNA breakage, exposition of phosphatidylserine, and the formation of minicells approximating apoptotic bodies, indicates the presence of a basic apoptotic mechanism in yeast (Madeo et al., 1997; Ligr et al., 1998). This is in accordance with the observation of chromatin condensation and nuclear fragmentation in *S. pombe* cells dying due to the expression of Bak (Ink et al., 1997) or CED-4 (James et al., 1997). Bax lethality in *S. cerevisiae* can be suppressed by a defect in mitochondrial F_0F_1-ATPase or by the expression of the mammalian gene BI-1. Significantly, BI-1 does not interact directly with Bax, suggesting that it acts on elements already present in yeast (Xu and Reed, 1998). Similarly, a mutant form of Bcl-X_L, an anti-apoptotic Bcl-2 family member, has been described, which in the absence of physical interaction can prevent Bax-induced death in yeast (Tao et al., 1997). In mammalian cells, both the inhibition of F_0F_1-ATPase by oligomycin and the expression of BI-1 prevent apoptosis (Matsuyama et al., 1998; Xu and Reed, 1998). Obviously, Bax, Bak, or CED-4 do not simply act as cytotoxic substances in yeast but seem to activate the same or a similar mechanism as in metazoan or-

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ganisms. Because of their absence in yeast, this mechanism can not depend on Bax relatives, caspases, or caspase-activated deoxyribonuclease.

The observation of cell death accompanied by apoptosis-like features in yeast suggests that apoptosis developed before the evolutionary separation between fungi and metazoans. Elements of the pathway conserved in yeast as well as animals should therefore belong to a basic, evolutionarily old mechanism. Yeast should be useful to trace the roots of apoptosis and solve some of the complications and apparent contradictions inherent to the various models of apoptosis.

Reactive oxygen species (ROS),[1] are formed in any organism exposed to molecular oxygen appear to be crucial players in apoptosis (Ghibelli et al., 1995). ROS or H_2O_2 can act as primary triggers of apoptosis (Hockenbery et al., 1993; Kane et al., 1993; Greenlund et al., 1995; Slater et al., 1995). The anti-apoptotic effect of Bcl-2 appears to be at least partly due to its antioxidant properties (Kane et al., 1993). In addition, oxygen radicals have been shown to act at a late step of the apoptotic pathway in certain neuronal cells, downstream of the effects of bax or caspases (Schulz et al., 1997).

We present proof that ROS accumulate in yeast cells after induction of apoptotic death by various stimuli, and show them to be necessary and sufficient to induce an apoptotic phenotype in yeast. We suggest that the formation of ROS is a key event in the evolutionary original apoptotic mechanism.

Materials and Methods

Strains and Plasmids

S. cerevisiae strain KFY437 (*MAT* a *cdc48*::*URA3 his4*-619 *leu2*-3,112 *ura3*-52 YEp52/cdc48^{S565G} containing mutant allele *cdc48*^{S565G} on vector YEp52), and the corresponding control strain KFY417 (*MAT* a *cdc48*::*URA3 his4*-619 *leu2*-3,112 *ura3*-52 YEp52/CDC48) with a wild-type *CDC48* on YEp52 have been described (Madeo et al., 1997). Strain YPH98gsh1 was constructed from yeast wild-type YPH98 (*MAT* a *ura3*-52 *lys2*-801 *ade2*-101 *leu2*-3,112 *trp1*-Δ1; Sikorski and Hieter, 1989) by deletion of the complete *GSH1* reading frame (Brendel et al., 1998). Cell division cycle mutants used as controls were Hartwell (1973) strains LH370 (*cdc2*^{ts}) and LH12021 (*cdc31*^{ts}), and rE24-15 (*cdc48*-3^{ts}; Moir et al., 1982). Plasmids pSD10.a-Bax and pSD10.a-Bcl-X_L contain murine bax, respectively, bcl-X_L under the control of a hybrid GAL1-10/CYC1 promoter (Tao et al., 1997) in a pRS316-based vector with a *URA3* marker (Dalton and Treisman, 1992). Plasmid pL009 was constructed by replacing the *URA3* marker of plasmid pSD10.a-Bcl-X_L by *LEU2*. Strain WCG4 (*MAT* α *his3*-11 *ura3*-52 *leu2*-3,112) was transformed with plasmid pSD10.a-Bax (strain WCG4bax), or with both pSD10.a-Bax and pL009 (strain WCG4bax/bcl-X_L), or with vector pRS316 as a control.

YEPD consisted of 1% yeast extract (Difco), 2% Bacto peptone (Difco), and 4% glucose. Synthetic complete medium (SC) consisted of 0.67% yeast nitrogen base (Difco) and 2% glucose (SCD) or 2% galactose (SCGal) supplemented with amino acids and nucleotide bases. Strains KFY437 and KFY417 were pregrown on YEP plates with 4% galactose and inoculated in liquid YEPD. WCG4bax, WCG4bax/bcl-X_L, and the vector control strain were pregrown in synthetic medium with 2% glucose to a cell density of about 0.5×10^6 cm^{-3}. To induce the expression of Bax or Bcl-X_L, cells were washed 3× and resuspended in synthetic medium with 2% galactose.

For growth under anaerobic conditions, strains were incubated in 100 ml culture medium in wash bottles under flow of N_2 (Messer Griesheim GmbH) at 100 ml/min. Viability was determined as the portion of cell forming visible colonies on YEPD plates after 3 d at 28°C.

Test for Apoptotic Markers

Electron microscopy, annexin V labeling, and DAPI staining were performed as described previously (Madeo et al., 1997). For the TdT-mediated dUTP nick end labeling (TUNEL) test, cells were prepared as described (Madeo et al., 1997), and the DNA ends were labeled using the In Situ Cell Death Detection Kit, POD (Boehringer Mannheim). Yeast cells were fixed with 3.7% formaldehyde, digested with lyticase, and applied to a polylysine-coated slide as described for immunofluorescence (Adams and Pringle, 1984). The slides were rinsed with PBS and incubated with 0.3% H_2O_2 in methanol for 30 min at room temperature to block endogenous peroxidases. The slides were rinsed with PBS, incubated in permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min on ice, rinsed twice with PBS, incubated with 10 μl TUNEL reaction mixture (terminal deoxynucleotidyl transferase 200 U/ml, FITC-labeled dUTP 10 mM, 25 mM Tris-HCl, 200 mM sodium cacodylate, 5 mM cobalt chloride; Boehringer Mannheim) for 60 min at 37°C, and then rinsed 3× with PBS. For the detection of peroxidase, cells were incubated with 10 μl Converter-POD (anti-FITC antibody, Fab fragment from sheep, conjugated with horseradish peroxidase) for 30 min at 37°C, rinsed 3× with PBS, and then stained with DAB-substrate solution (Boehringer Mannheim) for 10 min at room temperature. A coverslip was mounted with a drop of Kaiser's glycerol gelatin (Merck). As staining intensity varies, only samples from the same slide were compared.

Free intracellular radicals were detected with dihydrorhodamine 123, dichlorodihydrofluorescein diacetate (dichlorofluorescin diacetate), or dihydroethidium (hydroethidine; Sigma Chemical Co.). Dihydrorhodamine 123 was added ad-5 μg per ml of cell culture from a 2.5-mg/ml stock solution in ethanol and cells were viewed without further processing through a rhodamine optical filter after a 2-h incubation. Dichlorodihydrofluorescein diacetate was added ad-10 μg per ml of cell culture from a 2.5 mg/ml stock solution in ethanol and cells were viewed through a fluorescein optical filter after a 2-h incubation. Dihydroethidium was added ad-5 μg per ml of cell culture from a 5 mg/ml aqueous stock solution and cells were viewed through a rhodamine optical filter after a 10-min incubation. For flow cytometric analysis, cells were incubated with dihydrorhodamine 123 for 2 h and analyzed using a FACS® Calibur (Becton Dickinson) at low flow rate with excitation and emission settings of 488 and 525–550 nm (filter FL1), respectively.

Free spin trap reagents *N*-tert-butyl-α–phenylnitrone (PBN; Sigma-Aldrich) and 3,3,5,5,-tetramethyl-pyrroline *N*-oxide (TMPO; Sigma-Aldrich) were added directly to the cell cultures as 10-mg/ml aqueous stock solutions. Viability was determined as the portion of cell growing to visible colonies within 3 d.

To determine frequencies of morphological phenotypes (TUNEL, Annexin V, DAPI, dihydrorhodamine 123), at least 300 cells of three independent experiments were evaluated.

Inhibition of Protein Synthesis

Cycloheximide was added to exponentially growing yeast cultures at 5, 15, 50, 200 μg/ml, or 0 μg/ml as a control. After 30 min, 10 μCi 4,5-[^{3}H]-L-leucine (Movarek Biochemicals Inc.) was added to a 500-μl aliquot. After further incubation of 200 min at 30°C with shaking, 100-μl aliquots were spotted onto glass fiber filters and incubated in 10% TCA for 15 min in a boiling water bath. Filters were washed twice with 5% TCA, twice with ethanol and once with methanol, dried and counted with 5 ml Ultima Gold scintillation cocktail (Packard BioScience B.V.).

Results

H_2O_2 Induces an Apoptotic Phenotype in Wild-type *S. cerevisiae*

Exposure to H_2O_2 triggers apoptosis in numerous mammalian cells. To examine its effect in yeast, various amounts of H_2O_2 were added to wild-type cultures (strain YPH98) growing exponentially on YEPD. After a 200-min

1. *Abbreviations used in this paper:* PBN, *N*-tert-butyl-α–phenylnitrone; ROS, reactive oxygen species; SC, synthetic complete medium; TMPO, 3,3,5,5,-tetramethyl-pyrroline *N*-oxide; TUNEL, TdT-mediated dUTP nick end labeling.

incubation, cells were examined for markers of apoptosis. DNA cleavage was assayed using the TUNEL test (Gavrieli et al., 1992; Gorczyca et al., 1993), chromatin was visualized by fluorescence microscopy after DAPI staining as well as by electron microscopy.

We found that low concentrations of H_2O_2 result in a TUNEL-positive phenotype, which vanishes at higher concentrations. Incubation with 3 mM H_2O_2 produces a strongly TUNEL-positive phenotype (intense black nuclear stain) in 70% of the cells (Fig. 1 I), indicating massive DNA fragmentation. With 0.3 or 1 mM H_2O_2, only 20–40% of the cells are stained (Fig. 1 H). TUNEL-positive cells are often remarkably enlarged compared to TUNEL-negative cells from the same batch (Fig. 1, G and H, see also Fig. 3, G and H). Cells incubated in the absence of H_2O_2 showed unstained or only slightly grayish nuclei (Fig. 1 G). Increasing the H_2O_2 concentration above 5 mM did not intensify the TUNEL reaction. Instead, with 15 mM H_2O_2 the TUNEL staining is even much less intense and occurs in fewer cells than with 3 mM H_2O_2 (Fig. 1 J). Incubation with 180 mM H_2O_2 results in no detectable TUNEL staining (Fig. 1 K). To demonstrate that the DNA fragmentation is not a result of cell necrosis, membrane integrity was tested by incubating an aliquot of the protoplasts used for TUNEL staining with 23 μg/ml propidium iodide. Only 3–5% of the protoplasts from cultures treated with 0–5 mM H_2O_2 are stained (not shown). In cultures incubated with 180 mM H_2O_2, ~80% of the protoplasts are stained with propidium iodide, indicating the loss of plasma membrane integrity. Cells from stationary cultures tolerate higher concentrations of H_2O_2. After incubation with 5 mM H_2O_2 or less, no TUNEL staining is observed (not shown). On the other hand, incubation with 180 mM H_2O_2 results in a strong TUNEL staining (Fig. 1 L).

Analysis of isolated chromosomal DNA from H_2O_2 cells by agarose electrophoresis showed only a smear at low molecular weights, but not the DNA ladder pattern (not shown) that is found in many apoptotic systems as the result of DNA cleavage between nucleosomes. This confirms our result with the *cdc48* mutant showing an apoptotic phenotype (Madeo et al., 1997) and is probably caused by the *S. cerevisiae* chromatin structure with little or no linker DNA between the nucleosomes (Lowary and Widom, 1989). In addition, apoptosis without the occurrence of a DNA ladder has been described for several metazoan cell types (Oberhammer et al., 1993).

DAPI staining of cells incubated with 3 mM H_2O_2 shows chromatin fragments arranged in a half-ring (Fig. 1 D) or distributed nuclear fragments (Fig. 1, A, B, and E) in 10–50% of the cells. In some cells, nuclei seem to degenerate, showing protruding tubes (Fig. 1 C). In untreated cultures, all nuclei appear as single round spots in the cells. In contrast to nuclei or most nuclear fragments, mitochondria are predominantly located near the periphery of the cells and appear as dots of far less intensity and size (Fig. 1 D).

Electron microscopic investigation of cells incubated with 3 mM H_2O_2 revealed extensive chromatin condensation along the nuclear envelope typical for apoptosis (margination, Fig. 2, D and E), cells containing multiple nuclear fragments (Fig. 2 E), as well as tubular structures protruding from the nucleus (Fig. 2 F) that probably correspond to the tubular structures observed in DAPI-stained cells (Fig. 1 C). Some condensation is already visible after 30 min H_2O_2 treatment (Fig. 2 A) increasing gradually with time (Fig. 2, B and C). Nuclei of untreated cells are homogeneous in shape and density (Fig. 2 I). In addition, some cells show tiny vesicles on the outer side of the plasma membrane (Fig. 2, D and F). This could be an equivalent of membrane blebbing that is a characteristic marker of apoptosis and has not been observed in yeast cells before. At higher concentrations of H_2O_2, most intracellular structures are destroyed (Fig. 2 H), indicating necrotic cell death. This corresponds to the diminished TUNEL reaction in these cultures (Fig. 1 K).

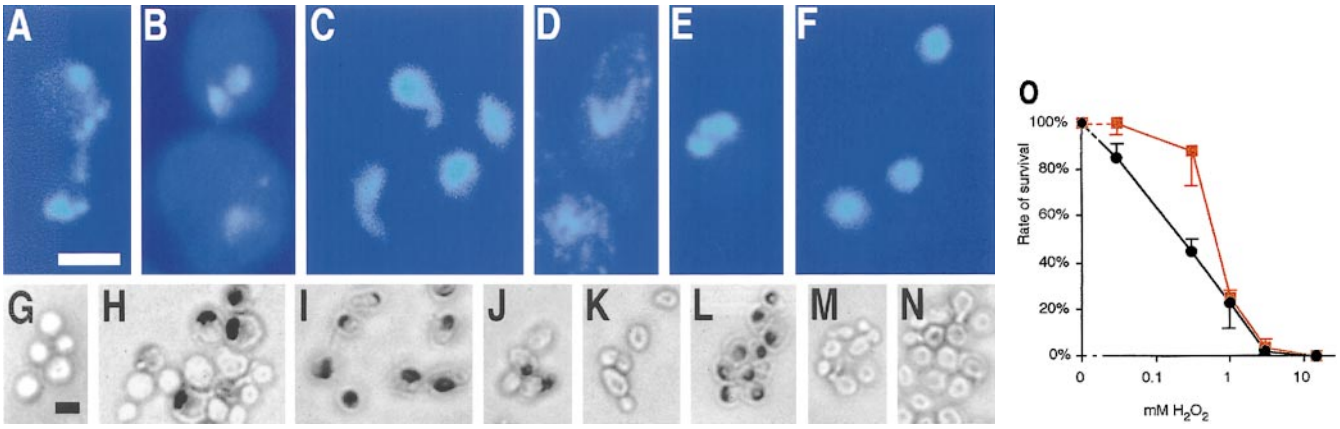

Figure 1. H_2O_2 induces an apoptotic phenotype in yeast. DAPI-stained wild-type yeast after incubation for 200 min without (F) and with 3 mM H_2O_2 (A–E) in YEPD. TUNEL reaction after 200 min treatment of exponentially growing cells with 0 mM (G), 1 mM (H), 3 mM (I), 15 mM (J), and 180 mM H_2O_2 (K), stationary cells treated with 180 mM H_2O_2 (L), exponentially growing cells treated with 3 mM H_2O_2 plus 15 μg/ml cycloheximide (M), and exponentially growing cells treated with 15 μg/ml cycloheximide for 230 min (N). Survival (colony formation on YEPD plates, 100% corresponds to the number of plated cells) of wild-type yeast incubated with H_2O_2 in the absence (black) or presence (red) of 15 μg/ml cycloheximide for 200 min (O). Results were averaged from three experiments. Bars: 5 μm (A–F, G–N).

Cycloheximide Prevents the Apoptotic Effects of H_2O_2 on Yeast Cells and Improves the Survival Rate

Apoptosis needs participation of the cell and can be prevented in many cell types by the inhibition of protein synthesis (Hiraoka et al., 1997; Sánchez et al., 1997). To address the question whether the H_2O_2-induced DNA breakage, chromatin condensation, and cell death are the result of basic radical chemistry or depend on participation of the cellular metabolism, we investigated the effect of cycloheximide on H_2O_2-treated yeast cells.

To establish which concentration of cycloheximide is sufficient for complete inhibition of protein synthesis, incorporation of labeled leucine into TCA precipitable material was determined. 5 μg/ml cycloheximide reduced leucine incorporation to 23% of the untreated control, 15 μg/ml cycloheximide, or higher concentrations reduced the incorporation to <8% of the untreated control.

Exponentially growing wild-type cultures were preincubated with 15 μg/ml cycloheximide for 30 min. After adding 3 mM H_2O_2, cultures were incubated for another 200 min. DNA fragmentation was visualized by TUNEL staining (Fig. 1 M). The resulting TUNEL assay stain is only slightly more intense than in untreated controls (Fig. 1 G) and far weaker than in cells treated with 3 mM H_2O_2 in the absence of cycloheximide (Fig. 1 I). Incubation only with cycloheximide for 230 min (Fig. 1 N) results in approximately the same enhancement of TUNEL staining as the combination of cycloheximide and 3 mM H_2O_2. This mi-

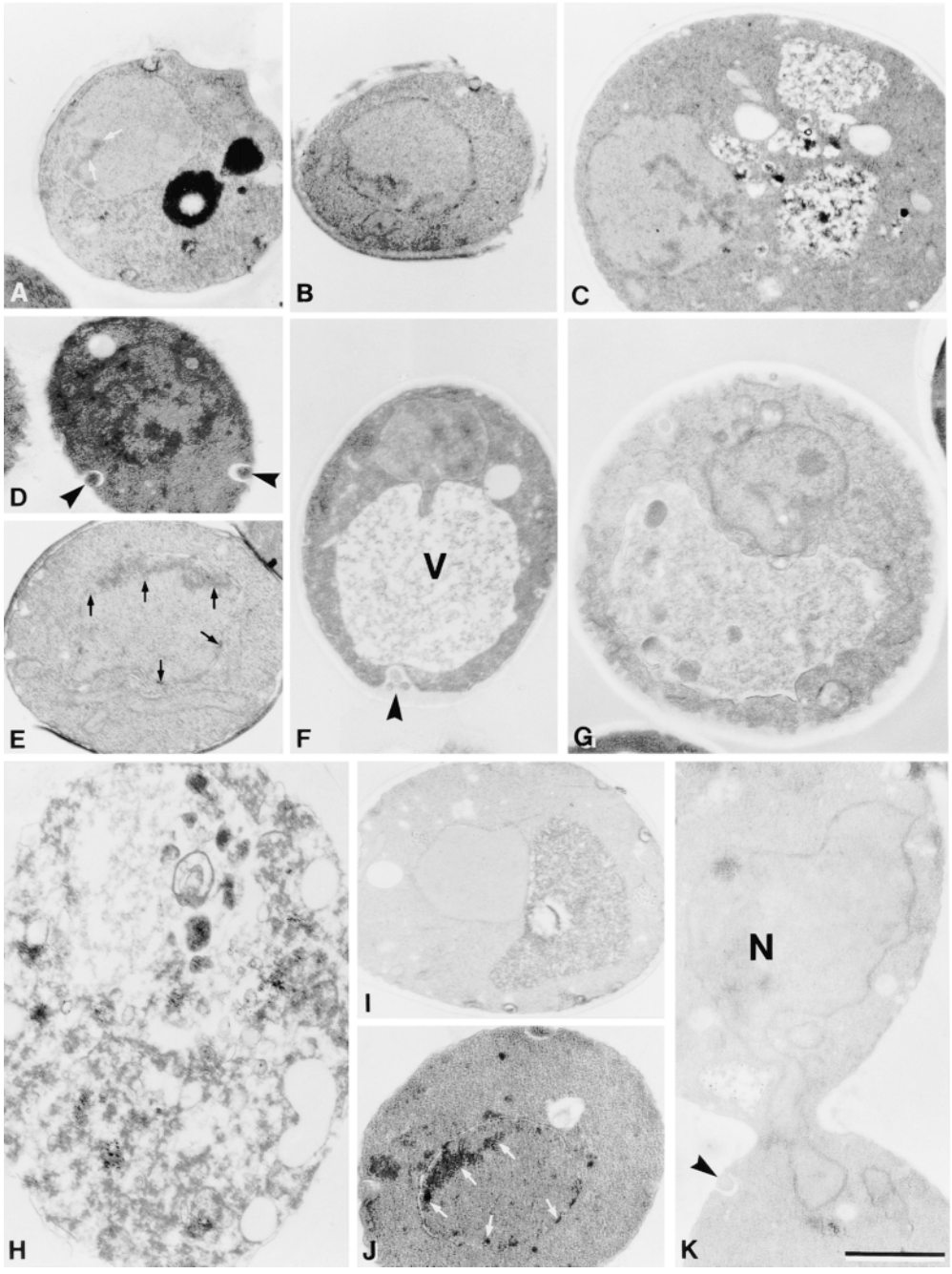

Figure 2. Low concentrations of H_2O_2 and glutathione depletion induce chromatin condensation and margination in *S. cerevisiae.* Electron micrographs of exponentially grown wild-type strain YPH98 treated with 3 mM H_2O_2 for 30 min (A), 60 min (B), 120 min (C), or 200 min (D–F), with 3 mM H_2O_2 plus 15 μg/ml cycloheximide for 200 min (G, nucleolus visible), with 180 mM H_2O_2 for 200 min (H), untreated control (I), and of *gsh1* deletion strain YPH98gsh1 grown on glutathione-free synthetic medium for 3 d (J and K). N, nucleus; V, vacuole; chromatin condensation is marked by arrows, extracellular vesicles (blebs) are marked by arrowheads. Bar, 1 μm.

nor increase in DNA fragmentation compared to untreated controls could be due to a reduction of DNA repair as a result of the inhibited protein synthesis.

Under the electron microscope, neither chromatin condensation, margination, nor nuclear fragmentation is detected in cells incubated with H_2O_2 in the presence of cycloheximide. However, numerous cells show vacuoles accumulating autophagic bodies, increased intracellular vacuolization, or misformed nuclei indicating necrotic damage (Fig. 2 G).

To investigate the effect of cycloheximide on yeast cell survival during oxygen stress, we incubated exponentially growing wild-type cultures with 15 μg/ml cycloheximide for 30 min, added various concentrations of H_2O_2, and incubated the cultures for another 200 min. Survival was determined as the portion of cells forming colonies after 3 d of incubation on YEPD plates. Cycloheximide considerably increased the cell survival rate in the range of 0.03 to 0.3 mM H_2O_2 (Fig. 1 O).

To investigate whether cycloheximide affects intracellular glutathione concentration by the inhibition of protein synthesis, the level of intracellular glutathione was determined as described (Schmidt et al., 1996). Untreated wild-type cells contained 1.55 μM glutathione per gram of wet twice-washed cells. Incubation with 15 μg/ml cycloheximide for 230 min increased the glutathione level to 140% of the control. The increase is probably caused by accumulation of cysteine. However, even an increase of the glutathione level to 250% of the wild-type level by overexpression of *GSH1* has no significant effect on H_2O_2 tolerance (Grey, M., unpublished result). Also, after 200 min incubation with 3 mM H_2O_2, cells retained a glutathione level of 1.24–1.36 μmol/g cells (~80% of the control level) indicating that the intracellular glutathione pool cell is far from exhaustion. Therefore, the observed increase of glutathione by cycloheximide can not be responsible for its protective effect.

A Yeast Strain Lacking Glutathione Exhibits the Characteristic Markers of Apoptosis

Glutathione plays a major role in the protection against ROS. Strain YPH98gsh1 lacks glutathione due to a deletion of the *GSH1* gene coding for γ-glutamylcysteine synthetase. It grows on YEPD due to the significant amounts of glutathione contained in the yeast extract, as well as on synthetic media supplemented with glutathione, but dies after 3 d on glutathione-free media (Brendel et al., 1998).

Strain YPH98gsh1 and the isogenic wild-type strain YPH98 were transferred from YEPD plates to glutathione-free synthetic medium plates and incubated for 3 d. Cells were examined for the phenotypic markers of apoptosis: DNA breakage, chromatin condensation and margination, and the exposition of phosphatidylserine.

The deletion strain shows strong staining (black nuclei) with the TUNEL test indicating massive DNA fragmentation in >80% of the cells (Fig. 3 G), while the control strain shows no TUNEL staining (Fig. 3 H). When the deletion strain is grown on YEPD or on synthetic medium supplemented with 20 μg/ml glutathione, nuclear staining is not observed with the TUNEL assay (not shown).

An aliquot of the protoplasts used for TUNEL staining was also tested for membrane integrity by incubation with 23 μg/ml propidium iodide. 10–20% of the protoplasts of both wild-type and glutathione deficient strain are stained (data not shown), confirming that the majority of the cells is intact and that the DNA fragmentation is not a result of cell necrosis.

The deletion strain exhibits an enhanced sensitivity towards H_2O_2. Incubation with 0.1 mM H_2O_2 induces a strong TUNEL reaction, as well as condensation and fragmentation of chromatin, whereas little or no TUNEL staining is observed after incubation with 3 mM H_2O_2 or higher concentrations (not shown).

Analysis of isolated chromosomal DNA from the deletion strain by agarose electrophoresis showed no indication of DNA laddering (not shown).

DAPI staining of the deletion strain shows a strong chromatin condensation with fragments arranged in a ring, a half-ring (Fig. 3, A and C), or as several randomly distributed nuclear fragments (Fig. 3 E). Electron micrographs show intense chromatin margination (Fig. 2 J) and fragmentation of the nucleus (Fig. 2 K). In cells of the deletion strain grown on YEPD medium or on synthetic medium supplemented with 20 μg/ml glutathione, no chromatin condensation or fragmentation occurs (not shown).

In mammalian cells, phosphatidylserine exposure at the outer leaflet of the cytoplasmic membrane is an early marker of apoptosis (Martin et al., 1995). The exposure can be detected with annexin V that specifically binds phosphatidylserine in the presence of Ca^{2+}. Like mammalian cells, *S. cerevisiae* has an asymmetric distribution of phospholipids within the cytoplasmic membrane with 90% of the phosphatidylserine on the cytoplasmic side of the membrane (Cerbón and Calderón, 1991). We have recently described the exposure of phosphatidylserine in a yeast *CDC48* mutant showing characteristics of apoptotic cells (Madeo et al., 1997).

Spheroplasts of the *GSH1* deletion strain and the isogenic control strain grown on glutathione-free synthetic medium for 3 d were examined for exposure of phosphatidylserine by incubation with FITC-labeled annexin V and for membrane integrity by incubation with propidium iodide. Approximately 20% of the protoplasts, both wild-type and mutant strain, take up propidium iodide indicating membrane damage. These protoplasts often exhibit annexin V staining of the whole cell. More than 15% of the protoplasts from the deletion strain show a strong fluorescence around the circumference of the cell (Fig. 3, I–K) of which none stain with propidium iodide, demonstrating that phosphatidylserine is indeed exposed at the outer layer of the cytoplasmic membrane (Fig. 3, K and L). All intact protoplasts of the isogenic control show little or no FITC staining in the cell lumen (Fig. 3 M). When the deletion strain was grown on YEPD or on synthetic medium supplemented with 20 μg/ml glutathione, no annexin V fluorescence is visible either (not shown).

The Apoptotic Phenotype of cdc48^{S565G} Mutant Strain KFY437 Is Triggered by an Accumulation of ROS

To investigate whether oxygen stress is involved in other apoptotic scenarios described in *S. cerevisiae*, we tested apoptotic mutant strain KFY437 for the occurrence of ox-

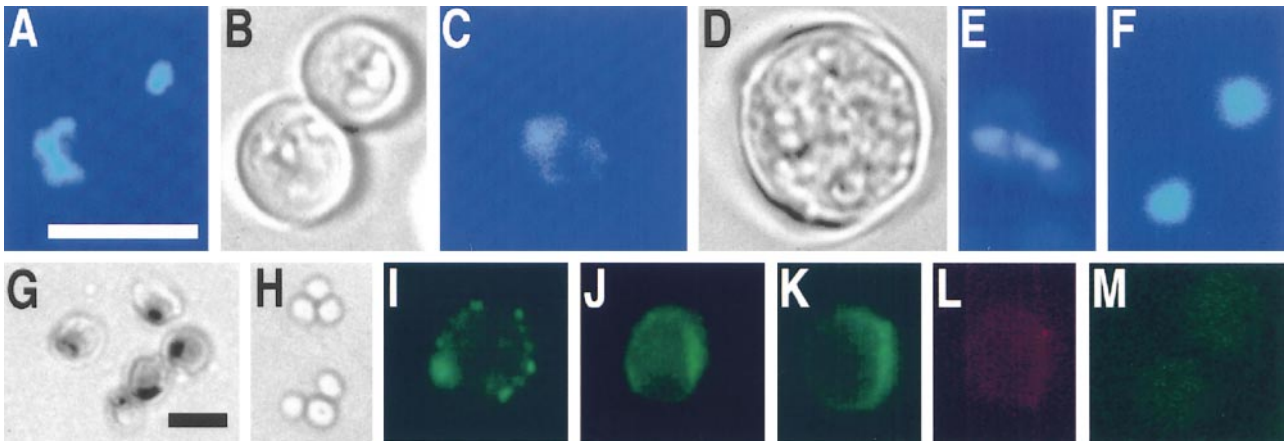

Figure 3. A yeast lacking glutathione exhibits the typical markers of apoptosis, nuclear breakage, DNA fragmentation, and exposition of phosphatidylserine. *gsh1* mutant (A, C, E) and wild-type control (F shows two cells) grown for 3 d on synthetic medium stained with DAPI; B and D are phase contrast pictures corresponding to A and C. TUNEL test of *gsh1* mutant (G) and wild-type control (H) grown for 3 d on synthetic medium. Annexin V binding assay of *gsh1* mutant (I–K) and wild-type control (M) grown for 3 d on synthetic medium; L shows the propidium iodide staining corresponding to K. Bars: 10 μm (A–F, I–M); 10 μm (G and H).

ygen radicals. Strain KFY437 expresses *cdc48*^{S565G}, a mutated allele of *CDC48* responsible for the homotypic fusion of ER-derived vesicles, and spontaneously develops an apoptotic phenotype during exponential growth at 28°C while other conditional alleles of *CDC48* result in cell death at the restrictive temperature without exhibiting any of these morphological markers (Madeo et al., 1997).

Strain KFY437 was tested for the production of ROS during apoptosis by incubation with dihydrorhodamine 123. This substance accumulates in the cell and is oxidized to the fluorescent chromophore rhodamine 123 by ROS (Schulz et al., 1996).

Strain KFY437 growing exponentially on YEPD was incubated with 5 μg/ml dihydrorhodamine 123 for 2 h at 28°C. More than 50% of the cells show a rhodamine 123 fluorescence (Fig. 4 B). Most of the cells of the corresponding wild-type strain show no fluorescence, appearing dark against the faint background fluorescence. Marginal fluorescence occurs in <1% of the cells (Fig. 4 A). Incubation for 2 h with 10 μg/ml dichlorodihydrofluorescein diacetate, which is deacylated to dichlorodihydrofluorescein and oxidized by ROS to fluorescent dichlorofluorescein (Hockenbery et al., 1993), or with 5 μg/ml dihydroethidium for 10 min, which is oxidized specifically by superoxide ions to the fluorescent ethidium (Budd et al., 1997), gave similar results (not shown). Flow cytometric analysis of dihydrorhodamine 123–stained cells confirms that strain KFY437 accumulates oxygen radicals. A majority of the mutants cells show an increased signal (Fig. 4 V), while all wild-type cells display a low fluorescence (Fig. 4 U).

We found that elevated temperature (37°C) induces apoptotic cell death in most cells of strain KFY437. After 4 h at 37°C, >80% of the KFY437 cells show a strong TUNEL reaction as well as condensed and fragmented chromatin. The elevated temperature induces a higher intensity of TUNEL staining as compared to cells grown at 28°C (Fig. 5, B and D). When strain KFY437 is incubated at 37°C for 4 h, more than 80% of the cells show a strong rhodamine 123 fluorescence (Fig. 4 D) of a significantly higher intensity than at 28°C. The majority of cells also show intense fluorescence after incubation with dichlorodihydrofluorescein (Fig. 4 N) or dihydroethidium (Fig. 4 P). The isogenic wild-type strain shows no increased fluorescence at 37°C with either dye (Fig. 4, M and O).

To verify that the accumulation of ROS is not an effect of any cell cycle arrest, temperature sensitive mutants in cell division cycle genes were arrested at 37°C for 4 h, incubated with dihydrorhodamine 123 and tested for fluorescence. Neither a non-apoptotic allele of *CDC48* (*cdc48*-3), nor mutants in *cdc2* (arresting as large-budded cells like *cdc48* mutants) or *cdc31* (arresting with an abnormal spindle-like *cdc48* mutants) show an increase of intracellular fluorescence (not shown).

Oxygen Radical Scavengers and Anaerobic Conditions Suppress Temperature Sensitivity and DNA Fragmentation of Apoptotic cdc48 Mutant KFY437

To discriminate whether the accumulation of ROS is just a byproduct of the induction of apoptosis in the *cdc48* mutant or whether it is essential for the process, oxygen radicals were scavenged with free radical spin traps (Knecht and Mason, 1993). Strain KFY437 and the corresponding wild-type strain KFY417 were grown on YEPD with shaking at 28°C and then incubated with various concentrations of PBN or TMPO for 4 h at 37°C. To evaluate effects on survival, cell viability was determined as the number of colonies formed after plating a defined number of cells. Aliquots of the same sample were used for TUNEL reaction and electron microscopic investigation. 5 mM PBN (Fig. 5 E) and 0.5 mM TMPO suppress TUNEL staining dramatically, whereas 0.5 mM PBN and 5 mM TMPO have less effect (not shown). Electron microscopy shows that the intense chromatin margination of strain KFY437 at 37°C (Fig. 5 J) is prevented almost

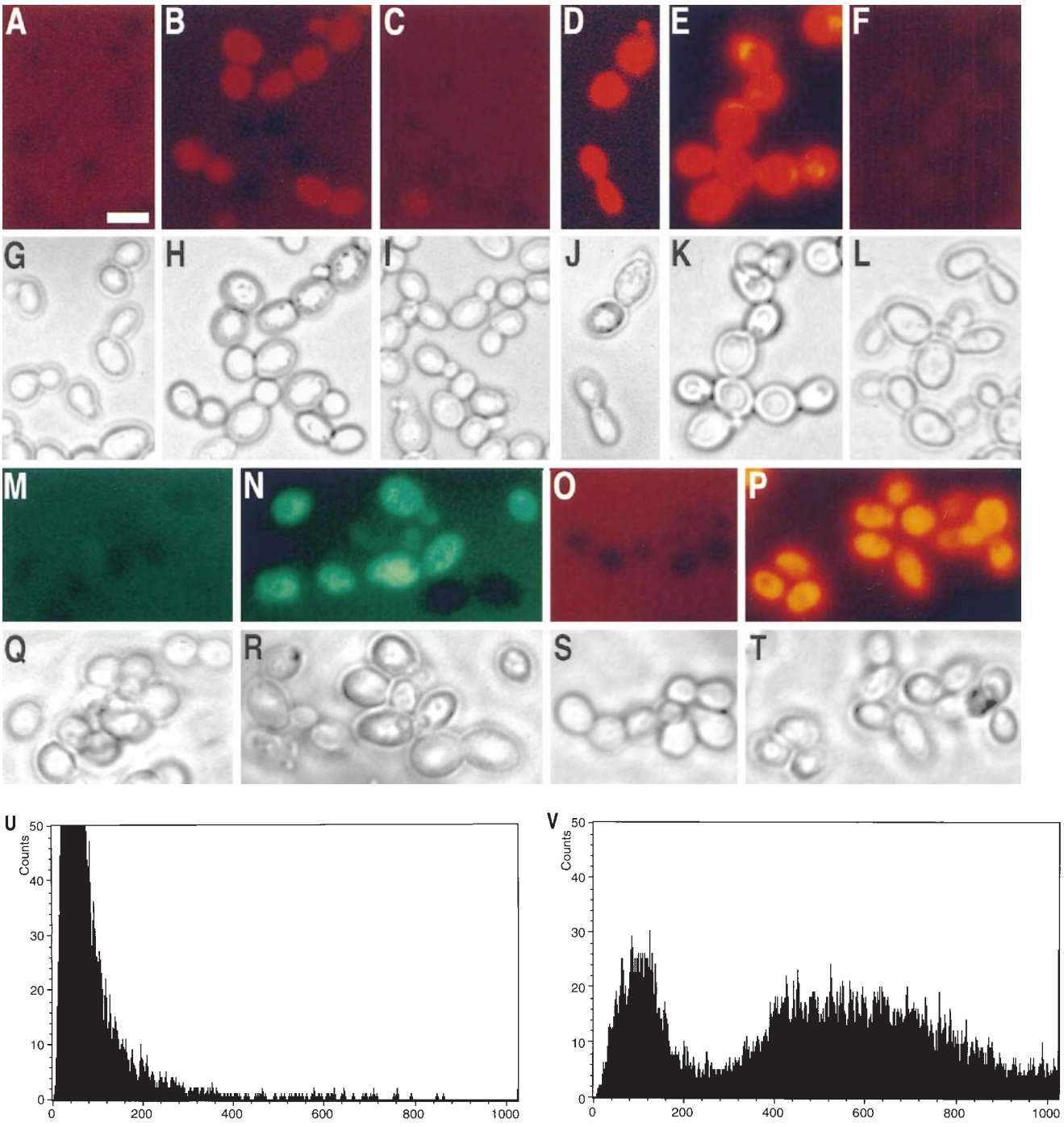

Figure 4. Yeast mutant KFY437 (allele *cdc48*^{S565G}) and yeast-expressing bax accumulate ROS. Rhodamine 123 fluorescence (A–F) and the corresponding phase contrast displays (G–L) after 2 h incubation with dihydrorhodamine 123. Wild-type control (A and G) and mutant KFY437 (B and H) grown at 28°C, wild-type control (C and I) and mutant KFY437 (D and J) grown at 37°C, strain WCG4bax-expressing bax (E and K), strain WCG4bax/bcl-X_L expressing bax and bcl-X_L (F and L). Fluorescence (M and N) and the corresponding phase contrast displays (Q and R) of wild-type control (M and Q) and mutant KFY437 (N and R) grown at 37°C after 2 h incubation with dichlorodihydrofluorescein diacetate. Fluorescence (O and P) and the corresponding phase contrast displays (S and T) of wild-type control (O and S) and mutant KFY437 (P and T) grown at 37°C after 10 min incubation with dihydroethidium. Flow cytometric analysis of wild-type control (U) and mutant KFY437 (V) after 2 h incubation with dihydrorhodamine 123.

completely by 5 mM PBN (Fig. 5 K). These observations correspond to the protective effect of the spin traps on cell viability at 37°C, which is strongest with 0.5 mM TMPO or 5 mM PBN. Viability of the wild-type control is inhibited by the spin trap substances, indicating a certain cytotoxicity (Fig. 6 A). At some concentrations of TMPO, proliferation of the control strain is even lower than that of the apoptotic strain. This might be due to

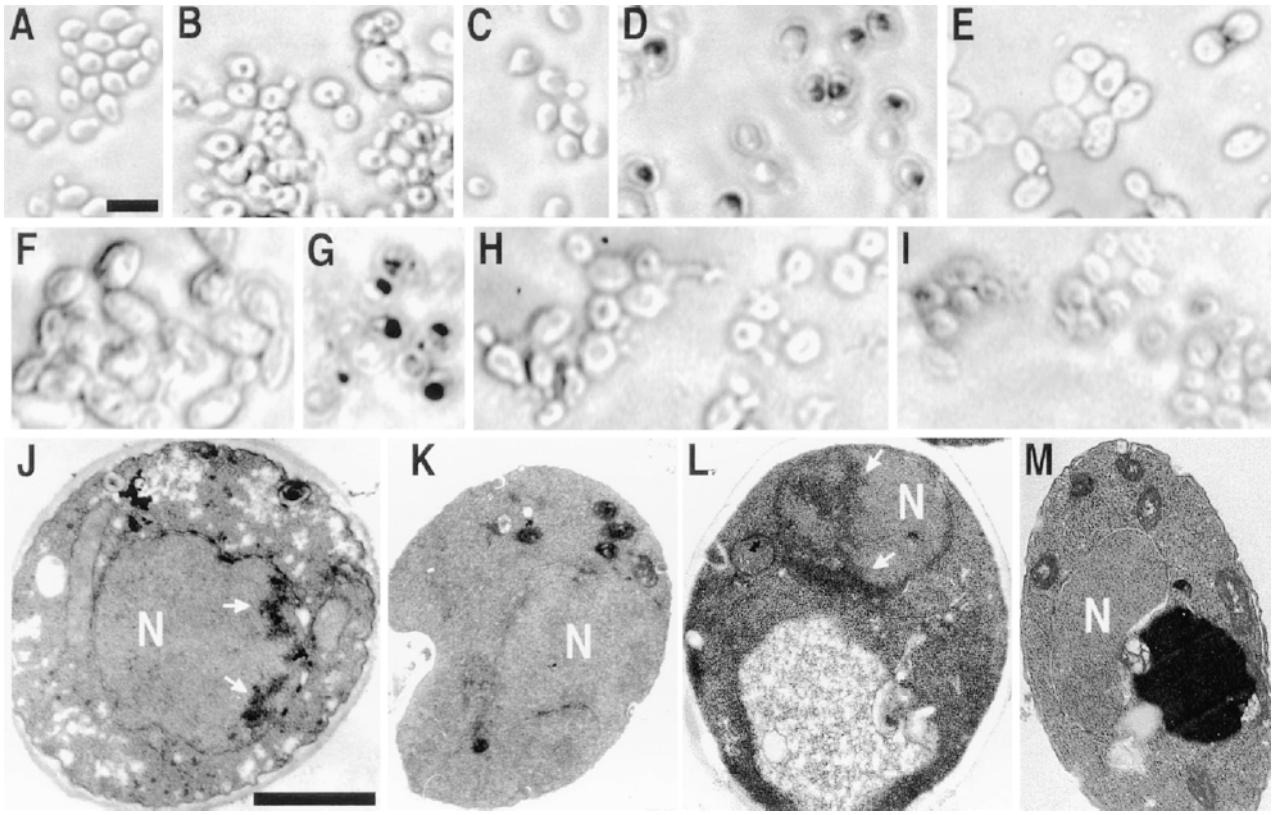

Figure 5. DNA strand breakage and chromatin margination in strain KFY437 and in bax-expressing WCG4bax is prevented by free radical spin traps or anaerobic culture conditions. TUNEL reaction of wild-type control (A) and mutant KFY437 (B) grown at 28°C, of wild-type control (C) and mutant KFY437 (D) incubated at 37°C for 4 h, of mutant KFY437 incubated at 37°C for 4 h in the presence of 5 mM *N*-tert-butyl-α–phenylnitrone (E), of mutant KFY437 incubated at 37°C in a nitrogen atmosphere (F), of bax-expressing WCG4bax (G), of bax-expressing WCG4bax in the presence of 5 mM *N*-tert-butyl-α–phenylnitrone (H), of bax-expressing WCG4bax incubated in a nitrogen atmosphere (I). Electron micrographs of mutant KFY437 incubated at 37°C for 4 h without addition (J) or in the presence of 5 mM *N*-tert-butyl-α–phenylnitrone (K), and of bax-expressing WCG4bax without addition (L) or in the presence of 5 mM *N*-tert-butyl-α–phenylnitrone (M). Bars: (A and J) 10 μm.

neutralization of radicals and spin traps in the apoptotic *cdc48* mutant.

A similar protective effect was observed after incubation in an anaerobic environment. Mutant cultures and controls were incubated in a nitrogen atmosphere at 28°C for 30 min and further incubated in a 37°C waterbath for 4 h under nitrogen. Their viability was tested in a plating assay. While the control was not affected by the anaerobic conditions, the mutant strain showed significant survival after anaerobic conditions (Fig. 6 B). Incubation in a nitrogen atmosphere also prevented accumulation of DNA strand breaks, as shown by TUNEL staining (Fig. 5 F).

Bax Expression Induces Accumulation of Reactive Oxygen Species in S. cerevisiae

Expression of bax in *S. cerevisiae* results in cell death with an apoptotic phenotype that is suppressed by the coexpression of bcl-X_L (Ligr et al., 1998). To determine whether Bax-induced apoptosis in yeast is also accompanied by oxygen stress, strain WCG4bax carrying a bax gene controlled by the *GAL1* promoter was used. Bax expression was induced on galactose medium for 4 h or 13 h. 5 μg/ml dihydrorhodamine were then added and the fluorescence determined after 2 more hours of incubation. After a total of 6 h induction, strain WCG4bax shows little fluorescence (not shown). After 15 h, >80% of WCG4bax cells show an intense rhodamine 123 fluorescence (Fig. 4 E), while the control strain (empty vector) shows no detectable fluorescence (not shown). Coexpression of bcl-X_L suppresses the bax-induced accumulation of radicals. The strain WCG4bax/bcl-X_L shows almost no rhodamine 123 fluorescence after 15 h incubation with galactose (Fig. 4 F). Incubation with dichlorodihydrofluorescein diacetate or dihydroethidium gave similar results (not shown). Flow cytometric analysis of dihydrorhodamine 123–stained cells confirms that a majority of bax-expressing cells show an increased signal, while all control cells display a low fluorescence (not shown).

To determine whether spin traps can prevent Bax-triggered cell death WCG4bax and the corresponding wild-type strain (empty vector) were incubated with the free radical spin traps PBN or TMPO at 28°C on galactose media for 15 h. Viability was determined as the portion of colony-forming units. While spin traps have a toxic effect on the control strain, WCG4bax survival is partially restored.

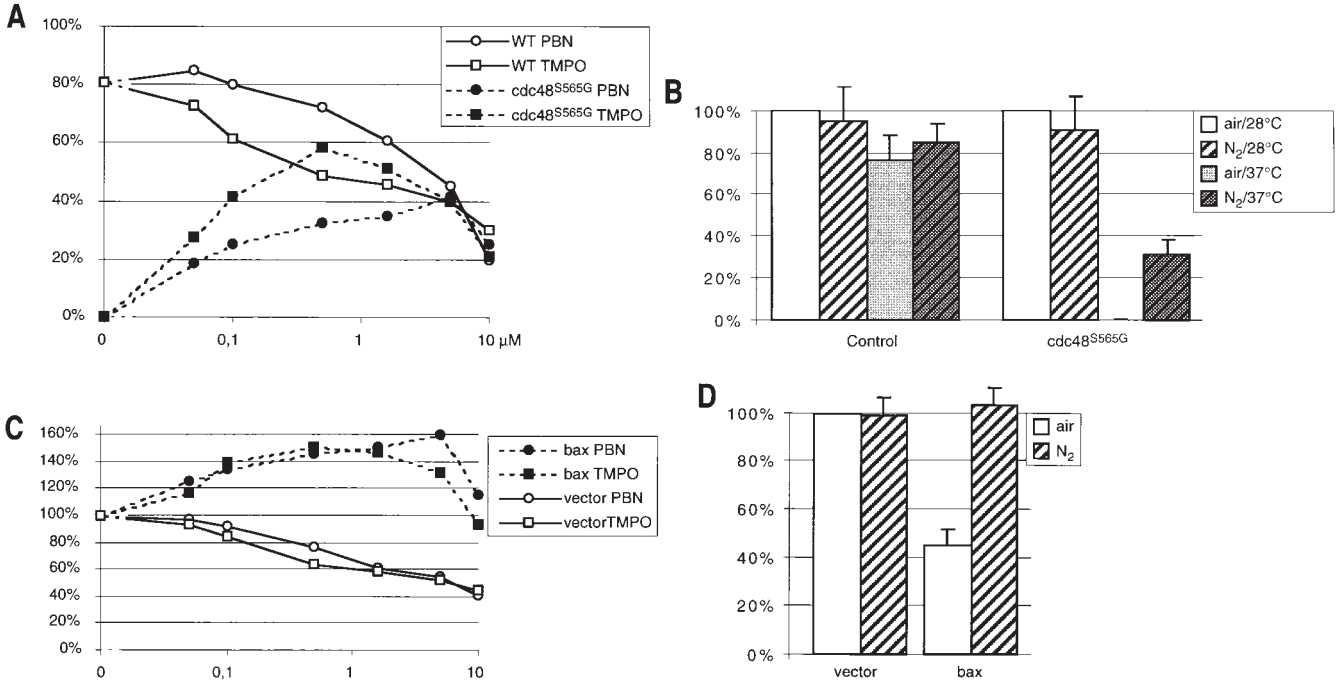

Figure 6. Free radical spin traps or anaerobic culture conditions partially suppress temperature sensitivity in strain KFY437 and bax lethality. (A) Strain KFY437 (allele *cdc48*^{S565G}) and the corresponding wild-type were adjusted to a cell titer of 2×10^6/ml and incubated with various concentrations of PBN or TMPO for 4 h at 37°C in YEPD. The portion of colony-forming units is plotted against spin trap concentrations. 100% corresponds to the respective strain grown at 28°C without spin traps. (B) Strain KFY437 (allele *cdc48*^{S565G}) and the corresponding wild-type were adjusted to a cell titer of 2×10^6/ml in YEPD, incubated with aeration or in a nitrogen stream at 28°C for 30 min, and further incubated at 37 or 28°C for 4 h. Viability was determined as the portion of colony-forming units. 100% corresponds to the respective strain grown aerobically at 28°C. (C) WCG4bax and the corresponding control strain grown on synthetic medium with glucose were transferred to synthetic medium with galactose, diluted to a cell titer of 2×10^6/ml, and incubated for 15 h either with various concentrations of PBN or TMPO. The portion of colony-forming units is plotted against spin trap concentrations. 100% corresponds to the respective strain grown without spin traps. (D) WCG4bax and the corresponding control strain grown on synthetic medium with glucose were transferred to synthetic medium with galactose, diluted to a cell titer of 2×10^6/ml, and incubated for 15 h with aeration or in a nitrogen stream. Viability was determined as the portion of colony-forming units. 100% corresponds to the aerobically grown control strain. The portion of colony-forming units was determined by incubating YEPD plates with 1,000 cells each for 3 d at 28°C and counting visible colonies. Results were averaged from three experiments each. Standard deviations are below 10% for all data points.

As with the apoptotic *cdc48* mutant, 5 mM PBN has the strongest protective effect on WCG4bax (Fig. 6 C). Accordingly, 5 mM PBN prevents DNA strand breakage (Fig. 5 H) and chromatin condensation (Fig. 5 M) occurring in bax-expressing WCG4bax in the absence of spin traps (Fig. 5, G and L).

Anaerobic conditions also strongly reduce cell death and DNA breakage after induction of bax. Strain WCG4bax and the vector control strain were incubated in galactose medium in a nitrogen atmosphere at 28°C for 15 h. Viability of the bax-expressing strain is restored to control levels under these anaerobic conditions (Fig. 6 D). The corresponding TUNEL test shows no indication of strand breaks (Fig. 5 I).

Discussion

In metazoan apoptosis, ROS have been shown to participate in both early and late steps of the regulatory chain. In several apoptotic models, for example in ischemia-induced apoptosis (Maulik et al., 1998), ROS act upstream of bax and caspases. In these models, radical trapping prevents the activation of caspases, and an inhibition of ensuing steps, e.g., with caspase inhibitors, prevents cell death indicating that the radicals act as signal molecules and do not simply cause lethal damage to DNA, lipids or proteins (Hara et al., 1997; Boggs et al., 1998; Tan et al., 1998). However, radicals have also been shown to play a role in the late steps of apoptosis. K^+ deprivation induces apoptosis in cerebellar granule neurons via an accumulation of ROS. ROS production is prevented by actinomycin D, cycloheximide, and caspase inhibitors Ac-YVAD-CMK, suggesting that ROS act downstream of gene transcription, mRNA translation, and activation of caspases (Schulz et al., 1997, 1996).

Two scenarios connecting yeast with metazoan apoptosis have been described previously. The expression of some metazoan proapoptotic genes (bak, bax, ced-4) results in cell death (Sato et al., 1994; Hanada et al., 1995; Greenhalf et al., 1996; James et al., 1997) accompanied by an apoptotic phenotype (Ink et al., 1997; Ligr et al., 1998). The mutant allele *cdc48*^{S565G} induces the appearance of

typical phenotypic markers of apoptosis (Madeo et al., 1997).

We found that in both cases ROS accumulate in the cell. Radical trapping or growth under strictly anaerobic conditions prevent cell death and the accompanying apoptotic effects, demonstrating that the radicals are not a byproduct but a promoter of the apoptotic-like features in these cells. In addition, apoptotic cell death could be induced by exposing yeast cells to oxidative stress, either with low concentrations of exogenous H_2O_2 or by growing a *gsh1* deletion mutant in the absence of glutathione. These results illustrate a central role of ROS in all cases of apoptosis in yeast known to date.

We observed that with low concentrations of H_2O_2, cycloheximide enhances the survival rate of *S. cerevisiae* cells. This phenomenon has been observed before, but at the time remained unexplained (Collinson and Dawes, 1992). Our electron microscopic investigation and TUNEL test show that cycloheximide prevents both apoptotic chromatin condensation and DNA fragmentation, and increases the number of necrotic cells. The prevention of cell death by inhibition of protein synthesis is a specific indicator to distinguish apoptosis from necrosis in metazoan systems (el Azzouzi et al., 1994; Hiraoka et al., 1997). Our findings indicate that yeast cell death triggered by low H_2O_2 concentrations is not caused by cell damage but involves an active cooperation of the cell. Massive corruption of cellular structures and metabolism prevents the cooperation of the cell in its death process. As we have shown, high concentrations of H_2O_2 result in cell death associated with a disintegration of intracellular structures but without the phenotypic markers of apoptosis. Stationary cells of *S. cerevisiae* are much less sensitive to oxidative stress than exponentially growing cells (Jamieson et al., 1994; Izawa et al., 1996). We find that they show no detectable effect at concentrations of H_2O_2 that cause an apoptotic phenotype in exponentially growing cells and give strong TUNEL staining at high concentrations of H_2O_2.

The observation that numerous cytotoxic substances that cause necrosis at high concentrations induce apoptosis at lower concentrations (Lieberthal and Levine, 1996) give a clue to the origin of apoptosis. ROS are natural inducers of fatal cell damage, aging, and cell death. A likely evolutionary mechanism for the development of apoptosis might be based on that phenomenon. Even if a cell survives the damage caused by radicals, it will probably become a useless mouth to feed, consuming resources but producing no or impaired offspring. For the total clonal cell population, the altruistic death of these cells spares energy sources for the undamaged cells and therefore poses an evolutionary advantage. A potentially altruistic death has been described for stationary cultures of *S. cerevisiae* that can survive for very long times in pure water, but quickly lose viability in nutrient depleted media, perhaps in order to keep the few resources for the best adapted isogenic relatives. Bcl-2 delays the loss of viability (Longo et al., 1997).

In evolution, more complex regulatory pathways probably developed upstream of the basic mechanism, resulting in a complex signaling network with a seemingly contradictory behavior in different apoptotic models (Fig. 7). The phenomenon that glutathione is actively extruded during apoptosis of human monocytic cells (Ghibelli et al., 1995) may be a strategy to enhance ROS signaling–induced apoptosis. Some apoptotic pathways retain the usage of ROS in early regulatory steps, other pathways still use it in late steps. In addition, alternative pathways lacking the involvement of ROS developed that cannot be blocked by radical trapping. But even in the case of the ROS-independent apoptotic pathway via CD95 (Hug et al., 1994), the expression of the proapoptotic CD95 ligand is induced by ROS (Hug et al., 1997).

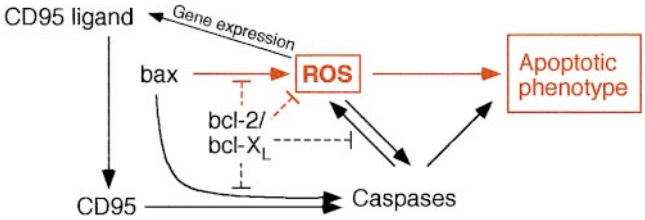

Figure 7. ROS participate in the regulation of apoptosis at various levels. Important established regulators of apoptosis. Components and functions present in yeast are marked in red.

While none of the established apoptotic proteins are encoded in the *S. cerevisiae* genome, some of them will perform similar functions when expressed in yeast cells. In mammals, one of the effects of Bax expression is a caspase-independent production of ROS (Xiang et al., 1996). Our finding that the expression of Bax protein in yeast also results in an accumulation of ROS that is prevented by the coexpression of Bcl-X_L indicates a mechanism for the induction of cell death and apoptotic phenotype by Bax in yeast. Anorganic ROS are therefore the first regulators of apoptosis found to be shared between yeast and higher animals (Fig. 7). Future research should address whether the mechanism downstream of this signal uses conserved elements.

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Appendix C: Ligr M, Velten I, Fröhlich E, Madeo F, Ledig M, Fröhlich K-U, Wolf DH, Hilt W: The proteasomal substrate Stm1 participates in apoptosis-like cell death in yeast. (submitted)

The proteasomal substrate Stm1 participates in apoptosis-like cell death in yeast

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Running head: Proteasome and Apoptosis in Yeast

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ABSTRACT

We have identified the yeast gene *STM1* in an overexpression screen for new proteasomal substrates. Stm1 is unstable in wild type cells and stabilized in cells with defective proteasomal activity, and thus a bona fide substrate of the proteasome. It is localized in the perinuclear region and is required for growth in the presence of mutagens. Overexpression in cells with impaired proteasomal degradation leads to cell death accompanied with cytological markers of apoptosis - loss of plasma membrane asymmetry, chromatin condensation, and DNA cleavage. Cells lacking Stm1 display deficiency in the apoptosis-like cell death process induced by treatment with low concentrations of H_2O_2. We suggest that Stm1 is involved in the control of the apoptosis-like cell death in yeast. Survival is increased when Stm1 is completely missing from the cells or when inhibition of Stm1 synthesis permits proteasomal degradation to decrease its amount in the cell. Conversely, Stm1 accumulation induces cell death. In addition we identified 5 other genes whose overexpression in proteasomal mutants caused similar apoptotic phenotypes.

INTRODUCTION

Multicellular organisms are in the state of dynamic equilibrium, sustained by the mutually opposing processes of cell division and cell death. The importance of programmed cell death to maintain the integrity of metazoans is widely appreciated, but is there a place for this process in the life cycle of single cell organisms?

The existence of programmed cell death in bacteria is now firmly established (Engelberg-Kulka and Glaser, 1999). Recently we have identified a translation-dependent programmed cell death process also in the unicellular eukaryote *Saccharomyces cerevisiae* (Fröhlich and Madeo, 2000). We observed that yeast cells underwent cell death due to presence of *cdc48-S565G* mutation (Madeo *et al.*, 1997), overexpression of the mammalian apoptotic cell death regulator Bax (Ligr *et al.*, 1998), or exposure to oxidative conditions (Madeo *et al.*, 1999). This process resembled apoptosis, a form of programmed cell death indispensable for development and homeostasis of metazoan organisms (Webb *et al.*, 1999). The occurrence of cytological markers of metazoan apoptosis in yeast, such as loss of plasma membrane asymmetry, chromatin condensation and margination, fragmentation of DNA, and membrane blebbing, as well as the identification of reactive oxygen species as a common regulator (Madeo *et al.*, 1999), led us to suggest that the basic mechanism of apoptosis is present already in this unicellular eukaryote (Fröhlich and Madeo, 2000). This view is further supported by recent reports that the orthologues of Cdc48 regulate the apoptotic pathways of *Caenorhabditis elegans* (Wu *et al.*, 1999) and humans (Shirogane *et al.*, 1999).

Cdc48 is an ATPase of the AAA family associated with a variety of cellular activities. Notably, Cdc48p is emerging as a factor involved in the regulation of the evolutionary conserved ubiquitin-proteasome system (Dai *et al.*, 1998; Ghislain *et al.*, 1996; Koegl *et al.*, 1999; Meyer *et al.*, 2000). Substrates to be degraded by this pathway are first covalently tagged with the small protein ubiquitin by an enzymatic cascade consisting of ubiquitin activating, and conjugating enzymes, in most cases in cooperation with additional substrate-specific recognition elements. Polyubiquitylated proteins are recognized and degraded by the 26S proteasome, a multisubunit multicatalytic protease (Hilt and Wolf, 1996). In mammals, inhibition of proteasome-dependent proteolysis leads either to repression or induction of apoptosis, depending on the proliferative status of the particular cell type (Drexler, 1998). It has been suggested that in proliferating cells the proteasome continuously degrades an activator of apoptosis. Curbing proteasomal activity is thought to result in accumulation of this hypothetical regulator and thereby activation of the apoptotic cell death cascade (Drexler, 1997).

Does proteasomal degradation play a similar role in the apoptosis-like cell death process in yeast? To answer this question, we screened for genes that cause this type of death when overexpressed in cells with defective proteasomes.

MATERIALS AND METHODS

Yeast strains, plasmids, and media

To construct plasmid pML1, a *PRE1*-containing *Bam*HI-*Xho*I fragment of p13/*PRE1* (a gift of W. Heinemeyer) was ligated into *Bam*HI-*Xho*I sites of pRS318 [*CYH2 LEU2 CEN6*](Sikorski and Boeke, 1991). The integrative plasmid pL090 was assembled from the *Nhe*I-*Mlu*I fragment of pYES2 (Invitrogen), a PCR fragment of the *STM1* terminator (flanked by *Sph*I and *Mlu*I sites), and the *STM1* ORF flanked by *Nhe*I at the 5' end and the IRS sequence (Luo *et al.*, 1996) followed by *Sph*I site at the 3' end. The *STM1* terminator region was amplified from yeast chromosomal DNA using primers AAAAGCATGCAAGCCTTATATATGAATAATTCCAA-CTG and AAAAACGCGTCGAACGGAAGAAGTGAATGG. The *STM1* ORF was amplified using primers AAAAGCTAGCATGTCCAACCCATTTGATTTG and AAAAGCATGCCTAAGA-ACGAATATAACGAGCCAAAGATGGCAAGTTAG, with an *STM1* cDNA library plasmid as the template. Plasmid pL092 [P_{GAL1}*::STM1::IRS URA3 2*μ] was made by inserting the *Nco*I-*Xba*I fragment of pYES2 (containing P_{GAL1} and 2μ sequences) between *Nco*I and *Nhe*I sites of pL090. All PCR and molecular cloning steps were done under standard conditions (Ausubel *et al.*, 1989).

S. cerevisiae strains used in this study are listed in Table 1. The strains YML1 and YML2 were constructed in two steps. First, YHI29-1 and YHI29-14 were selected for spontaneous mutations in the *CYH2* gene on YPD plates containing 10 μg.cm^{-3} cycloheximide (Sikorski and Boeke, 1991). Cyh^r clones were isolated and transformed with plasmid pML1, yielding strains YML1, and YML2, respectively. Complementation of the

pre1-1 mutation was confirmed by the restoration of proteasomal chymotrypsin-like activity, assayed by a substrate overlay test as described previously (Hilt and Wolf, 1999). Strains YL280 and YL286 were generated by pop-in/pop-out allele replacement using plasmid pL090 linearized with *Cla*I. The growth of YL280 and YL286 was indistinguishable from wild type on YPD plates supplemented with 12 mM caffeine or 10 $\mu g.cm^{-3}$ bleomycin, proving that Stm1-IRS construct was fully functional.

Yeast cells were grown at 30°C if not stated otherwise and liquid cultures were agitated at 200 rpm. Rich growth medium (YPD) contained 1% yeast extract, 2% Bacto-peptone, and 2% D-glucose. Synthetic complete (SC) medium (0.67% nitrogen base without amino acids and nucleotide bases) was lacking the appropriate auxotrophic factors for selection, and contained either 2% glucose or 2% galactose as required. Yeast transformations were carried out as described (Gietz *et al.*, 1995).

High expression lethality screen

A pYES2-based cDNA library (Espinet *et al.*, 1995) was transformed into YML1 and YML2 strains pregrown on YPD. Transformants were selected on SC glucose medium lacking leucine and uracil (SC ura^- leu^-). After three days of growth, colonies were replica plated onto SC glucose medium lacking uracil (SC ura^-) to enable loss of plasmid pML1. After additional two days of growth the colonies were replica plated onto SC glucose medium lacking uracil supplemented with 10 $\mu g.cm^{-3}$ cycloheximide (SC ura^- cyh^+). This step was repeated after two days of growth to ensure that colonies consisted of cells that had lost the plasmid pML1 complementing the *pre1-1* mutation. Loss of plasmid pML1 carrying *PRE1* was further confirmed by test for absence of the chymotrypsin-like activity (data not shown). Two days later, the colonies were replica plated onto SC galactose medium lacking uracil (SCgal ura^-). At the same time the original colonies from SC ura^- leu^- plates ("wild type") were also replica plated onto the SCgal ura^- medium to induce expression of the library genes. After two days the two sets of plates were compared and screened for clones able to grow on galactose in the presence but not in the absence of plasmid pML1. To confirm the phenotype candidates showing such features were picked from the original plates (SC glucose ura^- leu^-, resp. SC glucose ura^- cyh^+) onto SCgal ura^-. Plasmid DNA from positive clones (cured of pML1) was isolated and a restriction analysis was performed to ensure homogeneity of the colonies and to estimate the size of the cDNA inserts. Plasmids obtained by these means were transformed into the strains WCG4/a, YHI29-1, and YHI29-14 and retested for the ability of their encoded cDNAs to cause high expression growth arrest in cells with impaired proteasome by streaking onto SCgal ura^- plates.

Gene disruption

The *STM1* ORF was disrupted with a PCR-mediated method using the kanamycin resistance gene as a selection marker (Güldener *et al.*, 1996). PCR was performed using plasmid pUG6 as a template and primers designed to amplify the kanamycin cassette flanked by 40 base sequences corresponding to immediate down- and upstream region of the *STM1* ORF. Yeast cells were transformed with the PCR product and integrants were selected on YPD plates containing geneticin G418 (Gibco BRL) at 0.2 $mg.cm^{-3}$. Correct integration was confirmed by Southern blotting with the kanamycin cassette as a probe.

Analysis of DNA

Sequencing was performed using dideoxy sequencing the T7 Sequencing Kit (Pharmacia Biotech) and the Sequi-Gen GT Nucleic Acid Electrophoresis Cell (BioRad). For Southern blotting the semi-dry system and the Southern Gen Image kit (Amersham Pharmacia Biotech) were employed.

Immunofluorescence microscopy

Cells growing in logarithmical phase were fixed for 30 min (3.7% formaldehyde, 0.1 M PO_4^{3-} pH 6.5) and then washed 3 times in SP buffer (1.2 M sorbitol, 0.1 M PO_4^{3-} pH 6.5). The cell wall was digested with 15 $U.cm^{-3}$ Zymolyase 100T (Seikagaku Corporation) in 1.2 M sorbitol, 20 mM β-mercaptoethanol, 0.1 M PO_4^{3-} pH 6.5 at 30°C for 30 min. After washing 3 times in SP buffer spheroplasts were bound on poly-L-lysine-coated slides, washed 3 times with PBS (53 mM Na_2HPO_4, 13 mM NaH_2PO_4, 75 mM NaCl), and then incubated for 20 min at room temperature in PBT (1% bovine serum albumin, 0.1% Triton X 100 in PBS). The IRS-specific monoclonal antibody (BAbCO) was diluted in PBT 1:100 and applied to the samples for 2 h at room temperature in a humid chamber. The slides were washed 5 times in PBT and

incubated with goat anti-mouse IgG-AlexaFluor 594 conjugate (Molecular Probes) diluted 1:250 in PBT for 90 min in a dark humid chamber. The antibody was removed and the samples were washed 5 times with PBT and 5 times with PBS. A coverslip was mounted with 90% glycerol and 22.5 ng.cm^{-3} 4',6-diamidino-2-phenylindole (DAPI) in PBS.

Chromosome spreads

Immunostaining of spread chromosomes was performed as described earlier (Bishop, 1994) with modifications. Spheroplasts were prepared (see the previous section), and resuspended in ice cold 0.1 M 2-(N-morpholino) ethanesulfonic acid, 1 mM EDTA, 0.5 mM $MgCl_2$, and 1 M sorbitol. 20 μl of this suspension was placed on a glass slide and mixed with 40 μl of 4% paraformaldehyde in 3.4% sucrose. Afterward 80 μl of 1% Lipsol (Lip) was added, then after few seconds 80 μl of 4% paraformaldehyde in 3.4% sucrose. The mix was spread over the slide with a glass rod and allowed to dry overnight. The slide was submerged in PBS for 10 min and blocked for 10 min in 1% bovine serum albumin in PBS. The reaction with antibodies and mounting of the slides was performed as described above.

Annexin V assay

Externalization of phosphatidylserine was detected essentially as described previously (Ligr *et al.*, 1998). Cells were resuspended in digestion buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, 35 mM PO_4^{3-}, pH 6.8) and incubated for 2 h at 30°C with 15 U.cm^{-3} Zymolyase 100T (Seikagaku Corporation, Tokyo, Japan) and 5.5% Glusulase (NEN, Boston, MA). After cell wall digestion the cells were washed in binding buffer containing sorbitol (1.2 M sorbitol, 10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). The protoplasts were resuspended in 38 μl of binding buffer and incubated with 2 μl GFP-annexin V (Clonetech) and 2 μl propidium iodide (50 μg.cm^{-3}) in the dark for 20 min at room temperature. The cells were mounted on a slide and examined under the fluorescence microscope.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)

The TUNEL assay for detection of fragmented nuclear DNA in yeast was used as previously described (Ligr *et al.*, 1998). Cells were fixed in 3.7% formaldehyde for 1 hour and the cell walls were removed as described above. The protoplasts were then applied to polylysine-coated slides. The In Situ Cell Death Detection Kit POD (Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturers instructions. After mounting a coverslip with a drop of Kaiser's glycerol gelatin (Merck, Darmstadt, Germany) the cells were examined under the light microscope.

Electron microscopy

Yeast cells were fixed with phosphate-buffered glutardialdehyde, the cell walls were removed, and the cells were postfixed with osmium tetroxide and uranyl acetate and dehydrated as described for stationary-phase cells (Byers and Goetsch, 1991). Following the 100% ethanol washes, the cells were washed with 100% acetone, infiltrated with 50% acetone / 50% Epon for 30 min and with 100% Epon for 20 h. The cells were transferred to fresh 100% Epon, incubated at 56°C for 48 h and thereafter cut into thin sections and stained with lead acetate.

Promoter shut-off and cycloheximide-chase analysis, western blotting

Strains expressing plasmid encoded IRS-tagged *STM1* under the control of *GAL1* promoter were grown on SC glucose medium till A_{600} ~ 1 and then transferred to SC galactose to the final density of A_{600} ~ 0.5. After the culture reached A_{600} ~ 1.5 glucose and cycloheximide were added to the final concentration of 2% and 0.5 mg.cm^{-3}, respectively. Strains expressing Stm1-IRS from chromosome were grown on YPD till A_{600} ~ 1.5 and cycloheximide was added to the final concentration of 0.5 mg.cm^{-3}. The cells (5 A_{600} units) were harvested and lysed in 0.25 M NaOH and 1% β-mercatoethanol. The proteins were precipitated with 5.8% trichloracetic acid, pelleted, and washed with acetone. The dry pellet was resuspended in urea buffer (8 M urea, 5% SDS, 0.1 M EDTA, 0.02% bromphenol blue, 1% β-mercaptoethanol, 40 mM Tris/HCl pH 6.8). The proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. IRS-tagged Stm1 was detected with monoclonal anti-IRS antibody (BAbCO) and the ECL Kit (Amersham).

RESULTS

A screen for yeast cDNAs that cause growth arrest when overexpressed in cells with impaired proteasome mediated proteolysis

We developed a screen to search for proteins whose degradation by the ubiquitin-proteasome system is required for viability or growth. We reasoned that overexpression of such a protein should cause little effect in wild type cells with fully functioning proteasomes, but cause a growth defect in cells where proteasomal function is impaired. Proteasomal activity could not be eliminated completely, since knock-outs of proteasomal subunits are lethal. Cells with a *pre1-1* mutation residing in a β-type subunit of the proteasome are defective in chymotrypsin-like activity and show a significant defect in growth but only slightly impaired protein degradation. The *pre4-1* mutation locating in another β-type subunit causes loss of the PGPH-like activity of the proteasome, but cells otherwise behave phenotypically like wild type. When *pre1-1* and *pre4-1* mutations are combined, proteasomal protein degradation is significantly slowed down, and cells grow at reduced rate (Hilt *et al.*, 1993). A *pre1-1 pre4-1* strain was selected for spontaneous recessive mutations in the *CYH2* locus conferring cycloheximide resistance (Sikorski and Boeke, 1991). Plasmid pML1 carrying wild type *PRE1* and *CYH2* genes was introduced into this strain to complement the defect in the chymotrypsin-like activity of the proteasome. The resulting strain which was phenotypically wild type concerning proteasome dependent proteolysis (data not shown) and cycloheximide sensitive due to presence of *CYH2* was transformed with a 2μ-based cDNA library under the control of the *GAL1* promoter (Espinet *et al.*, 1995). Transformants were plated on selective medium with glucose and replicas were made on cycloheximide-containing plates to select for cells that had lost the *PRE1* encoding plasmid pML1. Original plates containing “wild type” cells and their copies containing clones with *pre1-1 pre4-1* background were then replica plated onto medium containing galactose to induce expression of the plasmid encoded cDNAs. Library plasmids that caused growth arrest in *pre1-1 pre4-1* mutants but not in the *PRE1 pre4-1* background (“wild type”) were isolated and their ability to induce growth arrest in cells with defective proteasomes was confirmed after retransformation of the isolated plasmids into the wild type, *pre1-1*, and *pre1-1 pre4-1* cells (Figure 1A). 125 library plasmids conferring the expected phenotype were isolated and sequenced, revealing 62 individual ORFs causing high expression lethality (*HEL* genes).

Overexpression of distinct HEL genes causes cell death and apoptotic phenotypes in proteasomal mutants

We noticed that overexpression of some *HEL* genes did not only halt growth, but also lead to decreased survival. Therefore we examined all isolated cDNAs for their ability to induce apoptotic-like cell death in *pre1-1 pre4-1* mutants.

In both *S. cerevisiae* and mammalian cells, 90% of phosphatidylserine is found under normal conditions in the inner leaflet of the plasma membrane, facing the cytosol (Cerbón and Calderón, 1991). Early during apoptosis in mammals (Martin *et al.*, 1995) and during apoptosis-like cell death process in yeast (Madeo *et al.*, 1997; Ligr *et al.*, 1998) the asymmetric distribution of phosphatidylserine is lost. This effect can be detected by binding of annexin V to the cell surface. We observed GFP-annexin V binding to yeast protoplasts derived from *pre1-1 pre4-1* cells that overexpressed 6 out of 62 *HEL* genes identified in the screen (Figure 2). Integrity of protoplasts was assessed by counter-staining with propidium iodide to exclude cells with GFP-annexin V bound to the cytosolic face of the plasma membrane (not shown). The highest rates of staining (about 40% of the cells) were observed in strains that overexpressed the *PPA1* or the *HEL13* gene. Lower but significant rates of annexin staining (15-20% of the cells) were found for *pre1-1 pre4-1* clones overexpressing *NSR1*, *SAR1*, *STM1*, or *HEL10* (data not shown). No staining was observed in *pre1-1 pre4-1* cells carrying an empty vector.

Another hallmark of apoptosis is DNA fragmentation (Collins *et al.*, 1997) that can be detected *in situ* by the TUNEL test. This assay detects the increased presence of free 3’ ends of DNA generated by fragmentation of chromosomes, and visualizes them via attachment of labeled nucleotides by terminal deoxynucleotidyl transferase. The 62 cDNAs identified in the high expression lethality screen were analyzed for their capacity to cause DNA fragmentation when overexpressed in *pre1-1 pre4-1* cells. Six cDNAs were detected that induced TUNEL staining in a significant fraction of nuclei in the respective cells (Figure 1B). Significantly, these were the same *HEL* genes that were identified to cause a positive signal in the annexin V test. Thus, the loss of plasma membrane asymmetry as indicated by annexin V staining was always associated with DNA fragmentation detected by TUNEL assay, and vice versa.

These results evidence that the identified genes (Table 2) were able to trigger an apoptosis-like process when overexpressed in *pre1-1 pre4-1* mutant cells.

To further support this interpretation, we analyzed the terminal phenotypes of *pre1-1 pre4-1* strains overexpressing one of the 6 detected cDNAs, each (listed in Table 2) with electron microscopy. In every one of these 6 strains cells were found that had abnormal nuclei with condensed and marginalized chromatin (Figure 3) as typically seen during mammalian apoptosis (Kerr *et al.*, 1972) and apoptosis-like cell death in yeast (Ligr *et al.*, 1998; Madeo *et al.*, 1997).

In addition, we confirmed that the appearance of apoptotic phenotypes in *pre1-1 pre4-1* cells overexpressing one of the 6 detected *HEL* genes is associated not only with growth arrest (Figure 1A), but also with cell death (Figure 1B). Overexpression of *NSR1* caused a strong growth defect already in wild type cells. However, the survival rate was still 20% and therefore we included it in further analyses. Moreover, because growth defects, reduction of survival, and appearance of apoptotic markers were significantly enhanced when *NSR1* was overexpressed in proteasomal mutant cells, *NSR1* was grouped together with the remaining 5 detected *HEL* genes.

Previously we have shown that the apoptosis-like process in yeast triggered in *cdc48-S565G* mutant cells depends on production of reactive oxygen species. We performed tests to detect oxygen radicals in *pre1-1 pre4-1* strains overexpressing every single one of the 6 cDNAs inducing apoptotic phenotypes as described (Madeo *et al.*, 1999). In no case any significant increase in reactive oxygen species production was observed (data not shown).

Stm1 is degraded by the proteasome

We were interested to see whether the toxic effect of overexpressed *STM1* in *pre1-1 pre4-1* proteasomal mutant was due to proteolytic stabilization and thereby accumulation of the gene product. To this end, wild type and proteasomal mutant cells were transformed with a multi-copy plasmid carrying the *STM1* ORF C-terminally tagged with a single IRS epitope under the control of the *GAL1* promoter. The Stm1-IRS construct was proved to be functional (see Materials and Methods). After inducing expression of *STM1::IRS* on galactose the synthesis of Stm1-IRS was stopped by repressing the *GAL1* promoter by addition of glucose and blocking protein synthesis by application of cycloheximide. In wild type cells the Stm1-IRS was rapidly degraded, whereas in *pre1-1 pre4-1* cells Stm1-IRS was completely stabilized (Figure 4, top). Similar results were obtained by cycloheximide chase analysis of C-terminally tagged Stm1 protein expressed from its endogenous chromosomal promoter (Figure 4, bottom), demonstrating that Stm1 is a natural substrate of the proteasome.

Stm1 is a perinuclear protein conferring resistance to mutagens

Analysis of the Stm1 sequence with PSORT (http://psort.nibb.ac.jp/) algorithm (Nakai and Kanehisa, 1992) revealed a putative nuclear localization sequence at the N-terminus. To find out if this domain is functionally relevant, localization of Stm1-IRS was determined by immunofluorescence (Figure 5A). We observed an intense staining in the perinuclear region and in some cases also weak diffuse cytosolic staining, suggesting the existence of two distinct populations of Stm1 in the cell. Notably, no Stm1-IRS signal was seen in the lumen of the nucleus. To uncover whether Stm1 is associated with nuclear envelope or directly with chromatin, chromosome spreading experiments were performed. After mounting chromosomes on glass slides and using a detergent to remove material not directly associated with DNA, the Stm1-IRS signal remained in a ring-shaped arrangement suggesting association of Stm1 with the periphery of nucleoids (Figure 5B).

Stm1 null mutant cells grow as wild type on rich medium at 30°C (Figure 6A) and display only marginally reduced growth at 37°C (Figure 6B; doubling time during logarithmic phase 2.5 h as compared to 2.1 h for wild type). Cells lacking Stm1 are also sensitive to caffeine. On plates containing 12 mM caffeine *stm1-Δ1* mutant cells showed about 100 fold reduced plating efficiency than wild type cells (Figure 6C). Sensitivity to caffeine is often associated with defects in PKC-MAP kinase pathway. However, staurosporine, a specific inhibitor of Pkc1, had no effect on *stm1-Δ1* cells in a halo assay (results not shown), arguing against direct involvement of Stm1 in the PKC pathway.

Given that caffeine is a purine analog, we explored the possibility that the sensitivity of *stm1-Δ1* cells to this substance may reflect a role of Stm1 in nucleic acid metabolism. We observed that mutant cells show 10 fold enhanced UV sensitivity as compared to wild type cells (Figure 6D). Bleomycin is a radiomimetic drug that induces single- and double-strand breaks through the production of free radicals (Hampsey, 1997). *stm1-Δ1* mutant cells

displayed only a slight growth defect on YPD plates containing 10 μg.cm^{-3} bleomycin at 30°C (not shown), but their plating efficiency on the same medium dropped approximately 10 times at 37°C compared to wild type (Figure 6E). In contrast, an alkylating agent, methyl methanesulfonate (MMS), did not cause any differential effect in wild type and *stm1-Δ1* strains in a halo assay (data not shown) or in a test on YPD plates containing 0.02% MMS, either at 30 or 37°C (Figure 6F).

Immunofluorescence experiments were performed to check whether treatment with caffeine or bleomycin leads to an alteration of Stm1 localization within the cell. No change in the localization pattern of Stm1-IRS was observed after 2.5 h growth of cells on YPD in the presence of 12 mM caffeine, or 750 μg.cm^{-3} bleomycin at 30 and 37°C as compared to cells grown on YPD at 30°C (data not shown).

Cells lacking Stm1 can recover from H_2O_2 treatment

As described in a previous section, accumulation of Stm1 leads to apoptotic-like cell death. Apoptotic phenotypes can also be induced in yeast by treatment with low concentrations of H_2O_2 (Madeo *et al.*, 1999). Therefore the question arose whether *stm1-Δ1* cells are as sensitive to H_2O_2 treatment as wild type. In a halo assay, both strains displayed the same level of sensitivity after incubation for 1.5 days. However, after 3 days *stm1-Δ1* cells started populating the zone that was up to that point devoid of any growth, thereby decreasing the size of the halo. In contrast, wild type cells did not extend their growth significantly towards the center of the halo (Figure 7A). To address the possibility that the *stm1-Δ1* cells growing in the halo were suppressor mutants several of them were isolated an tested again for H_2O_2 sensitivity using the halo assay. No increase in H_2O_2 resistance relative to the original *stm1-Δ1* strain was observed, thereby excluding appearance of suppressor mutations (data not shown). A possible explanation of the recovery of *stm1-Δ1* in the halo zone is that a portion of *stm1-Δ1* cells survived the otherwise lethal level of H_2O_2 and resumed their growth after decrease of H_2O_2 concentration (by diffusion and/or reaction with the components of the media). Therefore we analyzed plating efficiency of wild type and *stm1-Δ1* cells after exposure to various concentrations of H_2O_2 in liquid cultures. This experiment showed that as compared to wild type *stm1-Δ1* cells are slightly but significantly more resistant to treatment with low concentrations of H_2O_2, whereas higher concentrations of H_2O_2 (above 1 mM) are lethal to both mutant and wild type cells (Figure 7B). Quantification of TUNEL staining shows that the increase in survival rate of *stm1-Δ1* cells treated with low dose of H_2O_2 (0.05 mM) is accompanied by decrease in number of cells showing apoptotic phenotype (Figure 7C).

As previously shown cycloheximide treatment leads to increased survival of yeast cells after exposure to low concentrations of H_2O_2 (Collinson and Dawes, 1992) by preventing apoptotic-like cell death (Madeo *et al.*, 1999). However, cycloheximide treatment did not further increase resistance of *stm1-Δ1* cells to apoptotic-like cell death brought about by low concentrations of H_2O_2 (Figure 7C).

DISCUSSION

The ubiquitin-proteasome system has been proposed to control mammalian apoptosis by degrading a short-lived pro-apoptotic protein (Drexler, 1997). To find out if the ubiquitin system plays a similar role in yeast we performed a two layer screen. In the first step we looked for potential yeast proteasomal substrates that when overexpressed cause cells with defective proteasome to arrest. In the second step, we screened these putative substrates for their ability to cause cell death and to elicit diagnostical markers of apoptosis in yeast cells. Six proteins were found whose overexpression in the proteasomal mutant led to exposure of phosphatidylserine on the cell surface, chromatin condensation, DNA breakage, and cell death.

One of them was Stm1, a protein that was known to bind quadruplex DNA (Frantz and Gilbert, 1995) and purine-rich triplex DNA (Nelson *et al.*, 2000) in vitro. Quadruplex structures were suggested to be present at chromosome ends (Liu *et al.*, 1993). However, using a one-hybrid assay for telomere binding proteins (Bourns *et al.*, 1998) we could not detect any expression of the reporter gene that would indicate interaction of Stm1 with telomeric DNA (data not shown). Consistent with its predicted ability to interact with DNA, we found that *stm1-Δ1* cells are sensitive to UV light and treatment with bleomycin, a drug mimicking the effect of ionizing radiation. They are not, however, sensitive to the alkylating agent MMS, suggesting that Stm1 might function in a specific aspect of DNA repair. Stm1 shows weak diffused cytosolic and strong perinuclear staining in fixed cells. Its localization at the periphery of spread nucleoids suggest direct interaction with DNA. These results are consistent with the detection of Stm1 in the highly enriched nuclear envelope fraction (Rout *et al.*, 2000) and with the presence of a putative nuclear localization sequence in the protein.

Stm1 is an in vivo substrate of the proteasome, as evidenced by its rapid turnover in wild type cells and its complete stabilization in mutants with severely impaired proteasome. Because degradation of Stm1 is blocked also under non-lethal conditions (normal expression of Stm1 from its endogenous promoter) it can be excluded that Stm1 stabilization is a consequence of cell death. Therefore the data strongly suggest that the lethal effect of overexpressed Stm1 in *pre1-1 pre4-1* mutants is a result of accumulation of the stabilized protein.

The conspicuous feature of the *pre1-1 pre4-1* cells killed by overexpression of Stm1 is the appearance of phenotypes found in metazoan cells undergoing apoptosis and yeast cells killed for example by exposure to low concentrations of H_2O_2. We tested the sensitivity of *stm1-Δ1* cells to treatment with H_2O_2 using a halo assay and a survival test in liquid culture. In both cases a significant portion of *stm1-Δ1* cells survived exposure to low doses of H_2O_2 that are toxic to wild type cells. In addition, DNA cleavage as detected by the TUNEL assay was correspondingly reduced in *stm1* null mutant, indicating that increased survival of these mutants is due to suppression or absence of the apoptosis-like cell death. Cycloheximide treatment - and thereby blocking of protein synthesis - has a protective effect on wild type yeast cells exposed to low levels of H_2O_2 (Collinson and Dawes, 1992), but this phenomenon was absent in *stm1-Δ1* mutants. In a recent work we proposed that cycloheximide increases survival of H_2O_2 treated cells by inhibiting a translation-dependent apoptosis-like cell death process (Madeo *et al.*, 1999). Taken together, these findings led to the idea that protection against H_2O_2 induced cell death is based at least in part on depletion of Stm1 activity due to deletion of the *STM1* gene (*stm1-Δ1* cells) or blocking of its synthesis (application of cycloheximide). Hence, data presented here suggest that the Stm1 protein is an activator of the cell death process triggered by exposure of cells to low concentrations of H_2O_2. Control of its synthesis and/or degradation may be regulatory steps of H_2O_2 induced apoptosis-like cell death in yeast.

STM1 was originally identified as a multicopy suppressor of *tom1*, *htr1*, and *pop2* mutations, each of them being involved in an aspect of cell cycle control (for summary see Nelson *et al.*, 2000). In a genome-wide two hybrid screen, Stm1 was found to interact with a product of a predicted gene *YJR072C* (Uetz *et al.*, 2000), which has conserved orthologues in *Caenorhabditis elegans* and humans. Stm1 itself has a highly conserved orthologue in *Schizosaccharomyces pombe* and a putative orthologue in *Drosophila melanogaster* (Nelson *et al.*, 2000). This hints that Stm1 may regulate or participate in an evolutionary conserved process.

Apoptosis in mammalian cells has been tightly linked to activation of caspases (Rich *et al.*, 1999), that are missing in yeast. However, recent reports suggest that the appearance of apoptotic morphology can proceed in the absence of caspases, albeit in less efficient manner (Borner and Monney, 1999). Notable is the role of reactive oxygen species as

mediators of - in many cases - caspase independent apoptosis in mammalian cells (Carmody and Cotter, 2000; Xiang *et al.*, 1996). Recently a mammalian apoptosis inducing factor AIF has been identified that has closely related orthologues in all phyla (Susin *et al.*, 1999; Lorenzo *et al.*, 1999). Consequent to its translocation from mitochondria to the nucleus this factor triggers a cell death process with all the cytological hallmarks of apoptosis, but without the activation of caspases. Thus the target compartment of AIF and the major place of localization of Stm1 is the same - the nucleus - and both share similar function - induction of caspase independent cell death resembling apoptosis. The tempting hypothesis to be tested is that AIF, Stm1, and their respective orthologues participate in the same caspase-independent pathway in yeast and mammals.

A tentative link to apoptotic death can be assigned to at least two further *HEL* genes shown here to cause apoptotic phenotypes. Ppa1 is an essential subunit of the vacuolar membrane H^+-ATPase (Hirata *et al.*, 1997). *pre1-1 pre4-1* cells overexpressing Ppa1 display signs of leakage of vacuolar material to the cytosol (M. Ligr, unpublished observation). In mammalian cells, leakage of lysosomal content (such as cathepsin D – an ortholog of the yeast proteinase A) after mild oxidative stress has been proposed to lead to apoptotic death (Hellquist *et al.*, 1997; Brunk *et al.*, 1997; Fossel *et al.*, 1994). Lysosomes - the compartment analogous to the yeast vacuole - appear to integrate signals from the evolutionary conserved ceramide signaling pathway (Monney *et al.*, 1998; Heinrich *et al.*, 1999) implicated in control of apoptosis (Hannun and Luberto, 2000).

The GTP-binding protein Sar1 is a component of COPII coated vesicles involved in transport from the endoplasmic reticulum to the Golgi compartment (Kuehn *et al.*, 1998). Caspase-dependent cleavage of human BAP31 occurring during apoptosis leads to disruption of the endoplasmic reticulum - Golgi transport (Maatta *et al.*, 2000). Conversely, perturbation of the early secretory pathway by brefeldin A has been shown to trigger p53-independent apoptosis (Shao *et al.*, 1996) in a ceramide signaling pathway dependent manner (Linardic *et al.*, 1996). Ceramide levels rise in yeast during heat shock (Wells *et al.*, 1998), and preventing ceramide accumulation enhances survival of cells after heat treatment (Mandala *et al.*, 1998). The ceramide signaling pathway may thus turn out to be a player in regulation of apoptotic-like cell death also in yeast.

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Table 1. Yeast strains

Strain	Genotype	Reference/Source
WCG4/a	*MAT***a** *his3-11,15 leu2-3,112 ura3*	(Heinemeyer *et al.*, 1993)
YHI29-1	*MAT***a** *pre1-1*	(Heinemeyer *et al.*, 1991)
YHI29-14	*MAT***a** *pre1-1 pre4-1*	(Hilt *et al.*, 1993)
YML1	*MAT***a** *pre1-1 cyh2* [pML1]	This study
YML2	*MAT***a** *pre1-1 pre4-1 cyh2* [pML1]	This study
YIV2	*MAT***a** *stm1-Δ1::kanMX*	This study
YL280	*MAT***a** *STM1::IRS*	This study
YL286	*MAT***a** *STM1::IRS pre1-1 pre4-1*	This study

Table 2. Overexpressed ORFs causing apoptosis in *pre1-1 pre4-1* cells

ORF	protein function (Costanzo *et al.*, 2000)
NSR1	nucleolar protein involved in processing 20S to 18S rRNA
SAR1	component of COPII coat of vesicles involved in ER to Golgi transport; GTP-binding protein of the arf family
STM1	protein with specific affinity for G-rich quadruplex nucleic acids; multicopy suppressor of *pop2* and *tom1*
PPA1	proteolipid of the vacuolar H^+-ATPase (V-ATPase)
HEL 10	unknown
(YNL208W)	
HEL13	unknown
(YOR309C)	

LEGENDS

Figure 1: Several *HEL* genes cause growth arrest and cell death when overexpressed in proteasomal mutants. Wild type and *pre1-1 pre4-1* cells carrying pYES2 encoded, P_{GAL1} driven cDNAs of *HEL* genes (as indicated) were cultivated overnight in SC ura$^-$. Cells were harvested, and washed in water. (A) To test for growth arrest, 10-fold serial dilutions were spotted on SCgal ura$^-$ plates. Plates were incubated for 3 days at 30°C. (B) After 8 h induction in SCgal ura$^-$ liquid medium, cell death rate was scored as 100% - [plating efficiency on YPD medium]. Frequency of cells with TUNEL positive nuclei was determined by microscopic inspection. Results were averaged from 2 experiments.

Figure 2: Overexpression of *HEL* genes in proteasomal mutants leads to exposure of phosphatidylserine. *pre1-1 pre4-1* cells that carried pYES2 encoded cDNAs (as indicated) under control of the *GAL1* promoter were pregrown on SC ura$^-$ until logarithmic phase. Expression of cDNAs was then induced by incubation in SCgal ura$^-$ medium for 8 h at 30 °C. Only protoplasts excluding propidium iodide (and therefore intact) are shown.

Figure 3: Overexpression of *HEL* genes induces condensation and margination of chromatin in proteasomal mutants. For experimental conditions see the legend to Figure 2. Arrowheads point to peripherally condensed chromatin. N - nucleus.

Figure 4: Stm1-IRS is stabilized in proteasome mutant cells. Top: Expression of pL092 encoded Stm1-IRS was induced on SCgal ura$^-$ medium. Stm1-IRS synthesis was blocked by addition of glucose and cycloheximide. Bottom: Synthesis of chromosomally encoded Stm1-IRS was stopped by addition of cycloheximide. Stm1-IRS levels were followed at indicated chase times by western analysis. “0” is the time point when glucose and/or cycloheximide were added.

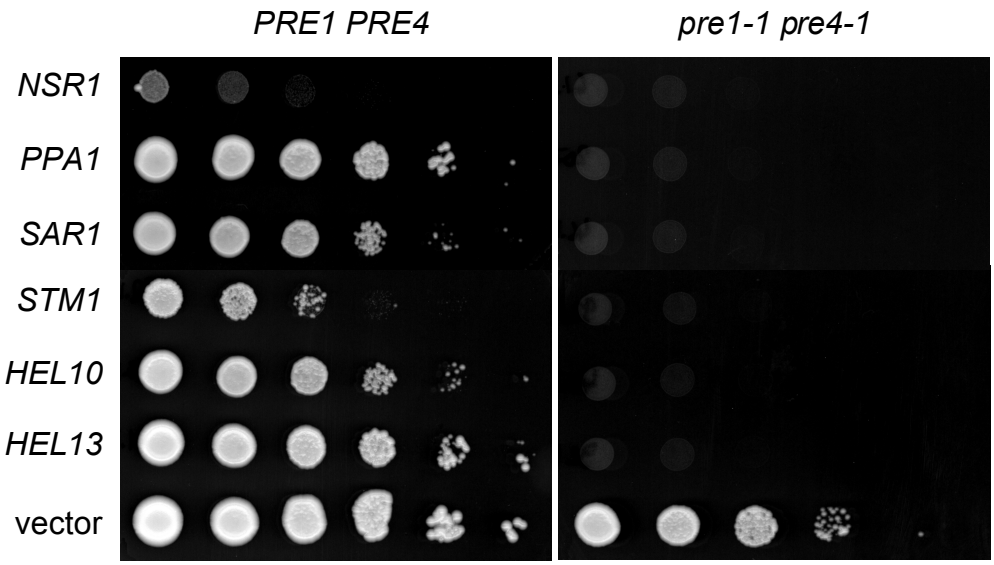
Figure 5: Stm1-IRS localizes to the periphery of nucleoids. *STM1* was chromosomally tagged at its C-terminus with IRS epitope. A strain with a wild-type copy of *STM1* was used as a negative control. Stm1-IRS was visualized in (A) fixed whole cells or in (B) chromosome spreads (here material unbound to DNA was removed by detergent treatment).

Figure 6: *stm1-Δ1* cells are sensitive to caffeine and DNA damaging agents. Cells were grown on liquid YPD to saturation and 5 μl of 10-fold serial dilutions were spotted on agar medium. Cells on YPD plates were incubated at (A) 30°C and (B) 37°C for two days. (C) Cells were spotted on YPD plates containing 12 mM caffeine and incubated at 30°C for 5 days. (D) Cells plated on YPD were exposed to UV light for 45 s and incubated in dark for 1.5 days at 30°C. (E) Cells were incubated for 4 days at 37°C on YPD plates containing 10 $\mu g.cm^{-3}$ bleomycin. (F) Cells were grown for 5 days at 30°C on YPD plates containing 0.02% MMS.

Figure 7: *stm1-Δ1* cells are resistant to low concentrations of H_2O_2. (A) Wild type and *stm1-Δ*1 cells were grown on YPD to saturation, mixed with 0.5% agar in YPD (40°C) and casted on YPD plates. Whatman 003 paper disks were soaked with 10 μl of 30% H_2O_2 and placed on the surface of the plates. Halo formation was recorded after 1.5 and 3 days of growth at 30°C. (B) Cells growing logarithmically on YPD were challenged with various concentrations of H_2O_2 for 200 min in the presence or absence of 15 $\mu g.cm^{-3}$ cycloheximide (CHX) as indicated. Equal numbers of cells were plated on YPD and colony forming units counted after 2 days of growth. Survival rate was expressed as a fraction of colony forming units relative to untreated cells. (C) Cells were treated with 0.05 mM H_2O_2 and/or 15 $\mu g.cm^{-3}$ cycloheximide when indicated. TUNEL frequency was determined by counting cells with TUNEL-positive nuclei under microscope. Results were averaged from 3 experiments.

Figure 1

A


PRE1 PRE4
pre1-1 pre4-1
NSR1
PPA1
SAR1
STM1
HEL10
HEL13
vector

B

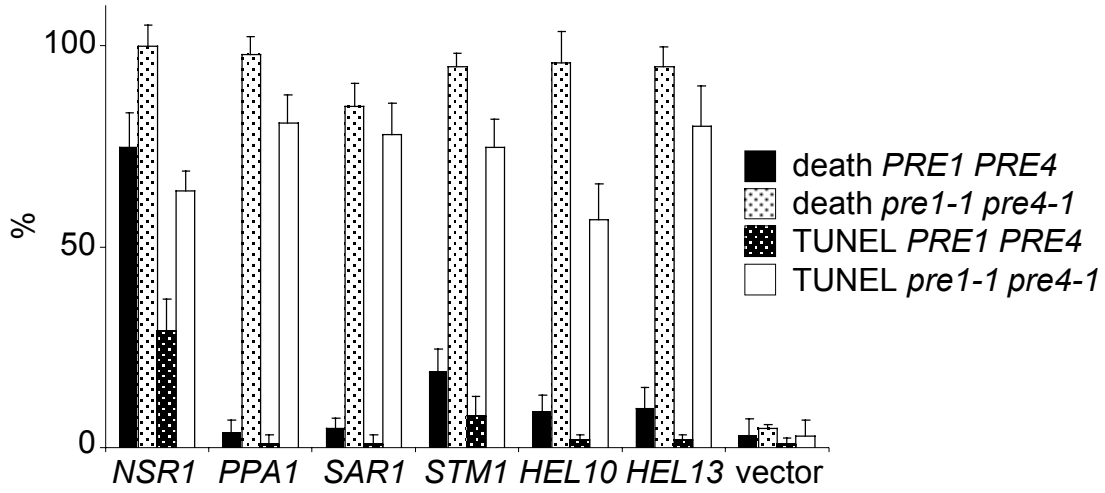

100
50
0
%
NSR1
PPA1
SAR1
STM1
HEL10
HEL13
vector
death PRE1 PRE4
death pre1-1 pre4-1
TUNEL PRE1 PRE4
TUNEL pre1-1 pre4-1

Figure 2

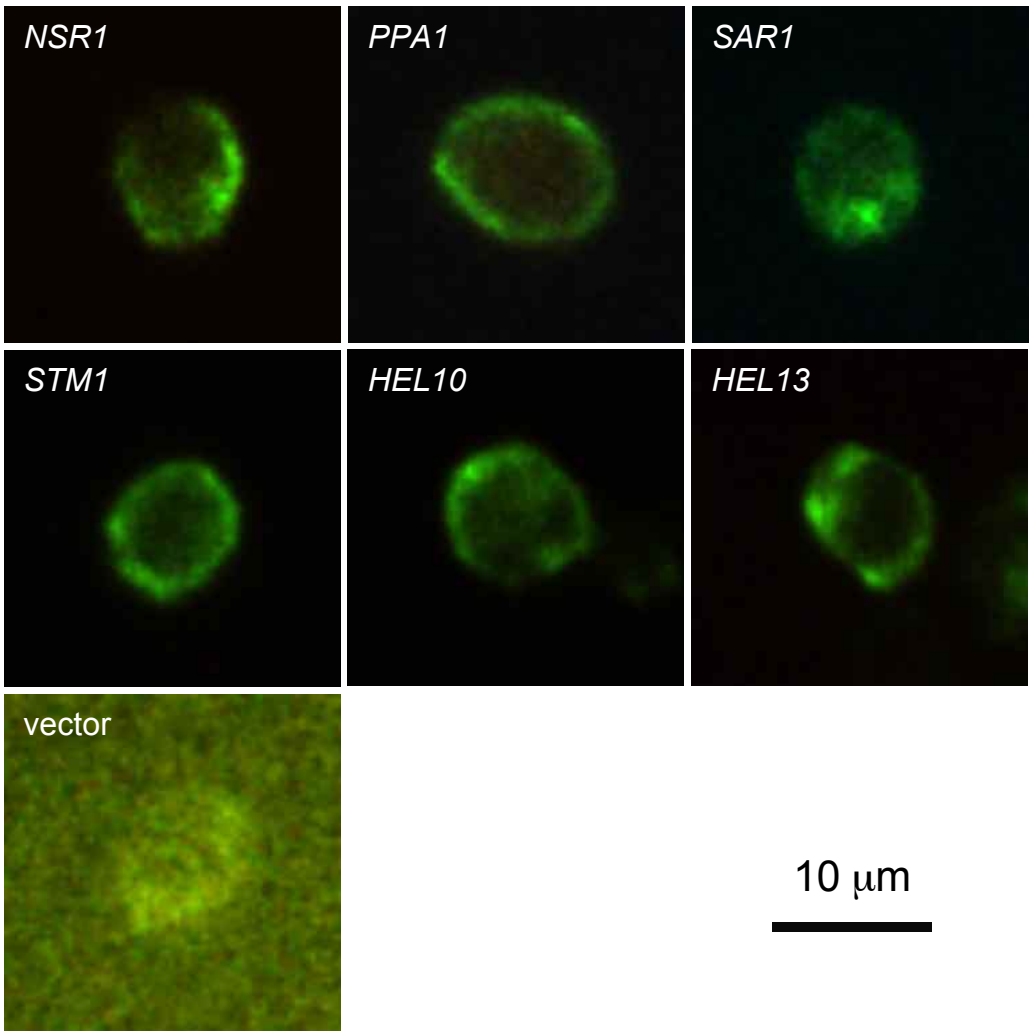


Figure 3

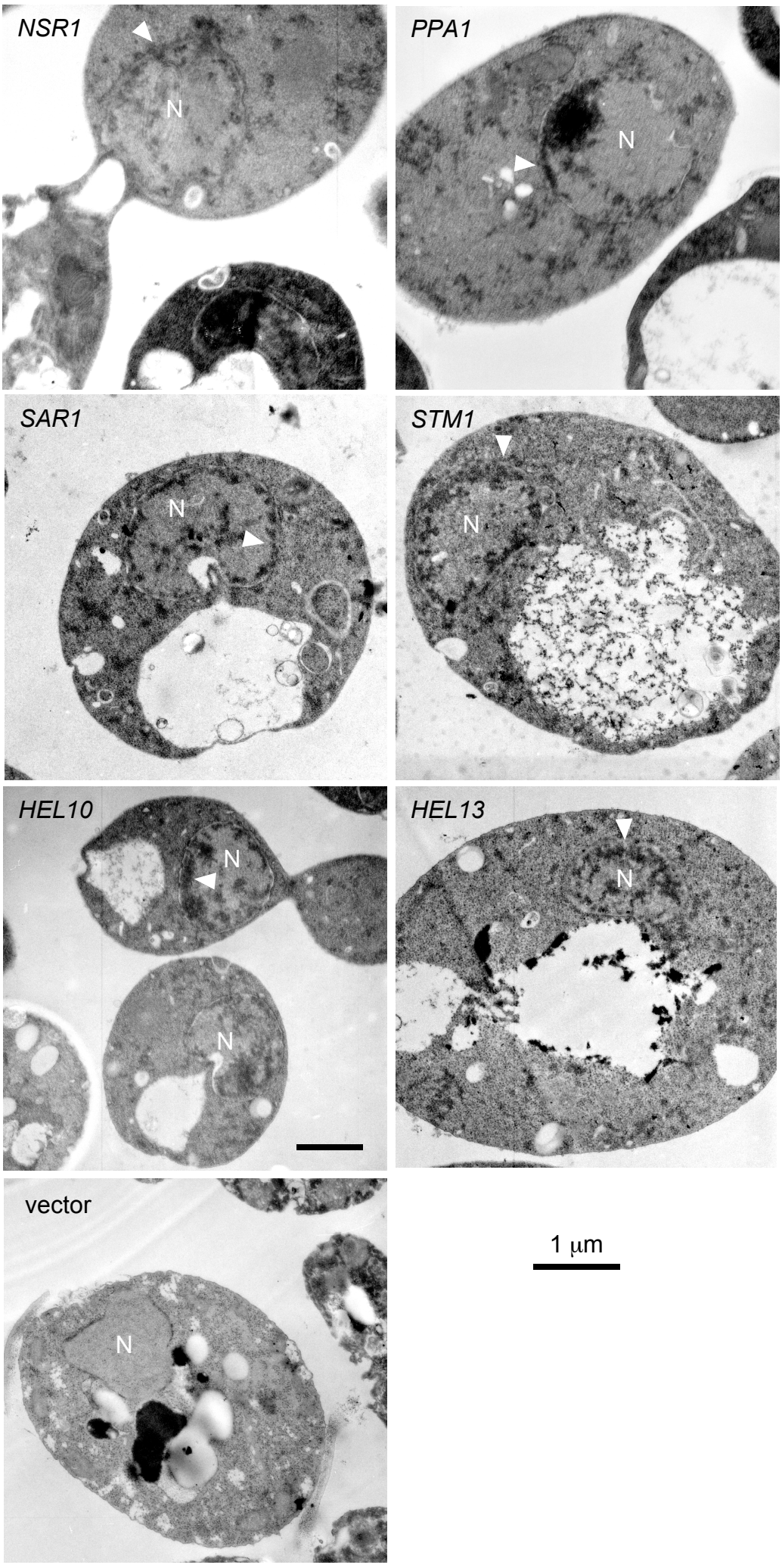


Figure 4

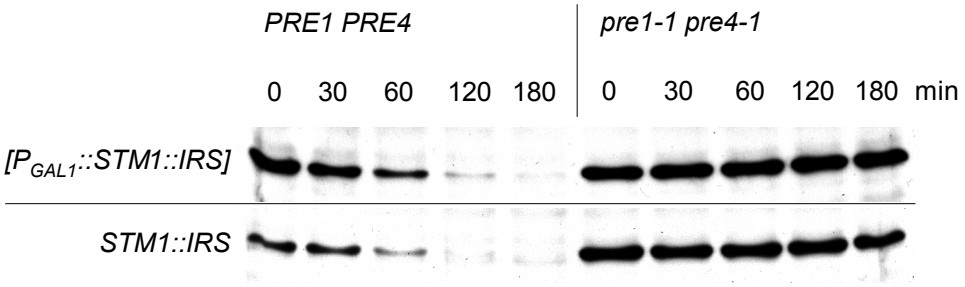

PRE1 PRE4
pre1-1 pre4-1
0 30 60 120 180 0 30 60 120 180 min
[P GAL1 ::STM1::IRS]
STM1::IRS

Figure 5

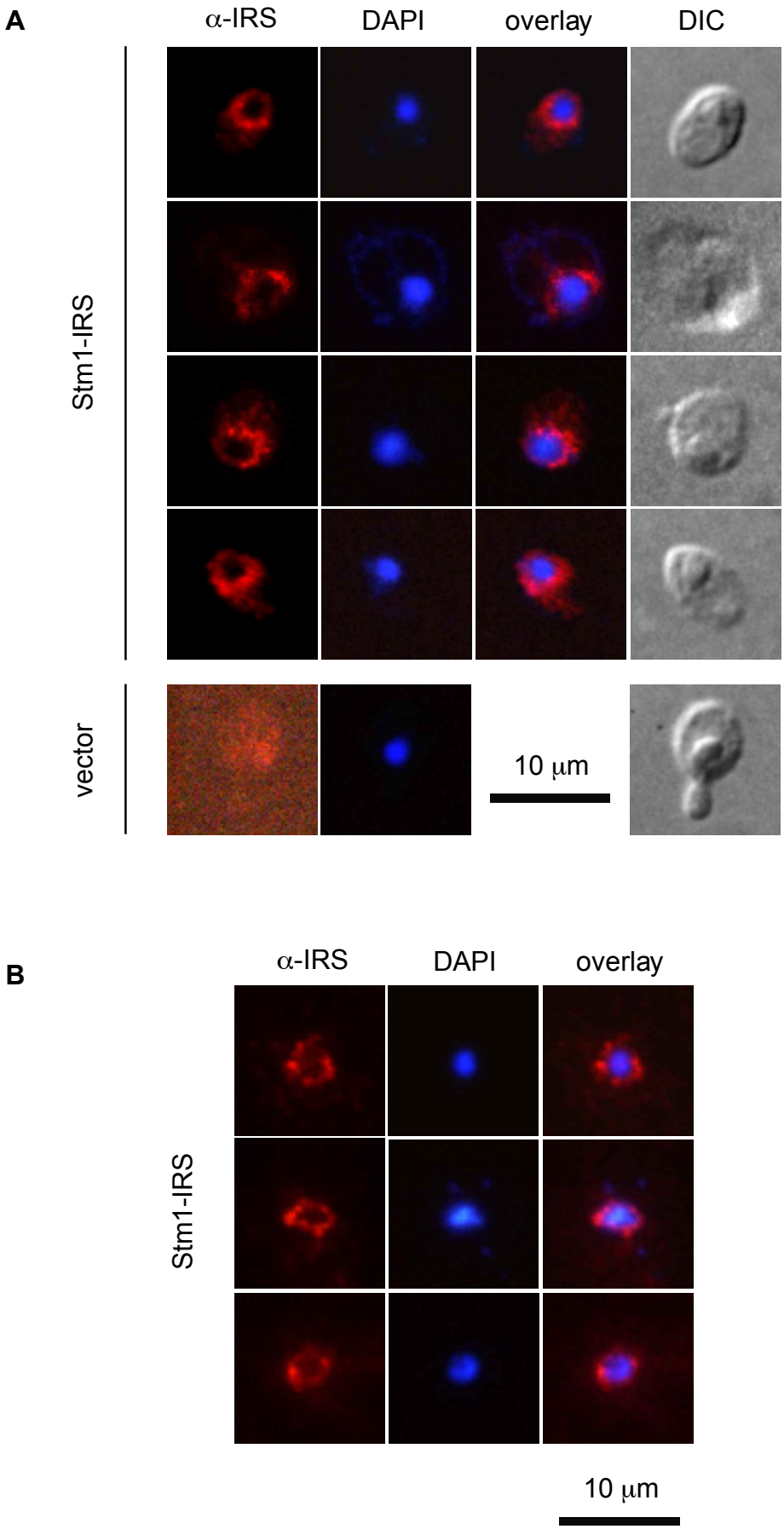

A
α-IRS
DAPI
overlay
DIC
Stm1-IRS
vector
10 μm
B
α-IRS
DAPI
overlay
Stm1-IRS
10 μm

Figure 6

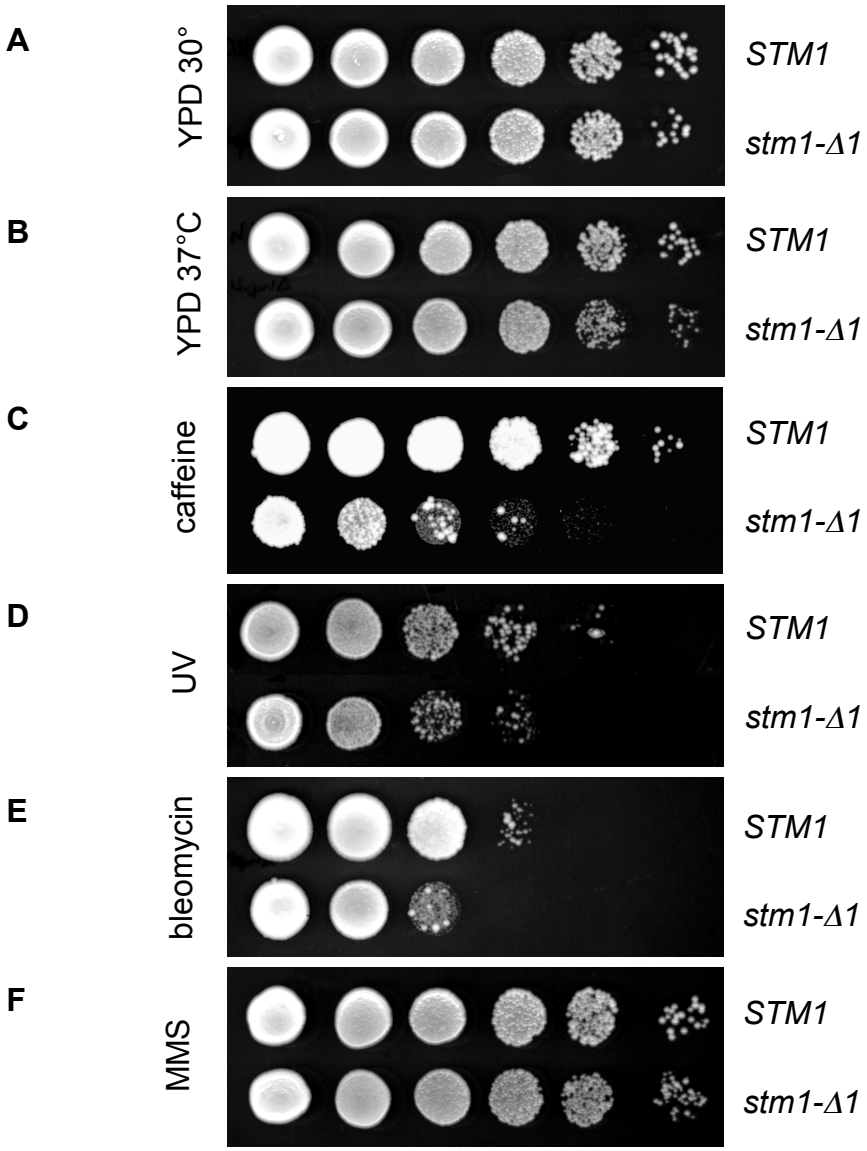
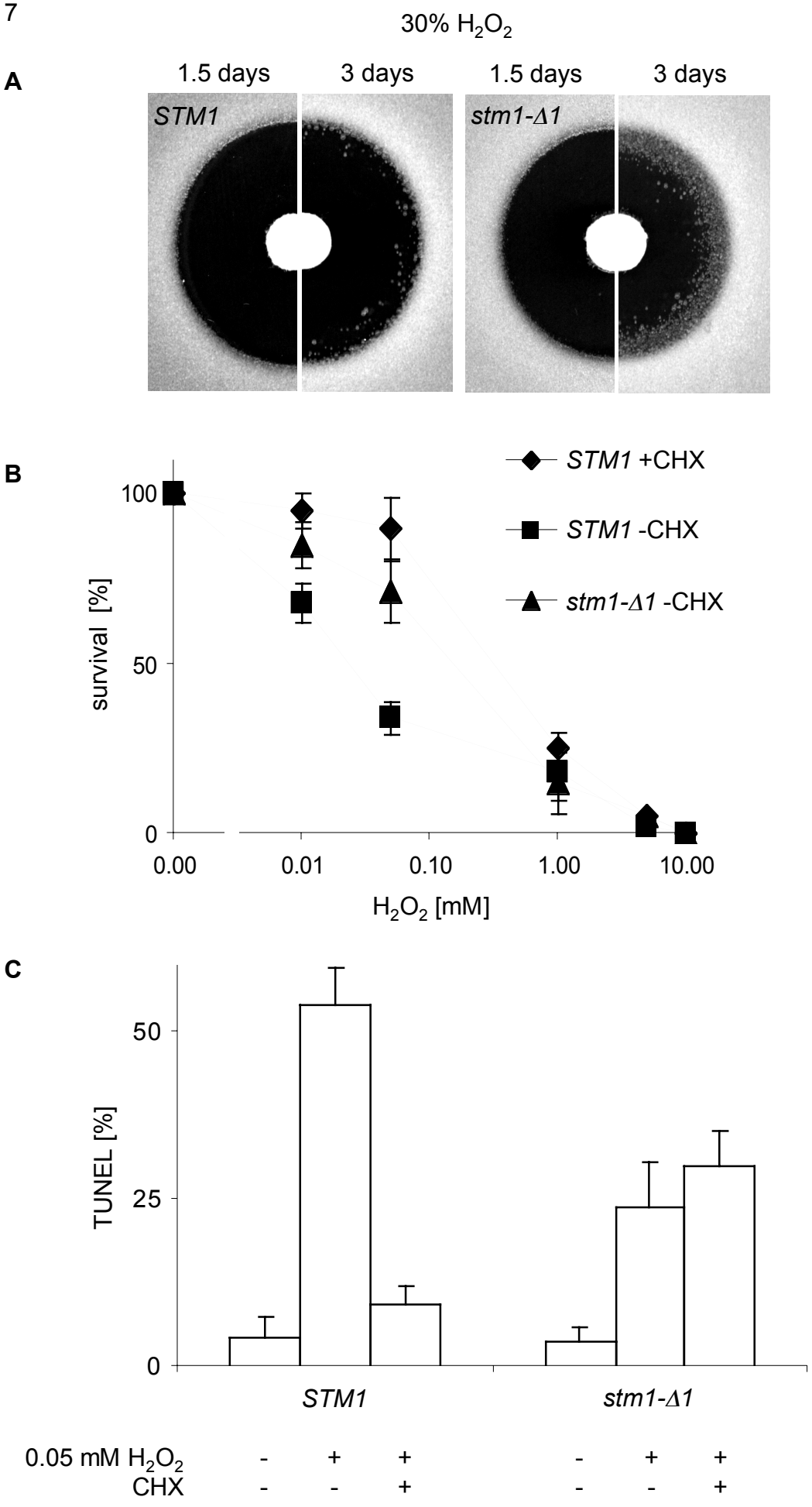

A
YPD 30°
STM1
stm1-Δ1
B
YPD 37°C
STM1
stm1-Δ1
C
caffeine
STM1
stm1-Δ1
D
UV
STM1
stm1-Δ1
E
bleomycin
STM1
stm1-Δ1
F
MMS
STM1
stm1-Δ1

Figure 7


30% H_2O_2
A
1.5 days
3 days
1.5 days
3 days
STM1
stm1-Δ1
B
STM1 +CHX
STM1 -CHX
stm1-Δ1 -CHX
100
50
0
survival [%]
0.00
0.01
0.10
1.00
10.00
H_2O_2 [mM]
C
50
25
0
TUNEL [%]
STM1
stm1-Δ1
0.05 mM H_2O_2 - + + - + +
CHX - - + - - +

Ich versichere hiermit, daß ich diese Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe. Alle Wissenschaftler, die zusammen mit mir an den veröffentlichten Projekten gearbeitet haben, sind in der Autorenliste der einzelnen Publikationen genannt.

Stuttgart, 22. Januar 2001