Functions of the yeast protein Stm1 and its involvement in apoptotic cell death

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Abbreviations

ADE	adenin
Amp	Ampicillin
Asp	Aspartic acid
ATP	adenosine triphosphate
bp	base pairs
BD	Becton Dickinson
Δ	deletion
DTT	dithiotreitol
ºC	degree celsius
CPY	carboxypeptidase
DHR123	dihydrorhodamine123
DiBaC4	bis-(1,3-dibutylbarbituric acid) trimethine oxonol
DMSO	dimethylsulfoxid
DSBs	double strand breaks
E. coli	*Escherichia coli*
EDTA	ethylenediamine-tetraacetic acid
e.g.	for example
FACS	fluorescence activated cell sorting
5-FOA	5-Fluoroorotic acid
FL	fluorescence
Gal	galactose
G-418	geneticin disulfate 418
Glu	Glutamic acid
h	hours
HRPO	peroxidase conjugate
Kan	kanamycin
kDa	kilodalton
(μ, m) l	(micro, milli) liter

(m,μ,p) g	(milli, micro, pico) gram
LYS	lysine
LB	Luria broth
min	minutes
MMS	methylmethanesylphoxyl
MV	synthetic minimal medium
MW	molecular weight
NHEJ	non-homologous end joining
nm	nanometer
OD_{600}	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
%	percentage
PGPH	peptidylglutamyl peptide hydrolysing
PI	propidium iodide
PMSF	phenylmethylsulfonyl fluoride
PCR	polymerase chain reaction
PBS	phosphate buffered saline
ROS	reactive oxygen species
rpm	rotations per minute
S. cerevisiae	*Saccharomyces cerevisiae*
SC	synthetic complete growth medium
$SC\text{-}URA^-$	synthetic complete growth medium lacking uracil
s	seconds
TCA	trichloro acetic acid
TEMED	N,N,N',N'-tetramethylendiamin
Tris	Tris [hydroxymethyl] aminomethane
ts	temperature sensitive
2μ	two micron
U	units
URA	uracil

Val	Valine
WT	wild type
YPD	yeast-peptone-dextrose (rich growth medium)

Zusammenfassung

Selektive, über das Ubiquitin-Proteasom-System vermittelte Proteolyseprozesse sind beteiligt an der Regulation einer Vielzahl von zellulären Prozessen. Die Identifizierung und Charakterisierung von proteasomalen Substraten ist wichtig zur Entschlüsselung der zellulären Funktionen des Proteasomen-Systems. Unter Verwendung eines Überexpressionsscreens war es gelungen ein neues proteasomales Substrat des Proteasoms der Hefe *Saccaromyces cerevisiae* zu identifizieren (Ligr et al., 2001). Die biologische Rolle von Stm1 sollte in dieser Arbeit näher untersucht werden.

Stm1 weist eine besondere Affinität zu Guanin-reicher Quadruplex-DNA auf und wird hauptsächlich in der Kernperipherie gefunden. Diese Befunde weisen auf eine mögliche Funktion von Stm1 an den Chromosomen, insbesondere deren Telomeren hin. Es wurden deshalb Untersuchungen durchgeführt, die klären sollten ob Stm1 eine Rolle im "Silencing" von Telomerregionen oder in der Reparatur von Doppelstrangbrüchen über den NHEJ (non-homologous-end-joining) Mechanismus spielt. Dies konnte anhand der Ergebnisse ausgeschlossen werden. Außerdem wurde mit einem speziellen Testsystem studiert, ob Stm1 an der Regulation der Telomeren-Elongation beteiligt ist. Auch dies konnte klar ausgeschlossen werden. Als interessanter Befund ergab sich aber dabei, dass die Spaltung von DNA durch HO-Endonuklease bei Abwesenheit von Stm1 beschleunigt abläuft, d.h. die DNA in *stm1*Δ Zellen wahrscheinlich leichter angreifbar ist.

Vorhergehende Studien hatten gezeigt, dass Stm1 bei Überexpression in Hefezellen mit defektem proteasomalem Weg die Induktion eines Apoptose-ähnlichen Zelltods bewirkt (Ligr et al., 2001). Dieser proapoptotische Effekt könnte durch Nebeneffekte, ausgelöst zum Beispiel durch die Stabilisierung zusätzlicher proteasomal kontrollierter Regulatoren, überhaupt erst möglich oder zumindest verstärkt werden. Deshalb war ein wichtiges Ziel der vorliegenden Arbeit eine "hyperaktive" Version von Stm1 zu generieren, die bei Überexpression schon in Wildtyp-Zellen einen Wachstumseffekt erzeugt. Dies gelang durch Isolation des Mutantenallels *stm1-9*, das bei Überexpression in Wildtyp-Zellen einen apoptotischen Zelltod induziert. Dabei entwickeln die Zellen typische zytologische

Merkmale, wie die Freisetzung von Cytochrom C aus den Mitochondrien oder eine extensive Fragmentierung chromosomaler DNA. Die Zellen zeigten sich im Verlauf dieses Zelltods färbbar mit FITC-VAD-FMK sowie aufnahmefähig für Propidium-Jodid, welches einen Verlust der Intergrität der Plasmamembran anzeigt. Zusätzlich konnte im Verlauf des *stm1-9* induzierten Todes der Hefezelle eine vermehrte Produktion von reaktiven Sauerstoffspezies (ROS) nachgewiesen werden.

"Cycloheximid-Chase"-Experimente zur Verfolgung des proteasomalen Abbaus von mutiertem Stm1-9 Protein ergaben im Vergleich zum Stm1 Wildtypprotein eine deutliche proteolytische Stabilisierung. Der proapoptotische Effekt von *stm1-9* ist damit mit hoher Wahrscheinlichkeit einer verstärkten Anhäufung des Proteins zuzuschreiben.

Zur Identifizierung von Komponenten, die zum Stm1-induzierten Zelltod beitragen, wurden Nullmutanten bekannter proapoptotische Komponenten der Apoptose in Hefezellen auf eine Suppression der *stm1-9* induzierten Effekte untersucht. Hierbei konnte gezeigt werden, dass die einzige bislang bekannte (Meta-)Caspase der Hefe, das Yca1 Protein (Madeo et al., 2002b) für die Induzierung des *stm1-9* vermittelten Zelltods gebraucht wird. In *yca1*Δ Nullmutanten war die durch *stm1-9* Überexpression ausgelöste Wachstumsinhibition deutlich supprimiert, das Auftreten apoptotischer Merkmale fast vollständig neutralisiert. Ähnliche Befunde wurden für Nullmutanten, des "apoptosis inducing factor" Aif1 (Wissing et al., 2004) erhalten. Auch hier kam es zu einer deutlichen Reduktion der durch Stm1-9 vermittelten pro-apoptotischen Effekte. Bei Kombination beider Deletionen, das heißt in *yca1*Δ *aif1*Δ Doppelmutanten, wurde bei Überexpression von *stm1-9* keine weitere Steigerung der Suppressoreffekte beobachtet. Dieses epistatische Verhalten zeigt, dass offensichtlich beide Proteine Aif1 und Yca1, im gleichen zellulären Weg - in noch nicht definierter Reihenfolge - operieren.

Weiterhin wurde die Auswirkung der *stm1-9* Überexpression in Nullmutanten des *MEC3* Gens untersucht. Mec3 kodiert für eine Kinase, die eine wichtige Rolle in DNA-Reparatur Signalprozessen spielt. Hier konnte gezeigt werden, dass auch Mec3 für den durch Stm1-9 getriggerten Zelltod benötigt wird. Coimmunopräzipitationsexperimente

beweisen außerdem, dass Stm1 und Mec3 *in vivo* miteinander interagieren. Diese Ergebnisse untermauern die Funktion von Stm1 in DNA-Reparaturprozessen. Eine weiterführende Untersuchung zeigte, dass *stm1-9 yca1* Mutanten eine erhöhte Sensitivität gegenüber UV-induzierter Mutagenese aufweisen. Dies kann als Hinweis gewertet werden, dass Stm1 und Yca1 gemeinsam für die korrekte Einleitung eines apoptotischen Zelltods nach Schädigung der DNA gebraucht werden. Die Untersuchung einer Serie bekannter Apoptose-stimulierender Gene der Hefe (die *HEL*-Gene *NSR1*, *SAR1*, *PPA1*, *YNL208w* und *YOR309c*) ergab, dass der durch Überexpression dieser Gene ausgelöste apoptotische Zelltod nicht von Stm1 abhängt. Stm1 operiert entweder in einem unabhängigen Apoptoseweg oder agiert "up-stream" zu diesen Apoptoseinduktoren.

Summary

Selective proteolysis conducted by the ubiquitin-proteasome system regulates many essential cellular processes. Therefore, identification and characterization of new proteasomal substrates is important to reveal cellular functions of the proteasome system. Using an overexpression screen a new proteasomal substrate, termed Stm1, had been found in the yeast *Saccharomyces cerevisiae* (Ligr et al., 2001). To better understand the biological role of Stm1, its reasonable contribution to different cellular functions ranging from telomeric silencing, DNA repair to apoptosis were elucidated in this work.

Regarding the specific affinity of Stm1 for guanine-rich quadruplex DNA and its localization at the nuclear periphery, the possible role of Stm1 in telomeric silencing, as well as its involvement in non-homologous end joining of double strand breaks (NHEJ) were investigated. The results exclude the direct involvement of Stm1 in such processes. Additionally, a possible role of Stm1 in the regulation of telomere elongation was analyzed. The data showed that *in vivo* telomere elongation was Stm1 independent. Surprisingly, however, chromosomal cleavage was accelerated in *stm1Δ* mutants.

It had been shown that Stm1 triggers apoptosis-like cell death when overexpressed in yeast mutants with impaired proteasomal activity (Ligr et al., 2001). However, defects in the proteasomal function might lead to the alteration of concentration or activity of some target proteins thereby causing possible side effects on the Stm1 function (Hilt and Wolf, 1996). Thus, in the present studies a mutated version, *stm1-9* was generated, which causes cell lethality when overexpressed in yeast cells harbouring intact proteasomal activity. Overexpression of *stm1-9* in wild type cells induces apoptotic cell death accompanied by cytochrome c release and extensive nuclear DNA cleavage. In addition, using bivariate flow cytometry, FITC-VAD-FMK staining paralleled by propidium iodide staining, indicating disintegration of the plasma membrane was observed under these conditions. As a further sign of apoptosis, enhanced production of ROS was found after *stm1-9* overexpression.

Using cycloheximide chase analysis, the hyperactive Stm1-9 protein was proven to be proteolytically stabilized. The pro-apoptotic effect of overexpressed Stm1-9 is, therefore, thought to be the result of the accumulation of the stabilized protein.

Investigation of potential suppressors of the *stm1-9* triggered cell death identified new downstream targets of this apoptosis pathway. One down-stream target is the caspase-like protease Yca1. This so far unique *S. cerevisiae* metacaspase is functionally involved in apoptotic cell death in yeast (Madeo et al., 2002b). In this work, it could be shown that cell death and induction of apoptotic phenotypes induced by overexpression of *stm1-9* is neutralized in the absence of Yca1. This result proves that the *stm1-9* induced cell death is Yca1 dependent uncovering that Yca1 is a down-stream element in the *stm1-9* mediated pathway.

Similar results were obtained for a recently discovered pro-apoptotic protein, the apoptosis-inducing factor Aif1 (Wissing et al., 2004). Deletion of the yeast *AIF1* gene leads to suppression of apoptotic phenotypes and cell death induced by *stm1-9* overexpression indicating that Aif1 functions as a down-stream element in the *stm1-9* pathway. The suppression effect of *yca1Δ* and *aif1Δ* could not be enhanced by combination of both deletions. This behavior of epistasis evidences that Yca1 and Aif1 are both part of the same *stm1-9* stimulated cell death pathway.

Further suppression studies revealed that Mec3, a protein functioning in DNA repair is also required for *stm1-9* triggered apoptosis. Co-immunoprecipitation analysis unraveled an interaction between Stm1 and Mec3. Thus, the present data suggest an additional function of Stm1 in DNA repair.

A closer inspection of *stm1Δ yca1Δ* double mutants revealed enhanced sensitivity to UV-induced mutagenesis. This might mean that Yca1 and Stm1 are required for proper execution of apoptotic cell death after DNA damage.

Investigation of a series of other apoptosis inducing genes (*HEL* genes: *NSR1*, *SAR1*, *PPA1*, *YNL208w* and *YOR309c*) uncovered that induction of cell death in these cases is Stm1 independent, placing Stm1 in an independent pathway or at an up-stream position.

1 Introduction

1.1 Cellular proteolysis

The maintenance of cellular homeostasis is necessary for cell survival. The delicate balance between cell cycle progression and cell death is highly dependent on selective protein degradation. In many cases, proteins can be degraded via the lysosomal/vacuolar pathway. In this specialized cellular compartment, degradation of proteins occurs via a variety of unspecific, highly active proteases (Seglen and Bohley, 1992). Another major proteolytic degradation process is the ubiquitin-mediated proteasomal pathway. Here, the proteins are modified by the attachment of chains made up from a small polypeptide called ubiquitin. This modification is performed by an enzymatic cascade consisting of ubiquitin activating (E1), conjugating (E2) enzymes and, in most cases, (E3) ligases, which either mediate E2-substrate interaction or covalently bind ubiquitin and transfer it to the substrate. Proteins that are marked by poly-ubiquitin chains are then recognized and degraded by the 26S proteasome. During substrate degradation, ubiquitin is released (Ciechanover and Schwartz, 1998).

The 26S proteasomes consist of a 20S core, the 20S proteasome and two 19S regulatory cap complexes attached to both ends of the 20S core complex (Glickman and Ciechanover, 2002; Peters, 1994). The 19S cap complexes of the proteasome are responsible for protein recognition and unfolding of target proteins. In contrast, cleavage of peptide bonds of substrate proteins occurs insite the 20S core, which bears defined proteolytically active sites, such as the chymotrypsin-like activity, the trypsin-like activity and peptidyl-glutamyl-peptide-hydrolysing (PGPH) activity (Hilt and Wolf, 1996).

It is known that proteasome dependent proteolysis is involved in activation and inactivation of many cellular processes such as cell cycle progression, regulation of gene expression and apoptosis. For example, dependent on the cell line used inhibition of proteasome-dependent proteolysis can lead to either induction or prevention of apoptosis in mammalian cells (Drexler, 1997). Therefore, identification and further characterization of proteasomal substrates is important to reveal detailed cellular

functions of the proteasome system. One newly identified proteasomal substrate in *S. cerevisiae* is Stm1, which was shown to be involved in the apoptosis-like cell death of yeast (Ligr et al., 2001).

1.2 Stm1 and its known functions

The *Saccharomyces cerevisiae* Stm1 protein, also called as Mpt4 is a 30-kDa basic protein (pI=10.5), which is a moderately abundant in the yeast cell (35,000 copies/cell). Stm1 was originally identified as a protein with a specific affinity for binding of guanine-rich quadruplex DNA and purine motif triplex nucleic acids (Frantz and Gilbert, 1995; Nelson et al., 2000). Immunofluorescence microscopic studies showed, that Stm1 is located at the nuclear periphery or at the cytoplasma of the yeast cell depending on the epitope tag or yeast strain used (Ligr et al., 2001; van Dyke, 2004).

Database search reveals two genes with similarity to *STM1*; one encodes an unknown protein in *Schizosaccharomyces pombe*, the other a probable nuclear antigen in *Arabidopsis thaliana*. In addition, the Stm1 protein sequence shows a region that is similar to histone-like protein H1 (Utsugi et al., 1995). Recently, it was reported, that an association factor that binds to ribosomal subunits shares 86% of sequence identity with Stm1 (Correia et al., 2004). Thus, it was suggested that both proteins might belong to a family of proteins, which have possible roles in the translation process.

Recent studies have proposed a role of Stm1 in a variety of biological processes, ranging from telomere maintainance to apoptosis (see below). The function of Stm1 as a multicopy suppressor of temperature sensitive *tom1* and *htr1* mutants, staurosporine-sensitive *pop2*, *ccr4*, and *pkc1* mutants, and a caffeine-sensitive *mpt5* mutant was proven by genetic studies (Hata et al., 1998). In addition to this, Stm1 was shown to interact genetically and physically with Cdc13, which functions as a telomere-binding protein (Hayashi and Murakami, 2002). However, in contrast to *CDC13*, *STM1* is not essentially required for telomere replication. Based on these findings, it was suggested, that the Stm1-Cdc13 interaction might help to maintain telomere structure.

A two-hybrid interaction between Mec3 and Stm1 was documented in a comprehensive two-hybrid analysis of yeast protein-protein interactions (Ito et al., 2001).

Mec3 is a DNA-binding protein, which is involved in DNA-damage, telomere silencing, and telomere length maintainance. The interaction of Stm1 with Mec3 indicates that a complex containing both proteins might function in DNA repair pathways. Using conventional immunoprecipitation, Stm1 was found to be primarily associated with ribosomal proteins and rRNAs. These findings strongly suggest a role of Stm1 in protein translation, folding or export (van Dyke, 2004). In addition, Stm1 was shown to be associated with telomere-proximal Y' element sequences by chromatin immunoprecipitation experiments. Therefore, one might speculate that Stm1 has a role at subtelomeric sequences of DNA.

Recently, the function of Stm1 in regulation of the apoptosis-like cell death in yeast has been elucidated (Ligr et al., 2001). Using an overexpression screen, Stm1 was identified as a new proteasomal substrate, as it was found to be unstable in wild type cells, but stabilized in cells with defective proteasomal activity. Overexpression of *STM1* in the proteasomal *pre1-1 pre4-1* mutant resulted in an apoptosis-like cell death showing typical cytological markers of apoptosis such as loss of plasma membrane asymmetry, chromatin condensation and cleavage of DNA. Cells lacking *stm1*, however, are partially rescued in the apoptosis-like cell death process induced by treatment with low concentrations of H_2O_2. Furthermore, *stm1Δ* cells are sensitive to certain DNA-damaging reagents suggesting a role of Stm1 in DNA repair.

Because Stm1 interacts with a variety of cellular proteins and is involved in a multitude of cellular processes, one may suggest that Stm1 is a multifunctional protein. Thus, advanced studies of the biological functions of Stm1 are necessary to elucidate its exact role in the cell.

1.3 Organization of telomeric chromatin

Chromosomes of eukaryotic cells are protected from DNA shortening, end-to-end fusions and thus, the genome instability by specialised end structures, called telomeres. It is known, that in most organisms, telomeres consist of tandemly repeated $TG_{1\text{-}3}$ sequence that varies between 225 and 375 bp e.g. in *S. cerevisiae* (Welinger and Sen, 1997; Zakian, 1996). These sequences are required for capping function of the chromosomes but are

simply not enough to provide the telomere function. A number of proteins that bind to the telomere DNA ends are also required for this task. One of these is the specialized reverse transcriptase called telomerase (Greider and Blackburn, 1985). This enzyme catalyzes the *de novo* addition of telomeric repeat sequences onto the 3' ends of the telomeric G-rich ends of chromosomes. Two essential components for the telomerase enzymatic activity were defined in *S. cerevisiae*. These are *EST2* encoding a catalytic subunit with similarity to reverse transcriptase and *TLC1*, which encodes an RNA component providing the template for telomere DNA addition (Lingner et al., 1997; Singer and Gottschling, 1994). Loss-of-function mutations in these genes lead to a progressive loss of telomere sequence with each round of DNA replication. Thus, telomerase provides the primary pathway for resolving the end replication problem in most eukaryotes. The complexity of telomeric DNA length regulation makes it difficult to understand, how telomerase functions and is regulated in cell. Recent studies have provided an *in vivo* assay in *S. cerevisiae*, where a precisely defined telomeric end is created *de novo*, which can be easily monitored for the addition of telomeric DNA repeats by telomerase (Diede and Gottsschling, 1999). In this assay, the telomerase-mediated telomere addition *in vivo* is controlled in a cell cycle dependent manner. These studies have shown that two essential DNA polymerases, α and β, as well as DNA primase, are required for telomerase activity *in vivo*. Moreover, the data indicated that the *de novo* telomere assay reflects the mechanism of normal telomere maintainance *in vivo*.

In the yeast *S. cerevisiae*, telomeres have been also implicated in gene silencing (Lustig, 1998). Telomeric yeast domains constitute the areas of transcriptional repression or local shutdown of transcription. Therefore, marker genes inserted near to the telomeres are transcriptionally "silenced" via control through the heterodimeric Yku70/Yku80 and Sir protein complexes, which have additional function in the repair of DNA double strand breaks (DSBs) (Gottschling et al., 1990; Weaver, 1998). Moreover, it was shown, that DNA damage triggers disruption of telomeric silencing and relocation of Sir3 from telomeres under control of the DNA damage-signaling components Ddc1 and Mec1 (McAinsh et al., 1999). Therefore, one might suggest that such subsequent

association of protein complexes can regulate transcription and participate in DNA repair and/or enhance genomic stability by other mechanisms.

1.4 DNA breaks and nonhomologous end joining of double strand breaks (NHEJ)

The repair of DSBs is primarily a function of genes associated with two discrete pathways that are conserved in all eukaryotic organisms from yeast to humans. Yeast cells repair the large majority of their DSBs by homologous recombination, which is well understood. This major and preferential pathway occurs without a loss of genetic information because repair by homologous recombination essentially copies the missing information from one homologous chromosome to the other in a diploid cell. In contrast, in the second pathway, so called non-homologous end joining or NHEJ mechanism, the diploid nature of cell is not utilized and, thus, not required. NHEJ involves modifying two broken ends to make them compatible prior to rejoining (Lieber, 1999). Thus, NHEJ is an imperfect mechanism to preserve any genetic information between two DNA ends. Indeed, the NHEJ mechanism is required to repair broken telomeric ends because it is problematic to find homology partners for repetitive sequences (Stellwagen et al., 2003). Moreover, NHEJ mechanism is not only implicated in repair of DSBs in haploid yeast cells. It is also required to protect efficiently haploid yeast cells against telomeric end fusions (Liti and Louis, 2003).

Recent studies have provided new insights into the mechanism of NHEJ. Regarding this mechanism, two broken ends occurred in DNA cannot be simply ligated. They must be modified prior ligation. The modification of two broken ends will proceed only if these ends are brought into their physical proximity. Repair factors such as Yku70 together with Sir and Rad50 must bind to the DNA ends to hold the ends together (Critchlow and Jackson, 1998; de Vries et al., 1989; Tsukamoto and Ikeda, 1998; Tsukamoto et al., 1997; Yaneva et al., 1997). When the two broken DNA ends are brought into proximity, they join at the exact point, which occurs at one to four nucleotides that are complementary between two ends. The excess of DNA beyond the points of microhomology are then removed by nucleases Rad27 and Exo1 and the

appeared gaps are subsequently filled-in by polymerases (Fiorentini et al., 1997). Finally, DNA ligases (DNA ligase I/Cdc9 and Lig IV) must carry out ligation of the two modified DNA ends (Teo and Jackson, 2000).

Studies in the yeast *S. cerevisiae* revealed that DNA-end joining can be measured *in vivo* by assessing the efficiency of re-circularization of linear plasmid DNA after cellular transformation, thus, providing the possibilities to analyze the NHEJ mechanism in details (Boulton and Jackson, 1996).

1.5 Apoptosis (programmed cell death) in the mammalian system

1.5.1 Apoptosis

Apoptosis (programmed cell death) is a complicated signal induced process leading to regulated cellular destruction. The activities of many proteins are involved in the execution of the multiple sub-programmes of apoptosis. Apoptosis is required for early stages of the development of a multicellular organism to make a correct shape of the tissue. Later apoptosis is important to maintain the tissues architecture. Defective regulation in apoptosis contributes to the development of severe diseases as cancer or neurodegenerative and autoimmune disorders (Thompson, 1995).

Besides apoptosis, two other types of programmed cell death with differing nature were found: autophagic degeneration and non-lysosomal vesiculate cytoplasmic cell death with newly formed intracytoplasmic vacuoles (Ohmuraya et al., 2005; Szende et al., 1995).

1.5.2 Cytological markers defining apoptotic cell death

Cells undergoing apoptosis show a carefully regulated set of cellular features including shrinkage of cells, membrane blebbing, chromatin condensation and nuclear fragmentation (Kerr et al., 1972). Moreover, during apoptosis of many cell types, the lipid phosphatidylserine, which is normally localized in the inner leaflet of the plasma membrane, is externalized to the outer leaflet of the plasma membrane (Fadok et al., 2000;

Martin et al., 1995). Finally, the apoptotic cell dissociates into a number of particles with a membrane as the envelope called apoptotic bodies. These are efficiently phagocyted and degraded.

1.5.3 Extra- and intracellular signals triggering apoptosis

Apoptosis in the mammalian system is triggered by a variety of external and internal signals mediated by cell surface receptors like FAS, cytotoxic T cells, distinct genes (e.g. p53) and mitochondrial reaction (Fig. I). Though apoptosis can be induced by different external and internal signals, normally all these signals lead to initiation of a very similar if not identical cellular process.

Key components of a shared apoptotic pathway were discovered due to the studies of genetics and cell biology of the nematode *Caenorhabditis elegans*. The important outcome of these studies was the identification of the *CED-3* gene (Yuan et al., 1993). Mammalian homologue for Ced3 was then discovered as interleukin 1β-converting enzyme protease (ICE) (Nicholson et al., 1995). This led to the identification of related proteases in the mammalian system, which are now called caspases and known to be as central elements of apoptotic machinery in mammals.

Caspases are a class of cysteine proteases having an active site cysteine and cleaving substrates at Asp-x sites. These death proteases are homologous to each other and highly conserved through the evolution. Caspases are synthesized as inactive zymogens, which are composed of three domains such as the N-terminal domain, the p20 and the p10 domain (Earnshaw et al., 1999). Most caspases are activated by proteolytic cleavage of the zymogen between the p20 and the p10 domain and usually also between the prodomain and the p20 domain. Thus, few active caspase molecules can induce the stimulation of many others in a positive feedback mechanism thereby strongly amplifying pro-apoptotic signals. This “caspase cascade” strategy of caspase activation is used by cells for the activation of the down-stream effector caspases. However, “caspase cascade” method of caspase activation does not explain how the first, most upstream initiator caspase, is activated. For example, caspase-8, as the key initiator caspase, becomes activated in response to apoptotic signal induced by death-receptor. Upon

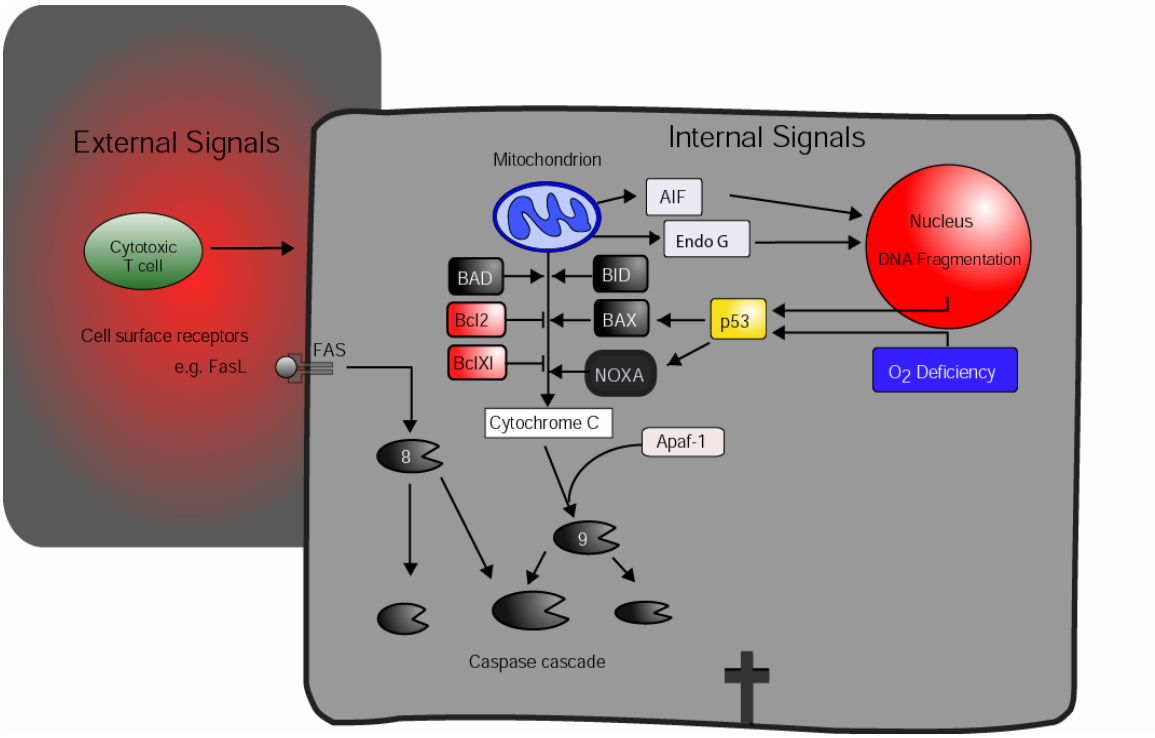


Figure I: Overview of apoptotic pathways in mammalian cells (Courtesy of Dr. W. Hilt).

Apoptosis in mammalian cells is triggered by external (left side of figure) and internal (right side of figure) signals. The external signals are arbitrated by death-receptor pathway or cytotoxic T-cells resulting in caspase-8 activation. The internal signals are mediated by mitochondria or activation of distinct genes (e.g. p53). After receiving a death signal mitochondria release a number of proteins from their intermembrane space such as cytochrome c, apoptosis inducing factor (AIF) and endonuclease G (endoG). This process is regulated by pro- and anti-apoptotic Bcl-2 family members. Released cytochrome c associates with Apaf1 followed by caspase-9 activation. Activated caspase-8 (-9) in turn, activates other caspases, which are responsible for the execution of a cell. [See text for more details].

ligand binding, death receptors such as Fas aggregate and recruit then several molecules of procaspase-8, resulting in an accumulation and, thus, high local concentration of caspase precursor. Under such induced proximity, the low protease activity of procaspase-8 is sufficient to allow the proenzyme molecules to mutually cleave and activate each other (Hengartner, 2000). There is a complex mechanism of caspase activation, which is used to activate another initiator caspase, caspase-9. The activation of caspase-9 is achieved by a mitochondria dependent apoptotic pathway. After receiving a death signal, or if mitochondria loose intactness due to some reason, mitochondria

release a number of proteins from their intermembrane space to the cytosol. These include such molecules as apoptosis inducing factor (AIF), endonuclease G (endo G) and cytochrome c (Li et al., 1997; Lorenzo et al., 1999). Released cytochrome c from mitochondria induces formation of complex made up from Apaf-1, which then recruit caspase-9.

The release of apoptotic effectors from mitochondria is regulated by the balance of anti-apoptotic (Bcl-2 and Bcl-X_L) and pro-apoptotic (Bax, Bad, Bid and Noxa) Bcl-2 family members. Bcl-2 family members have been classified into three functional groups according to the number of functional domains (Antonsson and Martinou, 2000). Anti-apoptotic members like Bcl-2 and Bcl-X_L are made up of four short domains called BH1-BH4 and a C-terminal hydrophobic tail localizing to the outer surface of mitochondria. In contrast, pro-apoptotic proteins (Bax or Bak) have only three functional BH1-BH3 domains and two large α-helices that have been proposed to participate in membrane insertion. The third group of Bcl-2 family members comprises a large variety of proteins, including Bid characterized by a common feature – the presence of a BH3 domain made up from 12-16 amino acids.

Three possible mechanisms of action of Bcl-2 family members were proposed (Gross et al., 1999; Reed, 1997). The first one is based on the structural similarity of Bcl-2. It was suggested that after receiving a death signal, pro-apoptotic members of Bcl-2 family might undergo a conformational change that enables them to integrate into the outer mitochondrial membrane, where they could form channels, through which cytochrome c and other intermembrane proteins can be released. The second mechanism uses one possibility that pro-apoptotic family members recruit other mitochondrial outer membrane proteins, such as voltage-dependent anion channel (VDAC). Here, several Bcl-2 family members can bind to VDAC and regulate its channel activity. Finally, in the third mechanism, it was suggested that Bcl-2 family members induce rupture of the outer mitochondrial membrane, resulting in the release of intermembrane proteins into the cytosol.

Although, many key apoptotic proteins have been identified, functional interplay of these components and molecular pathways of their action await further investigation.

1.6 Apoptotic cell death in the yeast system

1.6.1 Yeast cells undergo apoptosis

Evidence is accumulating that cell death with apoptotic cellular changes also exists in unicellular organisms such as in budding yeast *Saccharomyces cerevisiae*. An apoptosis phenomenon was described for yeast mutants with mutations in *CDC48* gene coding for an AAATP-ase and aging cells (Laun et al., 2001; Madeo et al., 1997). Apoptosis was also observed after heterologous expression of the mammalian apoptosis inducer Bax (Ligr et al., 1998). In an overexpression screen, Stm1 was found to induce apoptotic changes in *S. cerevisiae* (Ligr et al., 2001). Moreover, it was shown that starvation for an essential amino acid triggers cell death in auxothrophic *S. cerevisiae* cells (Eisler et al., 2004). In addition, treatment with weak acetic acid, H_2O_2, salt, UV-irradiation, glutathione-depleting chemicals, mating pheromone or amiodarone induces also apoptosis in yeast cells (del Carratore et al., 2002; Fabrizio et al., 2004; Ludovico et al., 2001; Pozniakovsky et al., 2005; Severin and Hyman, 2002). Dying yeast cells under these conditions display several cytological changes that are characteristic of mammalian apoptosis. These include membrane blebbing and phosphatidylserine externalization on the outer cell membrane, chromatin condensation along the nuclear envelope, nuclear fragmentation, and the degradation of DNA. These yeast cell death-associated changes were suppressed by the exposure of cells to the protein-translation inhibitor cycloheximide, indicating that cell death occurs in a protein-synthesis-dependent manner observed as well in mammalian cells.

Many biologists addressed the question why yeast should undergo apoptosis. They suggest that cells in old yeast cultures are subjected to apoptosis to save nutrients for the survival of the healthiest descendants (Herker et al., 2004). This idea is supported by the findings that yeast cells are dying when kept in minimal medium, but surviving in distilled water, suggesting that in complete absence of nutrients-a condition where prolonged survival of the fittest cells is no advantage-apoptosis is not induced.

1.6.2 Reactive oxygen species (ROS) are key regulators of apoptosis in yeast

Consistently, all apoptotic scenarios in yeast described above, are accompanied by the accumulation of reactive oxygen species (ROS). ROS can be generated by endogenous and exogenous signals and cause significant damage of macromolecules including DNA (Salmon et al., 2004). ROS production normally takes place in mitochondria. Mitochondrial ROS accumulation can lead to induction of cell death, indicating that, as in the mammalian system, ROS are key regulators of yeast apoptosis (Fleury et al., 2002).

Apoptotic cell death accompanied with ROS production can be ameliorated with free-radical scavenging chemicals and by hypoxia (Madeo et al., 1999). Nevertheless, in certain cell lines, apoptosis was shown to occur in almost complete absence of oxygen (Jacobson and Raff, 1995).

1.6.3 Mammalian Bcl-2 proteins are functionally integrated in yeast apoptosis

No Bcl-2 family members were found in the genomic sequences of both budding and fission yeast. Nevertheless, yeast cells can be used as a model to study the function of pro- and anti-apoptotic Bcl-2 proteins. For example, Bax was shown to induce apoptosis in *S. cerevisiae*, whereas the anti-apoptotic factor Bcl-2 opposes this effect (Ligr et al., 1998). Moreover, it was found that yeast cells have homologue to Bcl-2 interacting factor that is the regulator of autophagy-Apg6/Vps30 (Liang et al., 1999).

Striking parallels with mammalian cells have been noted in the mechanisms by which Bax induces apoptosis in yeast (Gross et al., 2000). For example, in both yeast and mammalian cells, Bax causes hyperpolarization of the proton gradient across the inner membrane of mitochondria (in association with matrix alkalinisation and cytosol acidification), triggers cytochrome c release and induces the production of ROS. These events can be followed later by depolarization of mitochondria.

However, important factors of Bcl-2 member induced apoptosis are absent in yeast. For example, in contrast to mammals, yeast cells lack genes encoding certain mitochondrial proteins, such as voltage-dependent anion channel (VDAC) of the outer membrane. Similarly, expression of Bcl-2 in yeast causes increase in the expression of catalase, which is not typical for mammalian cells (Longo et al., 1997).

1.6.4 Caspases in yeast

Caspases are defined as the central executioners of mammalian apoptosis. Recently, a new protein with caspase-like activity has been identified in *S. cerevisiae* (Yor197w) (Madeo et al., 2002b). This protein is implicated in the cell death that is induced by H_2O_2, acetic acid and aging and, therefore, called yeast caspase-1 (Yca1 or Mca1). Yor197w is a member of the metacaspase family. This family comprises putative proteases with a caspase-like fold found in plants, fungi and protists, but not in metazoans (Uren et al., 2000). Overexpression of *YCA1* was shown to induce apoptosis in yeast, whereas deletion of *YCA1* abrogates cell death. Yca1, like mammalian caspases, seems also to undergo proteolytic processing in a manner that is dependent on its active-site cysteine. Futhermore, extracts from *YCA1*-overexpressing cells cleave peptidyl-caspase substrates.

In addition, several studies revealed that apoptotic-like changes in yeast are caspase-dependent. For example, it was shown that apoptosis induced by valproic acid or by virally encoded killer toxins is Yca1 dependent (Mitsui et al., 2005; Reiter et al., 2005). Apoptosis stimulated by the aging process in yeast cells is also mediated by Yca1 (Gourlay et al., 2004; Herker et al., 2004). In addition, Yca1 was shown to be involved in the cell death of *S. cerevisiae* triggered by loss of *UBP10* encoding a deubiquitinating enzyme (Bettiga et al., 2004).

1.6.5 Search for other apoptotic regulators in yeast

Recently, an AIF orthologue named apoptosis-inducing factor1 (Aif1) was found in yeast (Wissing et al., 2004). Mammalian AIF is a flavoprotein with oxidoreductase activity normally localized in the mitochondrial intermembrane space (Lorenzo et al., 1999). During apoptosis, the mammalian AIF protein translocates to the nucleus and degrades DNA. The yeast Aif1 protein shows the same localization and displays similar apoptotic pathways as mammalian AIF. Moreover, *aif1* knock-out cells survive better during chronological aging, whereas overexpression of *AIF1* induces apoptosis in yeast. Aif1 triggered cell death in yeast is partially caspase-dependent.

Yeast cells also encode orthologue of the DNA endonuclease EndoG that is involved in cell death of mammals and *Caenorhabditis elegans* (Ikeda et al., 1996).

Recently, eight apoptotic endo/exo-nuclease candidates have been discovered in *S. cerevisiae*, which have sequence similarities to the apoptotic nucleases in *Caenorhabditis elegans* (Qiu et al., 2005). One of them is Tat-D, whose knockout results in better survival of yeast cells, whereas its overexpression facilitates induction of cell death.

In general, the yeast *S. cerevisiae* is a popular model to study apoptosis because that is not only a great medical relevance. Studies in yeast will also help to understand some of still unknown molecular mechanisms at the core of apoptosis.

2 Materials and methods

2.1 Materials

2.1.1 Media for yeast cultures

Standard yeast rich (YPD), synthetic complete (SC) supplemented either with 2% glucose, raffinose or galactose and necessary amino acids for growth and minimal media (MV) were prepared as described (Ausubel et al., 1995; Rose et al., 1990). (Pre)sporulation media were prepared as in (Guthrie and Fink, 1991). 5-FOA selection medium was made by adding 5-fluoroorotic acid (5-FOA) to SC medium to a final concentration of 1mg/ml. Geneticin (G418) resistant cells were grown on solid YPD containing 200 μg/ml of G-418.

2.1.2 Media for *Escherichia coli* cultures

Standard media for *E. coli* cultures were prepared as described (Sambrook et al., 1989). For growth of the *amp*^{r} bacterial clones, ampicillin was added to a medium to a final concentration of 50 μg/ml.

2.1.3 Chemicals and materials used in this study

Alexis Biochemicals, Lausen, Switzerland	Dihydrorhodamine 123
Amersham Biosciences, Buckinnghamshire, UK	ECL Immunodetection kit Hypobond-N+ Nylon membrane for DNA blots
Bachem, Bubendorf, Switzerland	Peptide substrates Suc-Leu-Leu-Van-Tyr-AMC (51) Cbz-Leu-Leu-Glu-4βNA (53) Cbz-Ala-Arg-Arg-4MβNA (54)
Becton Dickinson (BD) Biosciences, Heidelberg, DE	BD FACSFlow™, BDFACS Clean™ , BD FACSRinse™ , BD Cell Wash (PBS), BD Calibrite beads
BioRad, München, DE	Prestained (Protein) Standard marker

Calbiochem, Schwalbach, DE	Nocodazole
Merck, Darmstadt, DE	TEMED, APS, SDS, PMSF, KAISERS Gelatine
MWG Biotech, Ebersberg or Metabion, Martinsried, DE	Oligonucleotides
New England Biolabs, Hitchin, UK	Restriction enzymes and DNA polymerases
Pall Gelman, Rossdorf, DE	Nitrocellulose
Perkin Elmer Inc, Boston, USA	Glusulase
Promega, Madison, USA	Hering Sperm DNA, CaspACE™ FITC-VAD-FMK In Situ Marker
Quiagen, Hilden, DE	Plasmid prep, PCR clean and Gel extraction kit
Roth, Karsruhe, DE	Solvents, Agarose
Roche Diagnostics, Mannheim, DE	Nucleotides, Restriction enzymes, T4 DNA Ligase, T4 DNA polymerase, Complete ™ Inhibitor-Cocktail, RNA-ase, DNA-ladder standards, Lumi-light Western blotting substrate, TUNEL reaction and DAB substrate, Ampicilin, Complete protease inhibitor and Taq DNA polymerase
Schärfe System GmbH, Reutlingen, DE	CASYton, CASYClean solutions, CASYcups with caps
Serva, Heidelberg, DE	Coomassie Brilliant Blue G250 and Bromophenol blue
Sigma, Deisenhofen, DE	All other chemicals if not otherwise indicated
Toronto Research chemicals, Northyork, Canada	5-FOA

2.1.4 Antibodies used in this study

Source	Antibody	Dilutions	Buffer
Babco, Denver, PA, DE	anti-HA MMS-101p monoclonal antibody anti-HA antibody coupled to the affinity matrix	1 : 5000	T-TBST
	anti-MYC 9E10 monoclonal AB; anti-MYC 9E10 antibody coupled to the affinity matrix	1: 2000	T-TBST
Molecular Probes, Breda, the Netherlands	anti-CPY monoclonal antibody	1: 10000	T-TBST
Dianova, Hamburg, DE	AffiniPure Goat anti-Mouse IgG-HRPO monoclonal antibody	1:10000 +1% non-fat milk	T-TBST

where: T-TBST: Tris-buffered saline with 1% Tween20

2.1.5 Instruments used in this study

Source	Instrument
BD, Heidelberg, DE	BD FACS Calibur
BioRad, München, DE	Gel electrophoresis (Polyacrylamid Agarose) and Blotting apparatus
Midton Acrylics Ltd., Scotland, UK	Vacugene-vacuum blotting unit
Fröbel, Lindau, DE	Semi-dry blotting system
Jasco, Gross Umstadt, DE	Photometer V530
Pharmacia, Freiburg, DE	Gelelectrophoresis camera
Stratagene, La Jolla, USA	Robocycler Gradient 40 PCR grade
Nikon, Tokyo, Japan	Micromanipulator

Eppendorf, Hamburg, DE	Centrifuges T-5415
Kühner, Basel, Switzerland	Labshakers for liquid cultures
Zeiss, Oberkochen, DE	(Fluorescent-) Microscope

2.1.6 Yeast strains used in this study

Strains	Genotype	Source
WCG4a	Mat a *leu2-3,112 ura3 his3-11,15 Can^s GAL+*	W. Heinemeier
WCG4α	Mat α *leu2-3,112 ura3 his3-11,15 Can^s GAL+*	W. Heinemeier
WCG4a/α	Mat a/Mat α	W. Hilt
YHi29/14 a	Mat a *pre1-1 pre4-1*	W. Hilt
YHi30/14 α	Mat α *pre1-1 pre4-1*	W. Hilt
YJI001	Mat a *yca1Δ::KanMX4*	This study
YJI002	Mat α *yca1Δ::KanMX4*	This study
YJI003	Mat α *pre1-1 pre4-1 yca1Δ::KanMX4*	This study
YJI004	Mat a *pre4-1*	This study
YJI005	Mat a *pre4-1 yca1Δ::KanMX4*	This study
YJI006	Mat a *pre1-1*	This study
YJI007	Mat a *pre1-1 yca1Δ::KanMX4*	This study
YP4[a]	Mat a *TEL::URA3*	S.P. Jackson
YJI010[a]	Mat a *stm1Δ::KanMX4 TEL::URA3*	This study
TEL::ADE2[a]	Mat a *TEL::ADE2*	S.P. Jackson
YJI012[a]	Mat a *stm1Δ::KanMX4 TEL::ADE2*	This study
UCC5706[b]	*Mat a-inc*	D.E. Gottschling
YJI015[b]	Mat a *stm1Δ::KanMX4*	This study
YJI017	Mat α *STM1-HA$_3$*	This study
YR312	Mat a *his1-123*	Mat a-Tester strain
YR320	Mat α *his1-123*	Mat α-Tester strain
YMMF21/36[a]	Mat a *MEC3-MYC$_9$*	M. Muzi-Falconi
YJI027	Mat a *STM1-HA$_3$ MEC3-MYC$_9$*	This study
YLR288c	Mat a/α *mec3Δ::KanMX4*	Euroscarf

BY4741[c]	Mat a	Euroscarf
YJI030[c]	Mat a *mec3Δ::KanMX4*	This study
YJI036[c]	Mat a *yca1Δ::KanMX4*	This study
YL267	Mat a *stm1Δ::KanMX4*	M. Ligr
YIV002	Mat a *stm1Δ::KanMX4*	I. Velten
YJI040	Mat a *stm1Δ::KanMX4 yca1 Δ::KanMX4*	This study
YJI043	Mat a *yca1Δ::KanMX4 aif1Δ::KanMX4*	This study
YJI045	Mat a *aif1Δ::KanMX4*	This study
YJI046[c]	Mat a *aif1Δ::KanMX4*	This study
YJI048[c]	Mat a *yca1Δ::KanMX4 aif1Δ::KanMX4*	This study
YJI049[c]	Mat a *[GAL1]*	This study
YJI050[c]	Mat a *[GAL1::STM1]*	This study
YJI051[c]	Mat a *[GAL1::stm1-9]*	This study
YJI052[c]	Mat a *yca1Δ::KanMX4 [GAL1::stm1-9]*	This study
YJI053[c]	Mat a *aif1Δ::KanMX4 [GAL1 ::stm1-9]*	This study
YJI054[c]	Mat a *yca1Δ::KanMX4aif1Δ::KanMX4[GAL1::stm1-9]*	This study
YJI065	Mat a *[GAL1]*	This study
YJI067	Mat a *[GAL1::STM1]*	This study
YJI069	Mat a *[GAL1::stm1-9]*	This study
YJI071	Mat a *yca1Δ::KanMX4 [GAL1::stm1-9]*	This study
YJI073	Mat a *aif1Δ::KanMX4 [GAL1 ::stm1-9]*	This study
YJI074	Mat a *yca1Δ::KanMX4aif1Δ::KanMX4[GAL1::stm1-9]*	This study
YJI079	Mat α *pre1-1 pre4-1 [GAL1]*	This study
YJI080	Mat α *pre1-1 pre4-1 [GAL1::STM1]*	This study
YJI081	Mat α *pre1-1 pre4-1 [GAL1::stm1-9]*	This study
YJI082	Mat α *pre1-1 pre4-1yca1Δ::KanMX4 [GAL1::stm1-9]*	This study
YJI085	Mat a *pre1-1 pre4-1 [GAL1]*	This study
YJI086	Mat a *pre1-1 pre4-1 [GAL1::STM1]*	This study
YJI087	Mat a *pre1-1 pre4-1 [GAL1::stm1-9]*	This study
YJI091[c]	Mat a *[GAL1::stm1-9-HA$_2$]*	This study
YJI092	Mat a *[GAL1::stm1-9-HA$_2$]*	This study

YJI093[c]	Mat a *[GAL1::STM1-HA$_2$]*	This study
YJI094	Mat a *[GAL1::STM1-HA$_2$]*	This study
YJI095	Mat a *pre1-1 pre4-1 stm1Δ::KanMX4*	This study

Table 1: Yeast strains used in this study.

[a]: isogenic with the strain background W303: *ade2-1 leu2,3-112, his3-11,15 trp1-1 ura3-1 can1-100*; [b]: isogenic with the strain background UCC5706: *Mat a-inc ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1:LEU2-GALHO VII-L::ADE2-TG-HO site-LYS2 rad52::hisG*; [c]: isogenic with the strain background BY4741: *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0.* The rest of the yeast strains are isogenic with WCG4: *his3-11,15 leu2-3,112 ura3 Can^s Gal$^+$*.

2.1.7 Plasmid vectors and constructs used in this study

Plasmid	Insert details	Source
pYES2	*2μ URA3 GAL1* vector for yeast and E.coli (*amp^r*)	Invitrogen
pBTM116	*TRP1* vector used for NHEJ repair experiment	S.P. Jackson
pRS415	*LEU2* vector used for NHEJ repair experiment	S.P. Jackson
BR52	*URA3* vector used for Mat type switching	H. Rudolph
pUG6	*Kan^r* used as matrix for *KAN* deletion cassette	U. Guldener
pJI001[a]	*[GAL1::stm1-9]*	This study
92,4[a]	*[GAL1::STM1]*	M. Ligr
61,1[a]	*[GAL1::NSR1]*	M. Ligr
207,4[a]	*[GAL1::PPA1]*	M. Ligr
46,1[a]	*[GAL1::SAR1]*	M. Ligr
45,1[a]	*[GAL1::YNL208w]*	M. Ligr
31,1[a]	*[GAL1::YOR309c]*	M. Ligr
pL001	*[GAL1::HA$_2$TAG]*	M. Ligr

pJI020[b]	*[GAL1::stm1-9-HA$_2$]*	This study
pJI021[b]	*[GAL1::STM1-HA$_2$]*	This study

Table 2: Plasmid vectors and constructs used in this study. [a]: pYES2 derived plasmid constructs; [b]: pL001 derived plasmid constructs.

2.2 Methods

2.2.1 Molecular biological methods

PCR fragments were generated using standard conditions as described (White, 1993). Recombinant DNA techniques for generation of yeast strains or plasmid constructs, transformation of plasmids into bacterial or yeast cells were done as described (Ausubel et al., 1995; Guthrie and Fink, 1991; Sambrook et al., 1989). The new constructs were verified by sequencing (GATC, Konstanz, DE).

2.2.1.1 Gene disruption

The *ORFs* of *STM1*, *YCA1* and *AIF1* genes were disrupted using PCR fragment containing kanamycin resistance gene as a selection marker (Guldener et al., 1996). PCR was performed using plasmid pUG6 as a template and primers to amplify the kanamycin cassette flanked by 40 bp corresponding to immediate down- and up-stream region of the ORFs of the desired genes. Yeast cells were transformed with the PCR products and integrants were selected on YPD plates containing geneticin G-418. Correct integrations of the kanamycin cassette were checked by Southern blot analysis using kanamycin cassette as a probe, which was amplified using pUG6 as a template.

2.2.1.2 Southern blot analysis

To verify the correct integration of PCR fragment into chromosomal yeast DNA, which was used to generate the null yeast mutants, Southern blot analysis was applied. The

chromosomal DNA was isolated from potential yeast candidate strains and digested with appropriate restriction enzymes. The digested DNA fragments were separated by gel-electrophoresis. A vacuum blotting unit (Midton acrylics Ltd.) was used to transfer the separated DNA fragments from the agarose gel to a Nylon membrane (Hybond-N+, Amersham). The gel was soaked in depurination (250mM HCl) solution for 10min, then in denaturation (1.5M NaCl and 500mM NaOH) solution and neutralisation (3M NaCl and 50mM Tris/HCl) solution for each 15 min. 20×SSC buffer (3M NaCl and 0.3M Na_3 citrate pH 7.0) was used to transfer the DNA fragments from the gel to the membrane for 2h. After the transferring DNA procedure, the membrane was exposed to UV light for 5min. The Hybridization and detection of the desired DNA bands using the fluorescein-labelled probe was carried out according to the manufacture's instruction protocol (Amersham-Fluorescein gene images labelling and detection system, RPN 3340).

2.2.2 Cell biological methods

2.2.2.1 Telomeric silencing assay

The effect of *STM1* deletion on telomeric silencing was analysed using strain YP4a (provided by S.P. Jackson) containing *URA3* gene inserted into the subtelomeric *ADH4* site of chromosome VII. The drops of 10-fold serial diluted cultures were put on 5-FOA, SC-URA^- and YPD plates and incubated at 25°C.

2.2.2.2 Telomere addition assay

The effect of *STM1* on the telomere addition *in vivo* was analyzed using strain UCC5706a (provided by D.E. Gottschling) with a 6 kbp fragment consisting of the *ADE2* gene, 81 bp of $TG_{1\text{-}3}$ sequence and the recognition site for the *HO* endonuclease. The *LYS2* gene was placed approximately 10 kbp from both the natural telomere of VII-L and the *ADH4* locus and serves as a genetic marker to monitor *HO* cutting. The *ORF* of *STM1* was disrupted using the strain represented above. Cells were pre-grown in the synthetic complete media lacking lysine supplemented with 2% glucose, diluted and further grown

in the rich medium (YP) containing 2% raffinose for three or four generations, arrested in M phase using spindle depolimerizing drug (nocodazole) until more than 90% of cells showed the appropriate cell morphology. Cells were centrifuged and resuspended then in pre-warmed rich (YP) medium with 2% galactose to induce *HO* cutting. The samples were taken every 60 min, following by the isolation of the total amount of the genomic DNA. The cut by Spe I appropriate amounts of DNA were run on a 1.2% agarose gel and subjected to the Southern blot procedure. Southern blot was probed with the part of *ADE2* (563bp), which was made by PCR using the total amount of DNA isolated from the strain UCC5706a as a template and the primers 5'ADE2 5'-ATTTACAGTTTT-GATATCTTGGC-3' and 3' ADE2 5'-TTCTAATGTAGATTCTTGTTGTTCG-3'.

2.2.2.3 The non-homologous end joining (NHEJ) of double strand breaks or yeast plasmid repair assay

The role of Stm1 in the NHEJ mechanism was checked by the yeast plasmid repair assay as described in Boulton et al. with modifications. Undigested plasmid DNA or an equivalent amount of plasmid DNA that had been digested to completion with the appropriate restriction enzymes (pBTM116 or pRS415 bearing various types of complementary 5' and 3' overhanging DNA ends) was transformed into *stm1Δ* mutant or corresponding wild type cells under standard conditions. Transformants were then plated as serial dilutions onto appropriate selective media and colonies were counted after incubation of plates at 30ºC for 2 days. The used digestion procedure was as follows: 8µl plasmid DNA (pBTM116 or pRS415), 8µl buffer H, 64µl H_20 and 0.5µl enzyme (Eco RI, Xho I or Pst I) were carefully mixed, digested for 1h at 37ºC. The equal amount of each plasmid (5-10µl) was used for detection of complete digestion by gel-electrophoresis and the rest was used for transformations.

2.2.2.4 Growth conditions used for apoptotic markers detection and for induction of expression

All strains were grown in liquid synthetic complete (SC) medium supplemented with all necessary amino acids for growth but without uracil and with 2% raffinose till early

logarithmical phase (OD_{600} 0.3) followed by centrifugation and resuspended then (without washing) into the same volume of liquid SC medium containing the same amino acids but 2% galactose instead of raffinose. Strains were then shaken at 30°C for 26h, 40h or 45h. It was important that all strains had approximately the same OD_{600} before and after galactose shift. The harvested cells after the indicated time of induction were fixed for apoptosis tests.

2.2.2.5 Survival plating assay

For all survival platings an aliquot of culture was counted with the CASY1 system (Schärfe System), diluted 1:10000 in distilled water and 500 cells were plated on YPD. The number of colonies was determined after 2 days of growth at 30ºC.

2.2.2.6 FACS analysis

To determine the apoptotic markers using FACS analysis, 10^6 cells were harvested after defined time of *GAL1* induction, washed once in 1 ml PBS. To determine cell viability, cells were treated with DiBAC4 (0.1µg/ml) as described in Willetts et al., 1997; for *in vivo* FITC staining, cells were incubated with FITC-VAD-FMK staining solution (dilution 1:1000) as described in Madeo et al., 2002b. ROS production levels were measured by incubating cells with dihydrorhodamine123 (DHR123) (10 mg/ml) for 2h at 30°C. The stained cells were washed twice with PBS and analyzed by FACS (BD Biosciences) using CellQuest analysis software with excitation and emission settings of 488nm and 525-550nm (filter FL1), respectively. For detection of plasma membrane integrity, cells were briefly treated with propidium iodide (5 µg/ml) and subjected to FACS analysis (FL2).

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

Fragmented nuclear DNA in yeast was detected using TUNEL assay as described in Madeo et al., 1997 with modifications for cell wall digestion. For all cultures, 120 U lyticase and 75µl β-glucuronidase/arylsulfatase per ml cell suspension were used and cells were incubated for 2h at 30 ºC. The protoplasts were then applied to polylysine-coated slides. The In Situ Cell Death Detection Kit POD was used according to the manufactures instructions. After mounting a coverslip with a drop of Kaiser's glycerol gelatine cells were examined using phase contrast microscope with Ph2 Neofluar 40.

2.2.2.8 Cytochrome c release assay

The isolation of yeast mitochondria and detection of cytochrome c by immunobloting were done as described in Ludovico et al., 2002.

2.2.3 Biochemical methods

2.2.3.1 Co-Immunoprecipitation

Cells were grown at 30ºC on YPD medium till OD_{600} of around 1.0, washed once with the ice-cold water, centrifuged at 13000g for 1 min and resuspended into 0.4 ml yeast lysis buffer (50mM Tris/HCl, 5mM EDTA, 150mM NaCl, 1% (v/v) Triton X-100) with 0.4 g glass beads. Cells were lysed in a bead beater for 30 min and cell debris was centrifuged for 10 min at 13000g at 4°C. Protein supernatant fractions were incubated with the 15 µl of the appropriate antibodies already coupled with the protein A sepharose beads for 3h in an overhead shaker at 4ºC. Beads together with precipitated material were washed with PBS for four times, and proteins were resuspended with 30µl Laemmli buffer (50mM Tris/HCl pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol and 100mM β-mercaptoethanol), heated at 95°C for 15min and subjected to the Western blot.

2.2.3.2 Promoter shutt-off and cycloheximide chase analysis

Strains expressing plasmid encoded HA-tagged *STM1* under the control of *GAL1* promoter were grown in synthetic SC medium supplemented with all necessary amino acids for growth except uracil with 2% raffinose until the mid-logarithmic phase (OD_{600} 0.5-0.7) at 30ºC, centrifuged and resuspended (no washing) into SC containing the same amount of amino acids but 2% galactose. The strains were grown for 2h and glucose and cycloheximide were added to the final concentrations of 2% and 0.5 mg/ml, respectively. Cells (5 OD_{600} units) were harvested and lysed in 0.25 M NaOH and 1% β-mercaptoethanol. Proteins were precipitated with 5.8% trichloracetic acid. Dry pellets were resuspended in the Laemmli buffer (see above) and proteins were subjected to SDS-PAGE and immunoblotted using standard protocol.

2.2.3.3 Immunoblotting of proteins (Western blot)

Proteins were separated by SDS-PAGE (Laemmli, 1970). Protein samples were electro-transferred onto nitrocellulose membrane (Towbin et al., 1979) by using a semi-dry blotting system. Protein antigens on the membranes were probed with appropriate antibodies. The immunodetection procedure was carried out according to the manufacturer's protocol (ECL-Kit, Amersham Biosciences).

3 Results

3.1 Stm1 is not involved in telomeric silencing

Several studies revealed the involvement of Stm1 in the diverse cellular functions ranging from cell cycle to apoptosis (Ligr et al., 2001; van Dyke, 2004). The localization of Stm1 at the nuclear periphery (Ligr et al., 2001) and its specific affinity for guanine-rich quadruplex DNA (Frantz and Gilbert, 1995) suggested the possible involvement of Stm1 in telomeric silencing. This effect is based on a specific organization of silent telomeric chromatin leading to transcriptional repression of genes located nearby to the nuclear periphery. It was shown that deletion of some nuclear pore components cause release of telomeres from their perinuclear localization followed by de-repression of telomeric silencing (Galy et al., 2000). To analyze, whether Stm1 plays a role in the above function, an assay indicating the level of silencing at the telomere was applied. For this assay the strain YP4 (*TEL::URA3*) (kindly provided by S. Jackson) containing the *URA3* gene adjacent to the telomere of chromosome VII-L was used as a tester strain. Due to telomeric silencing, expression of the *URA3* gene is repressed making these tester cells resistant to the drug 5-FOA; if the *URA3* gene product is expressed, it converts 5-FOA to 5-fluorouracil, which (by inhibition of thymidilate-synthetase) is toxic for the cells. Therefore, in this strain, resistance to 5–FOA is a measure of telomeric silencing. To check whether Stm1 is involved in transcriptional repression of the *URA3* gene, 5-FOA resistance of *stm1* null mutant (strain YJI010) obtained by disruption of *STM1* inYP4 (*TEL::URA3*) strain was inspected for growth on 5-FOA medium (Fig. 1A). *TEL::URA3* cells containing *stm1* deletion (strain YJI010) and their corresponding wild type cells (YP4) were spotted as serial dilutions on solid YPD medium, synthetic complete medium lacking uracil (SC-URA$^-$) and SC containing 5-FOA medium and grown at 25°C for 3 days. No growth differences were found between the *TEL::URA3 stm1Δ* strain (strain YJI010) and the corresponding wild type strain YP4 on any of the given above media.

To confirm these findings, another strain containing *ADE2* as a reporter of telomeric position effect *TEL::ADE2* (a gift from S. Jackson) was used. Expression of the *ADE2* gene can be visualized at the clonal level by a colony color assay (Gottschling et al., 1990).

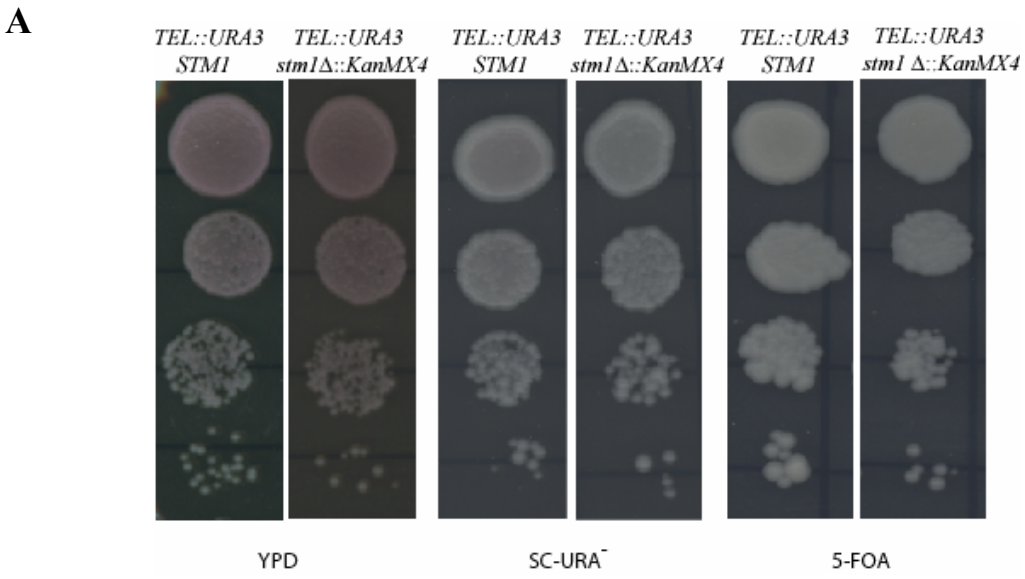


Figure 1: Stm1 is not involved in telomeric silencing.

A. An assay showing the telomeric silencing effect was determined in the strain YJI010 (*TEL::URA3 stm1Δ*) and its corresponding wild type (YP4a) containing *URA3* in the subtelomeric region of the chromosome VII. Cells were spotted as serial dilutions on the YPD, SC-URA⁻ and 5-FOA plates and grown at 25°C for 3 days.

B. The strain YJI012 (*TEL::ADE2 stm1Δ*) bearing *ADE2* at the telomeric region of chromosome VII and its wild type counterpart (*TEL::ADE2*) were streaked on YPD plate and grown at 30°C for 3 days.

When the *ADE2* gene is expressed, the resulting colonies are white, whereas its repression leads to accumulation of a red intermediate product in the adenine biosynthesis pathway and, thus the formation of red/pink colonies. To check, whether Stm1 is involved in the *ADE2* gene repression, the strain YJI012 harbouring a deletion of *STM1* (obtained by disruption of *STM1* in the *TEL::*ADE2 tester strain) and its corresponding wild type strain *TEL::ADE2* were streaked onto solid YPD medium and grown at 30°C

for 3 days. However, the strain carrying *stm1* deletion in combination with *TEL::ADE2* (strain YJI012) did not show any differences in the colony color when compared to the tester strain (Fig. 1B). Taken together the results strongly indicate that Stm1 is not involved in telomeric silencing.

3.2 Chromosomal cleavage by HO endonuclease is accelerated in *stm1Δ* cells

The above results may exclude that Stm1 has a role in telomeric silencing. Due to its interaction with Cdc13, which was shown as a regulator of telomere replication (Hayashi and Murakami, 2002), Stm1 be expected to provide an alternative function in the regulation of the telomeres as for instance telomeric elongation and, thus, the control of telomere repeat sequences at the ends of chromosomes. To clarify this, an *in vivo* telomere elongation assay was applied to visualize a possible effect of Stm1 in this process. This assay is based on the addition of telomeric sequence by telomerase *in vivo* activity onto a *de novo* generated telomere created by endonuclease cleavage (Diede and Gottsschling, 1999). The haploid tester strain UCC5706 (kindly provided by D. Gottschling) contains a construct consisting of the *ADE2* gene, 81 bp of TG_{1-3} repeats representing telomeric sequence, and a recognition site for the HO endonuclease, which is inserted into the *ADH4*, 20 kbp distant from the left telomere of chromosome VII (14910-16307bp). All genes distal to this construct are nonessential. The *LYS2* gene located in this region serves as an auxotrophic marker for the loss of sequences distal to the *HO* site. This strain, in addition, carries the *HO* endonuclease gene under the control of a galactose-inducible promoter and, to avoid the mating type switching, the *MAT*a-*inc* allele containing a point mutation in the *HO* recognition site at the *MAT* locus. If double strand breaks are induced at the telomeric TG_{1-3} repeats by HO endonuclease, the newly formed DNA ends recognized as a telomere and are, therefore, extended by telomerase-dependent elongation. Because telomerase activity seems to appear in M-phase of the cell cycle, the strain YIJ015 containing *stm1Δ* (obtained by disruption of *STM1* in the tester UCC5706 strain) and its corresponding UCC5706 wild type were arrested and held in M phase using the spindle depolymerizing drug nocodazole. Elongation of the telomeric repeats was then visualized using Southern blot analysis with a fragment from

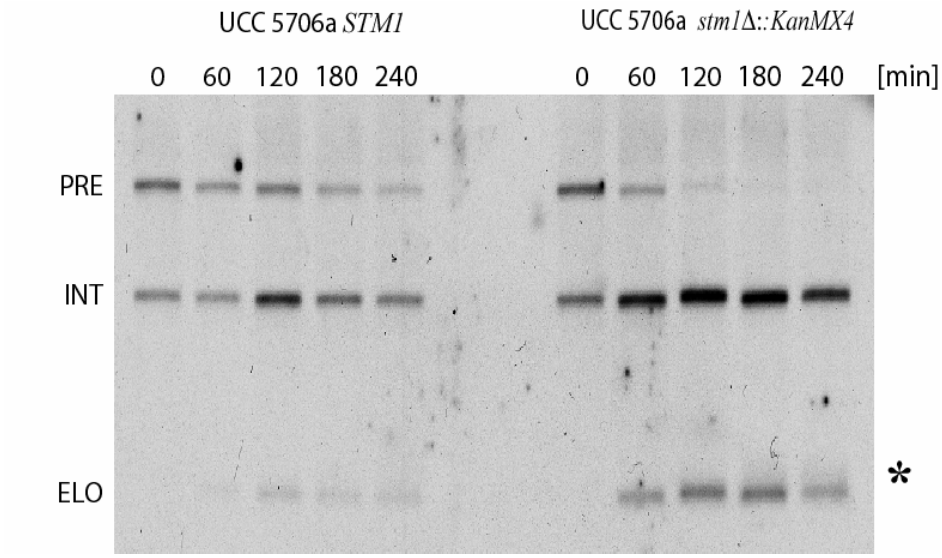


Figure 2: Chromosomal cleavage by HO endonuclease is accelerated in *stm1Δ* cells.

Southern blot analysis indicates the efficiency of HO endonuclease cleavage. Cells (UCC5706a *STM1* and YJI015 *stm1Δ*) were grown in liquid YPD raffinose medium, arrested in M phase using the drug nocodazole and induced to express HO endonuclease by shifting the cells into YPD galactose medium. After HO induction the cells were collected every 60 min. The total genomic DNA was digested by Spe I and separated by electrophoresis. The DNA was transferred to nitrocellulose membrane and probed with a labeled fragment of the *ADE2* gene (564476bp -566191bp). The 3 kbp Spe I fragment from the construct on chromosome VII-L consisting of *ADE2*, 81bp of TG_{1-3} telomeric sequence and a recognition site for HO endonuclease, is shown as PRE. Upon the chromosomal cleavage by HO endonuclease, the band PRE is converted into a 0.72kbp fragment (indicated as ELO). The 1.6 kbp Spe I fragment (INT) from the *ade2-101* locus detected by the *ADE2* probe served as a loading control. Appearance of higher molecular weight forms indicated by asterisk evidences that telomeric elongation takes place in *stm1* null mutant (YJI015).

the *ADE2* region as the probe. The strain YJI015 harbouring the deletion of *STM1* showed similar telomerase activity as the tester strain UCC5706 (Fig. 2). However, surprisingly, the telomeric cleavage appeared to be accelerated in the strain YJI015 carrying the *stm1Δ* mutation than in the wild type (strain UCC5706). These results indicate that telomere elongation is independent of Stm1 but that chromosomal cleavage by HO endonuclease is accelerated in the absence of Stm1.

3.3 Stm1 does not contribute to non-homologous end joining of double strand breaks (NHEJ)

stm1Δ mutants were shown to be sensitive to DNA damaging agents, particularly to bleomycine damage leading to induction of double-strand breaks (Ligr et al., 2001). These results led to the idea that Stm1 might contribute to DNA repair. In haploid yeast cells non-homogolous end joining (NHEJ) is the prominent mechanism in repair of DNA double strand breaks (Critchlow and Jackson, 1998; Lieber, 1999). This type of rejoining does not require homologies between two recombining molecules, such as DNA telomeric ends, where the broken ends are modified to make them compatible prior to rejoining.

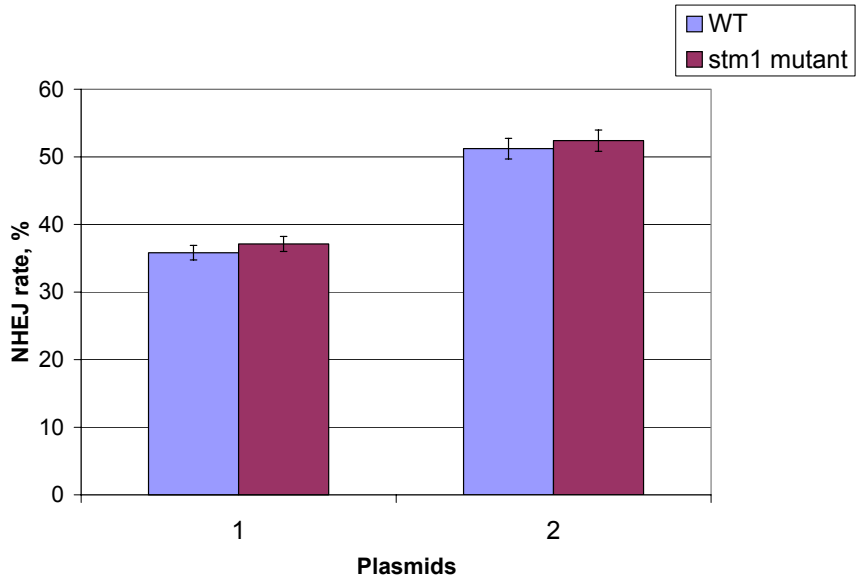


Figure 3: Stm1 does not contribute to non-homologous end joining of double strand breaks (NHEJ).

The measure of *in vivo* NHEJ activity is shown for the *stm1Δ* mutant (YL267) and the wild type strain (WCG4a). The number of transformants obtained with linearized plasmids (pRS415 [**bar 1**] cut with Xho I or pBTM116 [**bar 2**] cut with Eco RI) was compared to the number of transformants received with respective uncut plasmids in each of the indicated strains.

To examine the potential role of Stm1 in NHEJ, an *in vivo* plasmid-rejoining assay was applied (Boulton and Jackson, 1996). In this assay, a measure of *in vivo* NHEJ activity is scored as the number of transformants obtained with linearized plasmid DNA

compared to the number of transformants received with uncut plasmid DNA. For this, restriction endonucleases (e.g. Eco RI or Xho I) were used to produce double strand breaks within the regions of the tester plasmids (pBTM116 or pRS415) that are not homologous to chromosomal sequences. The linearized plasmids were introduced into *stm1Δ* mutants in either YL267 (WCG4a *stm1Δ*) or YJI010 (*TEL::URA3 stm1Δ)* backgrounds and into corresponding wild type cells, respectively. To control the transformation efficiencies, uncut versions of the respective plasmids were introduced into the indicated above yeast strains by transformation. The number of transformants, grown on selective media for 3-4 days at 30°C, was obtained by counting the number of colonies. In these experiments, the *stm1Δ* mutants in the different strain backgrounds (YL267 orYJI010) did not show any alteration of transformation rates and, thus, no differences in the rate of non-homologous end rejoining when compared with wild type counterparts (Fig. 3). These data could exclude a possible role of Stm1 in the NHEJ mechanism.

3.4 Isolation of a mutant version of *stm1* whose overexpression induces cell lethality in wild type cells

Apoptosis-like cell death was found to exist in the yeast cells as proven by the appearance of cellular morphological alterations typically found in apoptosis of higher eukaryotes (Frohlich and Madeo, 2000; Madeo et al., 1997). The endogenous *S. cerevisiae* Stm1 was uncovered to contribute to apoptosis in yeast cells. Overexpression of *STM1* was shown to induce an apoptotic-like cell death in cells that are defective in proteasomal degradation due to presence of the *pre1-1 pre4-1* mutations (Ligr et al., 2001). It is known that the ubiquitin-proteasome system regulates a large variety of cellular processes by negatively controlling the levels of defined target proteins (Hilt et al., 1993). Therefore, any major defects in proteasomal function will lead to the alteration of concentration or activity of a broad range of substrates including some that might interfere with Stm1 function. To avoid such side effect, availability of a mutated version of *stm1* that shows the lethal effect even when overexpressed in cells with normal proteasomal activity was analyzed. It was hypothesized, that such *stm1* version may

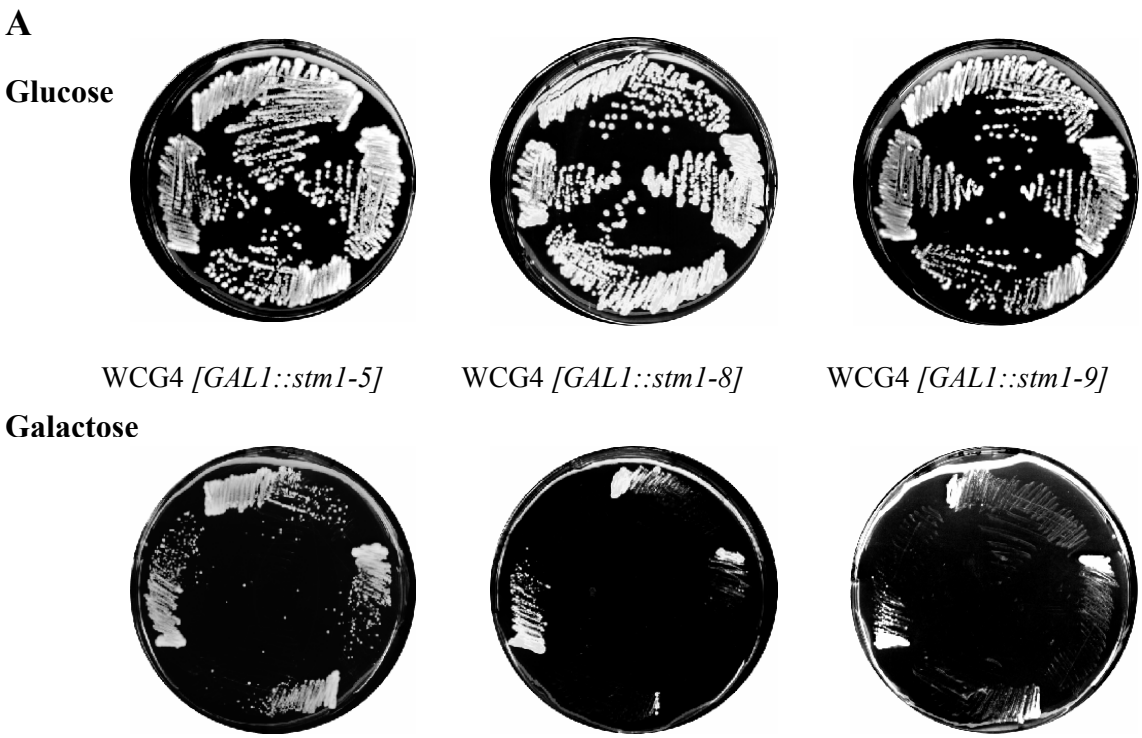


Figure 4: Overexpression of a mutant version of *stm1* in wild type cells leads to significant growth defect.

A. The effect of overexpression of different *stm1* mutant versions (*stm1-5*, *stm1-8* or *stm1-9*) under the *GAL1* promoter on growth of wild type cells (WCG4) is compared on solid SC-URA$^-$ medium with galactose (for induction of *stm1*) or glucose (for repression of *stm1*) medium. The plates were incubated at 30ºC for 3 days.

B. Wild type cells (BY4741a) show severe growth defects upon *stm1-9* overexpression. BY4741a wild type cells bearing plasmid encoding *stm1-9* allele (pJI001) were grown on solid SC-URA$^-$ galactose (for induction of *stm1-9*) or on solid SC-URA$^-$ glucose (for repression of *stm1-9*) at 30 ºC for 3 days. For control, the growth rate of the wild type cells carrying *STM1* wild type [*GAL1::stm1-9*] or empty vector [*GAL1*] (see SC-URA$^-$ galactose) is presented.

show hyperactivity after overexpression due to increased proteolytic stability. *stm1* versions with the expected effect were created by using mutagenic PCR. The mutant alleles were inserted into the high copy plasmid pYES2 under the control of the *GAL1* promoter. The constructs were then introduced into wild type yeast strains with WCG4a or BY4741a background by transformation. Strains carrying mutant *stm1* constructs were grown on solid SC medium lacking uracil (SC-URA$^-$) supplemented either with glucose (for repression of *stm1*) or with galactose (for induction of *stm1*) and grown at 30ºC for 3 days. When compared with *STM1* wild type (plasmid 92,4), several *stm1* mutant versions indeed showed an increased inhibitory growth effect when overexpressed in wild type backgrounds (Fig. 4A). The strongest effect was found with the allele *stm1-9* (plasmid pJI001) (Fig. 4B). Sequencing of this allele uncovered a single mutation at position 644 leading to a Glu – Val exchange.

3.5 Overproduction of Stm1-9 in wild type cells leads to apoptotic cell death

Overexpression of *stm1-9* caused a significant growth defect and most probably cell lethality in wild type cells (see above). Therefore, it was tested whether *stm1-9* induced a cell death in wild type background (WCG4a or BY4741a), which is indeed of apoptotic nature. For this purpose, several subcellular markers indicating apoptosis were examined.

Wild type cells carrying plasmid encoded either the *stm1-9* mutant allele (pJI001) or the *STM1* wild type allele (92,4) both under the control of the *GAL1* promoter were grown in liquid SC-URA$^-$ medium containing raffinose to low cell density. The expression of *stm1-9* and *STM1* wild type was induced then in liquid galactose medium. For each analysis, empty vector (pYES2) was used as a control. At defined time points after induction, cells were plated to check the survival rate, or to test on apoptotic markers such as cell viability assays, FITC-VAD-FMK staining accompanied with propidium iodide staining, tests on DNA fragmentation (TUNEL) and cytochrome c release as well as detection of reactive oxygen species produced (see below).

3.5.1 Overexpression of *stm1-9* in wild type cells leads to enhanced lethality

First, *stm1-9*-induced cell death was tested by checking cell survival using a plating assay. In this assay, the strains (WCG4a and BY4741a) overproducing mutated Stm1-9 (plasmid pJI001) or wild type Stm1 protein (plasmid 92,4) were grown in liquid SC-URA$^-$ galactose medium for 4 days at 30°C. Cell number in aliquots of the cultures was counted at different time points before plating on YPD medium. The number of colonies was determined after 2 days of incubation at 30°C. Notably, the wild type strain BY4741a expressing *stm1-9* (YJI051) showed significantly reduced survival rate starting from 30h of *stm1-9* induction. This effect was enhanced in the wild type strain WCG4a (YJI069). Here, overexpression of *stm1-9* dramatically reduced cell survival to a rate of 13% of surviving cells starting from 45h of *stm1-9* induction, when compared to respective strains (BY4741a and WCG4a) that are carrying empty vector as a control or wild type *STM1* (Fig. 5A / B).

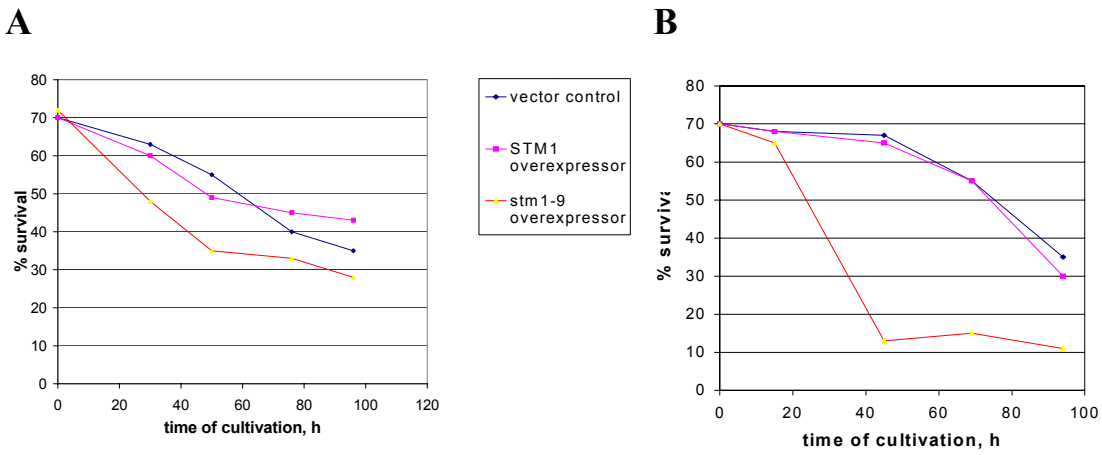


Figure 5: Overexpression of *stm-9* allele in wild type cells leads to reduced survival rate.

The graphs indicate cell survival rate of wild type cells of the strain BY4741a (**A**) or the strain WCG4a (**B**) overexpressing *stm1-9* allele, *STM1* wild type gene or containing empty vector as a control. The cells were grown in liquid SC-URA$^-$ raffinose medium until low cell density. Expression of *GAL1* controlled genes (*stm1-9*, *STM1*) was then induced in liquid SC-URA$^-$ galactose medium (0-time point). After induction, equal number of cells was plated at different time points. Number of colonies formed on YPD plates was counted and compared with the number of viable cells obtained at 0-time point.

To examine the influence of *stm1-9* overexpression on cell viability, another approach was applied as described in Willetts et al., 1997. For this purpose, cells that are

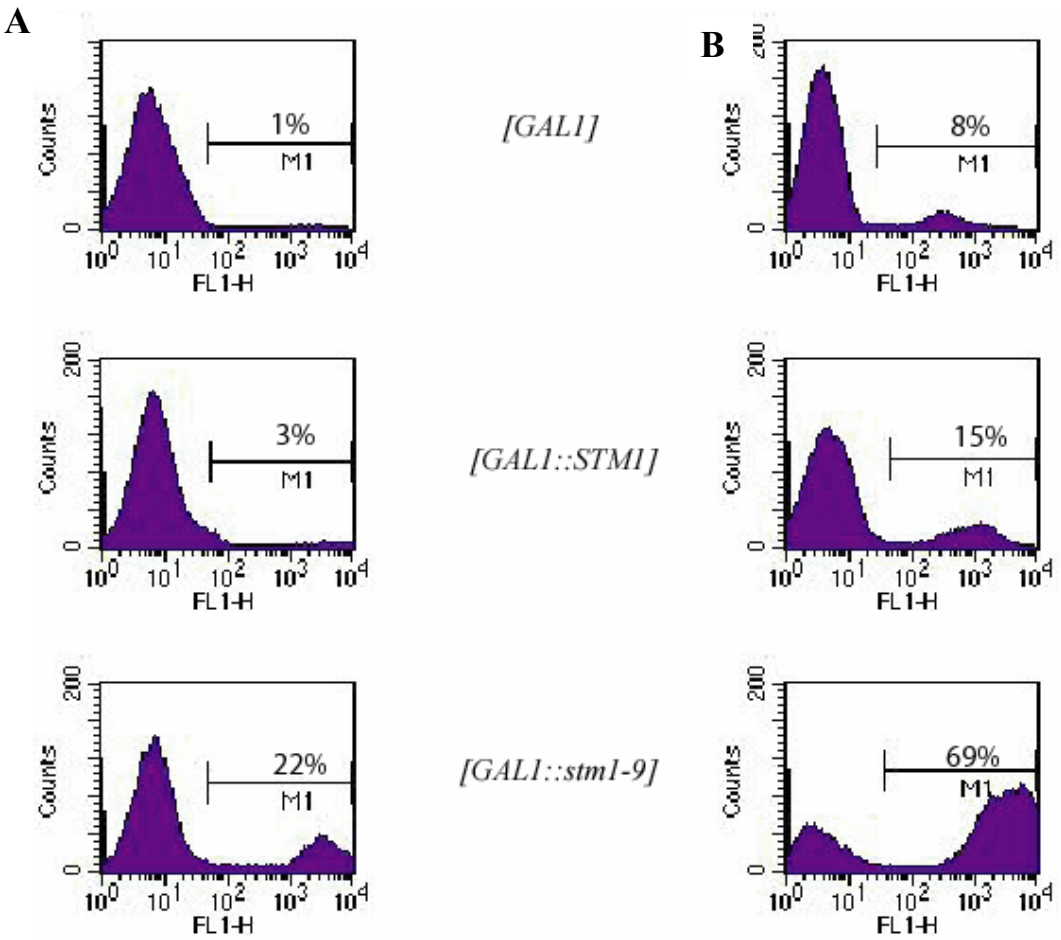


Figure 6: Overexpression of *stm1-9* in wild type cells causes lethality.
Cell viability of strain BY4741a (**A**) or strain WCG4a (**B**) bearing *stm1-9*, *STM1* wild type under control of the *GAL1* promoter or empty vector as a control was determined after 26h (**A**) or after 40h (*B*) of induction in liquid SC-URA$^-$ galactose medium. Cells were treated with DiBaC4 and the fraction of dead cells showing fluorescence was analyzed by flow cytometry (M1).

induced to express mutant allele *stm1-9* or the corresponding wild type *STM1* were briefly treated *in vivo* with the anionic voltage-sensitive oxonol dye DiBaC4 (bis-(1,3-dibutylbarbituric acid) trimethine oxonol), as an indicator of loss of the plasma membrane electrochemical potential and subsequently analyzed by flow cytometry. The strain carrying empty vector was used as a control. In this method, the viable cells exclude the fluorophore, so that only dead (non-viable) cells become fluorescent. The *stm1-9* allele in the strain BY4741a (YJI051) was induced in liquid SC-URA$^-$ galactose medium for 26h resulting in 22% of cell death after incubation with DiBaC4 reagent,

whereas overexpression of *stm1-9* in the strain WCG4a (YJI069) for 40h caused almost 70% of cell death (Fig. 6A / B). The strains bearing empty vector as a control did show very low rates of lethality (1% in the strain BY4741a (YJI049), 8% in the strain WCG4a (YJI065), respectively). Lethality was moderately enhanced by overexpression of wild type *STM1* (3% in BY4741a (YJI050), 15% in WCG4a (YJI067)).

3.5.2 Overexpression of *stm1-9* in wild type cells leads to FITC-VAD-FMK-positive staining

It is known, that cysteine proteases, so called caspases, function as central regulators and effectors in mammalian apoptosis. Recently, a new protein with caspase-like activity was found in yeast (Madeo et al., 2002). This protein named Yca1 or Mca1 (the product of the gene *YOR197w*) was proposed to be a bona fide caspase. Like mammalian caspases, Yca1 is activated through proteolytic cleavage of an inactive precursor. The proteolytic activity of Yca1 like other caspases can be inhibited by the Z-VAD-FMK substrate. Binding of the valyl-alanyl-aspartyl (VAD) sequence to an activated mammalian caspase induces the fluoromethyl ketone (FMK) moiety to react with the active site cysteine of the caspase. This mechanism leads to inactivation and labeling of the protein with the fluorescent dye (Grabarek and Darzynkiewicz, 2002). To monitor the potential activation of endogenous caspase in response to overexpression of *stm1-9* allele, FITC-VAD-FMK staining assay was used. Thus, cells expressing *stm1-9* or *STM1* wild type for control were incubated *in vivo* with FITC-labeled VAD-FMK (FITC-VAD-FMK) and then, the amount of cells showing fluorescence due to VAD-FMK binding, was analyzed by flow cytometry. More than 20% of FITC-VAD-FMK positive cells were scored in BY4741a cells that overexpressed *stm1-9* (strain YJI051) for 26h on a galactose medium. In contrast, a lower amount of fluorescent cells were detected after overexpression of *STM1* wild type gene (14%) and only a minor amount (7%) was measured in cells harbouring empty vector as a control (Fig. 7A). Notably, after a 40h *stm1-9* induction in strain

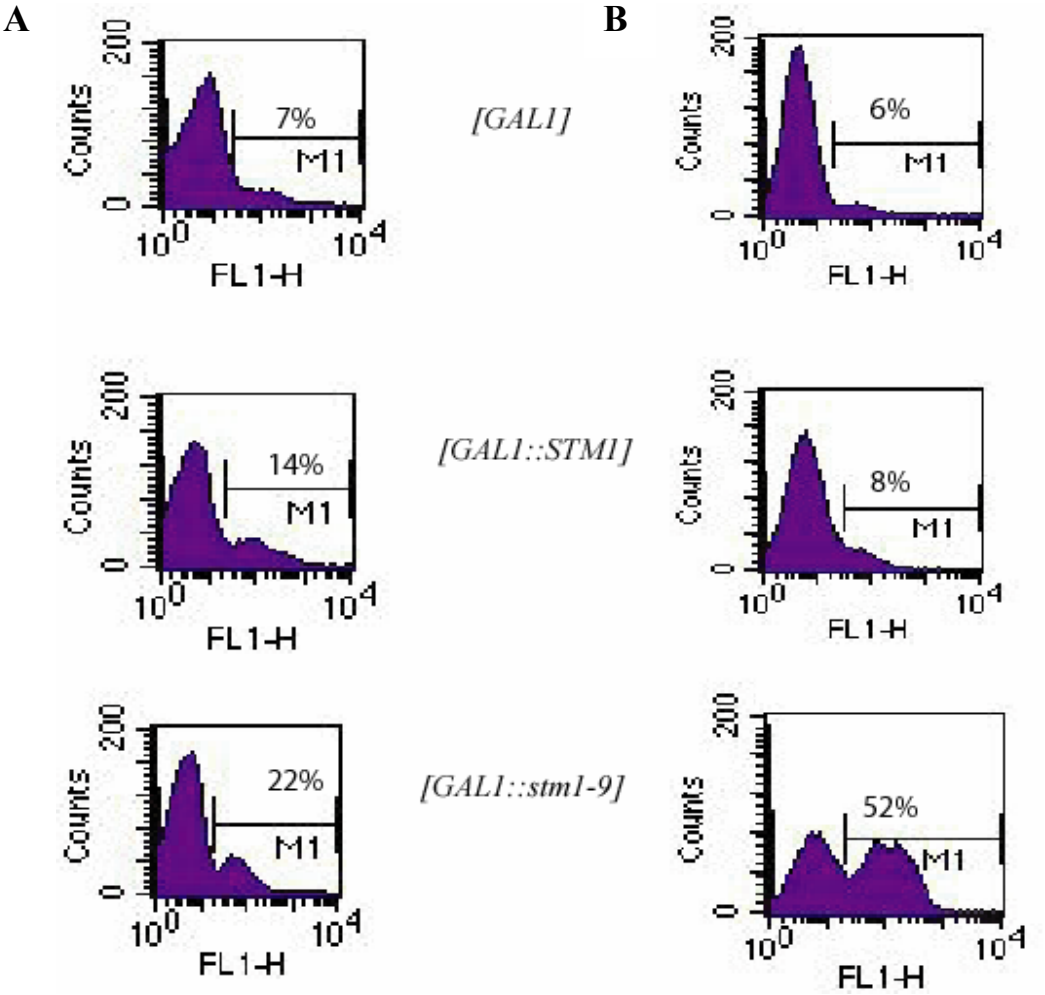


Figure 7: Overexpression of *stm1-9* allele in wild type cells leads to FITC-VAD-FMK-positive staining.

Histograms demonstrating the rate of FITC-VAD-FMK staining in strain BY4741a (**A**) or strain WCG4a (**B**) carrying plasmid derived *GAL1* inducible *stm1-9*, *STM1* wild type or empty vector. Cells were induced in liquid SC-URA$^-$ galactose medium for 26h (**A**) or 40h (**B**). The cells were labeled with FITC-VAD-FMK and the fraction of cells showing fluorescence was determined by flow cytometry (M1).

WCG4a (YJI069), a remarkable number of FITC-VAD-FMK positive cells (52% from whole population) were detected in comparison to 8% for cells that overexpressed *STM1* wild type gene and 6% for cells containing empty vector as a control (Fig. 7B).

3.5.3 Overexpression of *stm1-9* version in wild type cells leads to propidium iodide (PI) positive staining

To check the integrity of cells overproducing Stm1-9, an assay showing staining of cells with propidium iodide (PI) as a reporter of plasma membrane integrity was applied in parallel to FITC-VAD-FMK staining (see above). In this assay of bivariate flow cytometry analysis, cells are plotted by red fluorescence (FL2) on the X-axis to report propidium iodide staining, and green fluorescence (FL1) on the Y-axis to report FITC-VAD-FMK binding. BY4741a cells expressing *stm1-9* allele (strain YJI051) for 26h were analyzed for double staining with FITC-VAD-FMK and propidium iodide. In parallel, the amount of cells showing fluorescence either after solely FITC-VAD-FMK staining or after propidium iodide staining alone were determined. BY4741a cells bearing empty vector (strain YJI049) were used as a control. Four distinct populations of the cells expressing *stm1-9* was identified on the scatter plots: the unstained cell population was expected in the lower left quadrant of the scatter plot (Fig. 8.1 A). When cells were solely stained with FITC-VAD-FMK, fluorescent cells were found in the upper left quadrant (Fig. 8.1 B2). In contrast, cells solely stained with propidium iodide appeared in the lower right quadrant (Fig. 8.1 C2). Cells showing both fluorescent signals due to double FITC-VAD-FMK and PI staining were found in the upper right quadrant (Fig. 8.1 D2). Single staining with FITC-VAD-FMK (FITC-positive) or PI (PI-positive) revealed similar number of cells (21% FITC-positive/22% PI-positive cells (Fig. 8.1 B2 / C2)). Significantly less amount of FITC-positive or PI-positive stained cells were detected in the cells harbouring empty vector as a control (5.5% and 8%, respectively (Fig. 8.1 B1 / C1)). Single FITC-VAD-FMK staining alone yielded cells in the two upper quadrants of the scatter plot (Fig. 8.1 B1 / B2), whereas cells solely stained with PI were found in the lower right quadrant (Fig. 8.1 C1 / C2). After double staining all cells were found in the overlap zone of the FITC and PI signal indicating that FITC-VAD-FMK staining was accompanied by PI staining or vice versa.

To confirm these findings, identical experiments were performed with WCG4a cells overexpressing *stm1-9* allele. After a 40h *stm1-9* overexpression, WCG4a cells were analyzed for FITC-VAD-FMK and PI staining. As expected, all unstained cells were detectable in lower left quadrant of the scatter plot (Fig. 8.2 A). FITC-stained cells

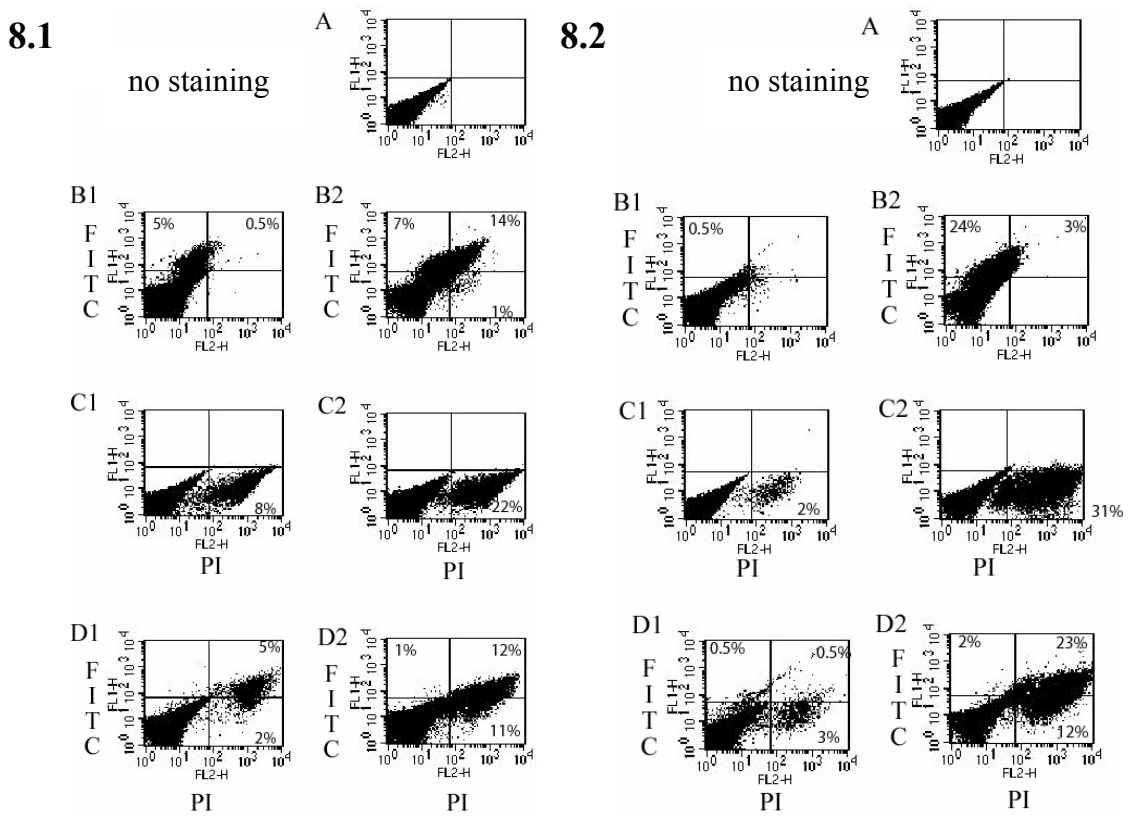


Figure 8: Cells expressing *stm1-9* allele show enhanced staining with FITC-VAD-FMK paralleled by propidium iodide staining.

BY4741a (**8.1**) or WCG4a (**8.2**) cells were induced to express *stm1-9* (right scatter plots) for 26h (**8.1**) or for 40h (**8.2**) in liquid SC-URA$^-$ galactose medium. For control, corresponding wild type strains containing empty pYES2 vector were treated in the same manner (left scatter plots). The cells were single stained with FITC-VAD-FMK (**8.1 B1 / B2**; **8.2 B1 / B2**) or propidium iodide (PI; to visualize membrane disintegration (**8.1 C1 / C2**; **8.2 C1 / C2**)) or double stained with both dyes (**8.1 D1 / D2**; **8.2 D1 / D2**) and analysed using flow cytometry.

were found in two upper quadrants (27%) (Fig. 8.2 B2), whereas PI stained cells were concentrated in the lower right quadrant (31%) (Fig. 8.2 C2). WCG4a cells harbouring empty vector for control showed very minor amount of positive staining for both, FITC-VAD-FMK (0.5%) and PI (2%) (Fig. 8.2 B1 / C1, respectively). Double staining with FITC-VAD-FMK and PI revealed a major population of cells showing both FITC and PI signals (23% and 12%, respectively) and small amount of cells that only exhibited the

FITC signal (less than 2%) (Fig. 8.2 D2). In contrast, no remarkable staining were detected in empty vector control cells (Fig. 8.2 D1). Taken together the results suggest that FITC-VAD-FMK staining even after Stm1-9 overproduction paralleled by PI staining. These findings lead to the idea, that cells expressing *stm1-9* allele are accessible for FITC-VAD-FMK staining only when they had lost plasma membrane integrity. Wysocki et al. have shown that cells that have lost plasma membrane integrity show positive FITC-VAD-FMK staining independent of the presence of the caspase Yca1. In agreement with these data, the positive FITC-VAD-FMK signal found after *stm1-9* overexpression may be caspase independent. Nevertheless, the data proof that under these conditions induced cell death is accompanied by the accessibility of cells to PI and FITC-VAD-FMK.

3.5.4 Expression of *stm1-9* in wild type cells causes DNA fragmentation

Another hallmark of apoptosis is extensive fragmentation of chromosomal DNA. This process can be analyzed by the TUNEL test (terminal d-UTP nick end labeling) and has been successfully applied to yeast cells undergoing an induced cell death (Herker et al., 2004; Ligr et al., 1998; Madeo, 2002). In the TUNEL procedure, terminal deoxynucleotidyl transferase attaches FITC-conjugated d-UTP moieties to free 3´ ends of DNA in a template-independent fashion. To amplify the signal of FITC-labelled DNA an immunostaining using anti-FITC antibody was used. In the apoptotic cells, during fragmentation of DNA, the number of free 3´ ends increases resulting in TUNEL-positive nuclei. After 26h *stm1-9* induction in strain BY4741a (YJI051), approximately 30% of TUNEL positive cells appeared, whereas only minor number of TUNEL positive cells (7%) were observed in cells carrying an empty vector for control (Fig. 9). A similar rate of TUNEL staining was found in strain WCG4a expressing the *stm1-9* allele (data not shown).

BY4741
[*GAL1*]

BY4741
[*GAL1::stm1-9*]

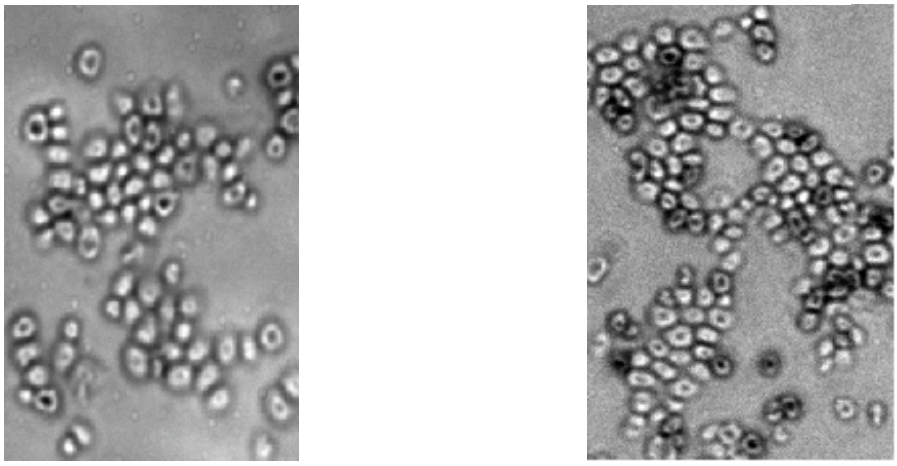


Figure 9: Overexpression of *stm1-9* in wild type cells causes DNA fragmentation.

Fragmentation of chromosomal DNA was analyzed by TUNEL reaction followed by immunostaining of FITC-labelled DNA. TUNEL test was performed in BY4741a cells that were induced to express *stm1-9* (right panel) for 26h in liquid SC-URA$^-$ galactose medium. Phase contrast microscope was used for visualization of fragmented DNA. BY4741a cells expressing empty vector were used as a control (left panel).

3.5.5 Overexpression of *stm1-9* in wild type cells leads to release of cytochrome c

During apoptosis, a variety of pro-apoptotic proteins including cytochrome c are released from the mitochondria. This mechanism was also proven in yeast cells using a cytochrome c release assay (Ludovico et al., 2002). The assay is based on the isolation of mitochondria from spheroplasts followed by cytochrome c detection in the cytosolic and mitochondrial fraction using Western blot analysis. Expression of *stm1-9* was induced in BY4741a cells (strain YJI051) until flow cytometry revealed 25% of FITC-VAD-FMK positive cells and 5% of FITC-VAD-FMK positive cells in a control strain containing empty vector (data not shown). After separation of mitochondrial and cytosolic fractions, cytochrome c was shown to accumulate in the cytosolic space of the cells overexpressing *stm1-9* (Fig. 10 lanes 7 / 8). Smaller but remarkable amount of cytochrome c was detected in the cytosolic fraction of control strain bearing empty vector. (Fig. 10 lanes 5 / 6). In these samples, the cytosolic fraction was contaminated with a tiny amount of

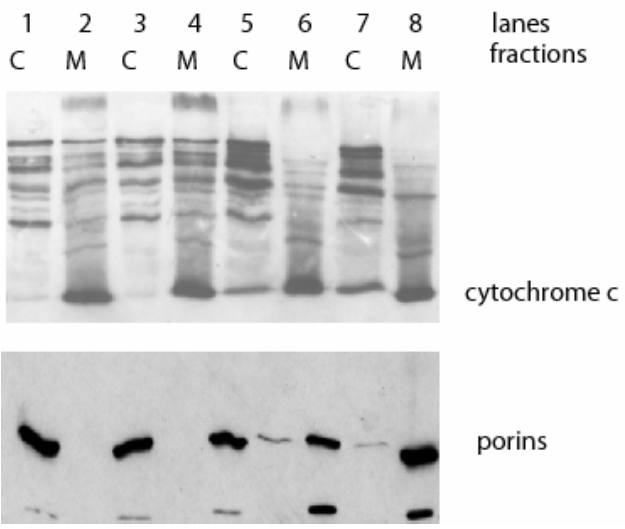


Figure 10: Overexpression of *stm1-9* allele in wild type cells leads to cytochrome c release.

The immunoblot representing release of cytochrome c from the mitochondrial (M) to the cytosolic (C) fraction in BY4741a cells that overexpressed *stm1-9* allele for 45h in liquid SC-URA$^-$ galactose medium (lanes 8 and 7, respectively). In parallel release of cytochrome c from M to C fraction in BY4741a cells expressing empty vector as a control was detected (lanes 6 and 5, respectively). No migration of cytochrome c from M to C fraction for cells containing *stm1-9* or empty vector was seen after immediate shift to galactose medium (lanes 4 and 3 or 2 and 1, respectively). Mitochondrial porins served as a control for separation of M and C fractions.

porins, indicating that either fractionation did not work perfectly or nuclear porins are released from the nuclear membrane under these conditions (Fig. 10 lanes 5 / 7).

In summary, the results presented above show that the cell death induced by overexpression of *stm1-9* allele in wild type cells undergoes with the apoptotic-like features.

3.6 *stm1-9* - dependent cell death is accompanied by increased ROS production

Reactive oxygen species (ROS) provide important signaling and effector functions in apoptosis. Elevated ROS production was observed in yeast cells in response to various

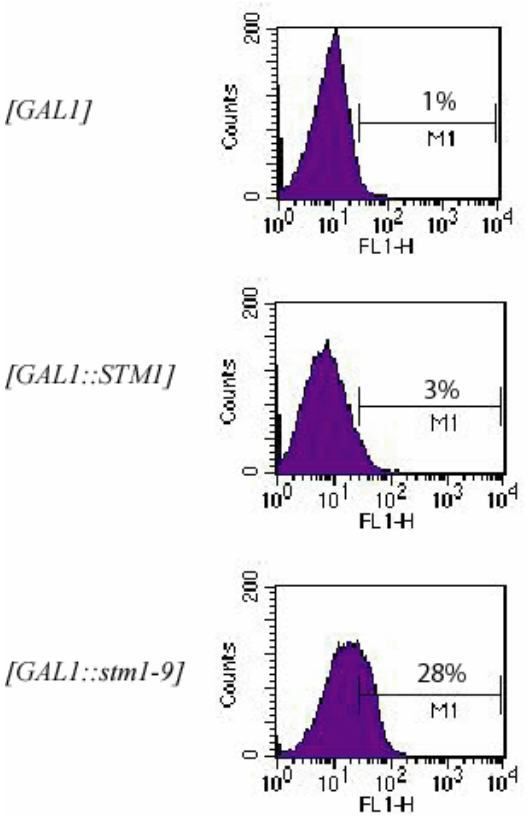


Figure 11: *stm1-9* – dependent cell death is accompanied by increased ROS production.

ROS levels were detected in wild type cells WCG4a that overexpressed *stm1-9* for 40h in liquid SC-URA$^-$ galactose medium. For control, corresponding wild type cells carrying empty vector or *STM1* wild type were treated in the same manner. The cells were incubated with dihydrorhodamine123 for 2h at 30°C and subjected to FACS analysis (filter FL1). M1 denotes the fraction of ROS positive cells.

death-inducing signals (Madeo, 2002a,b; Madeo et al., 1999)). To examine whether cell death induced by *stm1-9* overexpression is accompanied with accumulation of ROS, WCG4a cells expressing *stm1-9* (strain YJI069) for 40h were incubated with the reagent dihydrorhodamine123 (DHR123) for 2h. In the presence of ROS, DHR123 is oxidized, so that rhodamine123 accumulates, as a green fluorescent product, in the mitochondria of the living cells. Cells that have accumulated the fluorescent dye can be detected by flow cytometry (Henderson and Chappell, 1993). FACS analysis of WCG4a cells after 40h *stm1-9* overexpression (strain YJI069) showed, that approximately 28% of the cell population accumulates rhodamine 123 (Fig. 11). In contrast, only 3% rhodamine 123 positive cells were found after 40h induction of *STM1* wild type gene, whereas cells harboring empty vector for control contained 1% fraction of rhodamine positive cells. These data show that *stm1-9*-induced cell death is accompanied by the production of ROS.

3.7 The toxic effect triggered by overproduction of Stm1-9 protein in wild type cells occurs due to its proteolytic stabilisation

Previous work by Ligr et al. showed that cell death induced by overexpression of wild type *STM1* in the proteasomal *pre1-1 pre4-1* mutant was most probably due to proteolytic stabilisation of Stm1. Thus, it was reasonable that a proteolytically stable version of Stm1 would cause growth defect when expressed in wild type cells containing an intact proteasomal degradation system. Therefore, mutated Stm1 protein was examined for its proteolytic stability. For immunodetection an epitope derived from influenza hemagglutinin (2x HA) followed by a stop codon was introduced into the C-terminus of *STM1* or the *stm1-9* allele sequence. The resulted plasmid encoded *GAL1* promoter controlled *STM1::HA*$_2$ or *stm1-9::HA*$_2$ (pJI021 and pJI020, respectively) was introduced into wild type cells by transformation. After inducing expression of

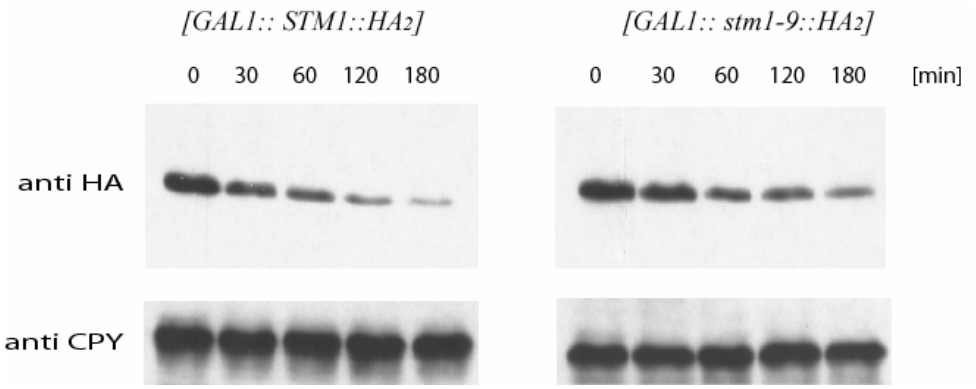


Figure 12: Stm1-9 is partially stabilized in wild type cells in comparison to its wild type counterpart.
An immunoblot analysis exhibits the levels of Stm1-HA or Stm1-9-HA in wild type cells at the different time points. Cells carrying plasmid encoded *GAL1* promoter controlled *STM1::HA*$_2$ (strain YJI093) or *stm1-9::HA*$_2$ (strain YJI091) were grown in liquid SC-URA$^-$ medium containing raffinose up to the logarithmic phase. The expression of *stm1-9::HA*$_2$ and its wild type counterpart was induced then in liquid SC-URA$^-$ medium containing galactose. After 2h of induction, the expression of *STM1::HA*$_2$ or *stm1-9::HA*$_2$ in wild type cells was repressed by shifting the cells to SC-URA$^-$ medium containing glucose. Simultaneously, protein synthesis was stopped by the addition of cycloheximide (0 time point). The protein levels were followed at indicative chase time by Western analysis. The steady-state level of the vacuolar protease, carboxypeptidase, (CPY) was followed as a loading control using anti-CPY antibody.

STM1::HA$_2$ in strain YJI093 or *stm1-9::HA*$_2$ in strain YJI091 on galactose, the synthesis of Stm1-HA$_2$ or Stm1-9-HA$_2$ was blocked by addition of glucose repressing the *GAL1*

promoter and cycloheximide inhibiting the protein synthesis. Equal amounts of the cells were collected at different time points and analyzed by immunoblotting. As compared to the wild type Stm1-HA_2, the degradation of Stm1-9-HA_2 was notably reduced (Fig. 12). The results indicate that the cell death induced by overproduction of Stm1-9 protein in wild type cells is the result of its stabilization and, therefore, partial accumulation.

3.8 Apoptotic cell death induced by overexpression of *stm1-9* in wild type cells requires the yeast caspase Yca1

The so far unique caspase Yca1 was shown to contribute to apoptosis in yeast cells (Madeo et al., 2002). To examine whether Yca1 is involved in the apoptotic cell death induced by *stm1-9* overexpression, the effect of *stm1-9* overexpression was examined in *yca1Δ* null mutant with different genetic backgrounds (WCG4a and BY4741a). Therefore, region encoding the *YCA1 ORF* was disrupted in WCG4a derived strain (YJI071) carrying the *stm1-9* allele. To study the effect in BY4741a background, wild type control (strain YJI051) and the *yca1Δ* null mutant (strain YJI052) were transformed with the *stm1-9* encoding plasmid. The growth of *YCA1* wild type strains and the *yca1Δ* mutant strains overexpressing *stm1-9* either in WCG4a (strains YJI069 and YJI071, respectively) or BY4741a (strains YJI051 and YJI052, respectively) background was examined after streaking on solid SC-URA^- medium containing either glucose (for repression of *stm1-9*) or galactose (for induction of *stm1-9*). Interestingly, the growth defect caused by *stm1-9* overexpression was significantly suppressed in *yca1Δ* mutant strains with both genetic backgrounds (Fig. 13A / B). These data show that Yca1 is important for generation of *stm1-9* induced growth defect.

Furthermore, to analyze whether Yca1 is required for the efficient appearance of apoptotic phenotypes in wild type cells expressing *stm1-9*, several features as cell viability, FITC-VAD-FMK staining and formation of ROS were examined (for details see above). For this purpose, *YCA1* wild type and *yca1Δ* strains were induced to express *stm1-9* on galactose. At defined time points of *stm1-9* overexpression, wild type and

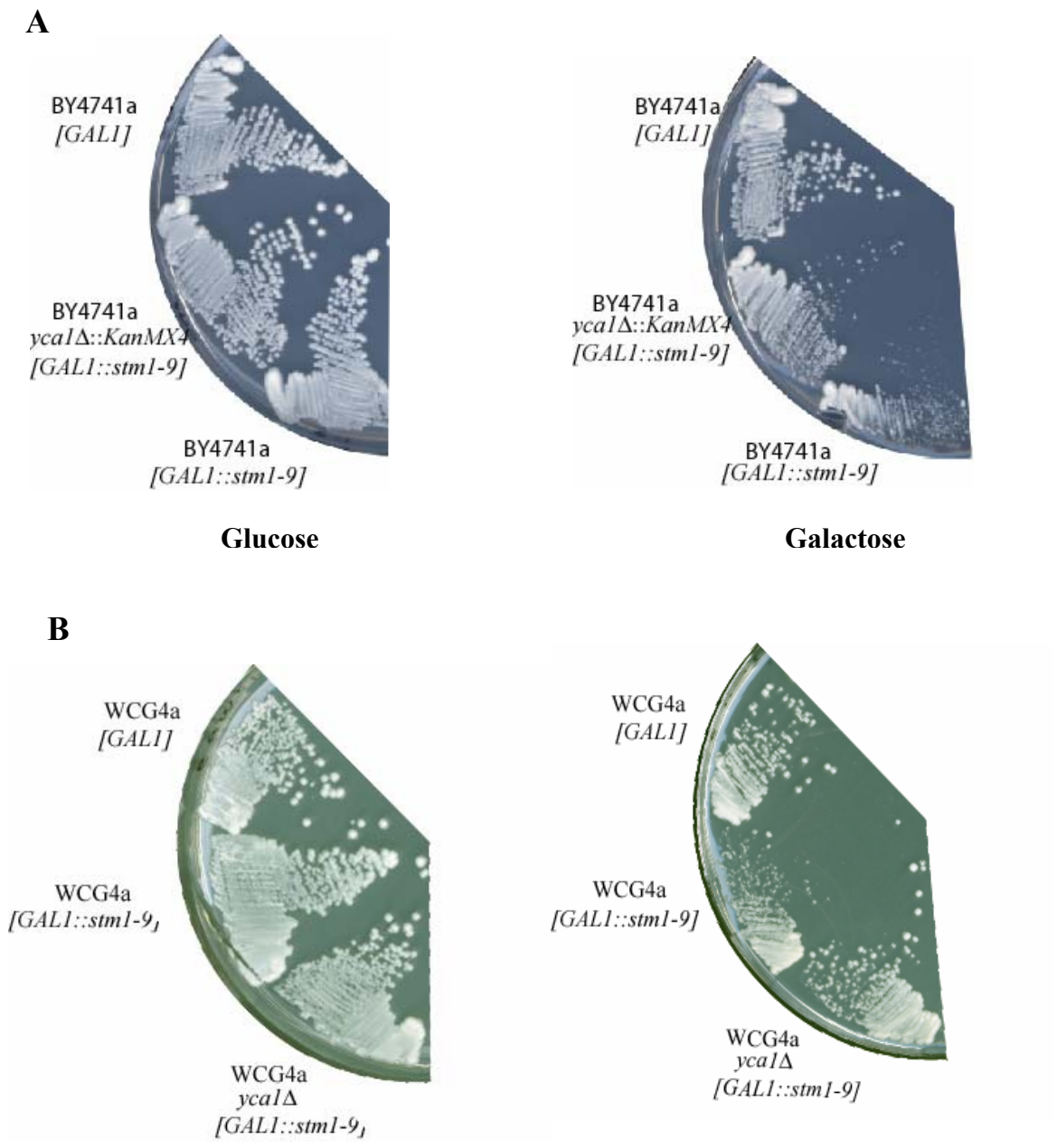


Figure 13: Growth defects induced by *stm1-9* overexpression are suppressed by deletion of caspase Yca1.

A. BY4741a wild type cells and corresponding *yca1Δ* mutant expressing *GAL1* driven *stm1-9* were streaked on SC-URA$^-$ medium containing either glucose (repressive conditions, left plates) or galactose (induction of *stm1-9*, right plates). Wild type cells containing empty vector were used as a control. The plates were incubated at 30ºC for 3 days.

B. WCG4a wild type cells and corresponding *yca1Δ* mutant expressing *GAL1* driven *stm1-9* were streaked on SC-URA$^-$ medium containing either glucose (repressive conditions, left plate) or galactose (induction of *stm1-9*, right plate). Wild type cells containing empty vector were used as a control. The plates were incubated at 30ºC for 3 days.

yca1Δ cells were analyzed for several subcellular markers by flow cytometry. Cells carrying empty vector were used as a control. To determine the rates of lethality after *stm1-9* overexpression, wild type and *yca1Δ* cells were stained with the DiBaC4 reagent and analyzed by flow cytometry. After 26h of *stm1-9* induction in cells with BY4741a background, a significant lower amount of dead cells were scored in *yca1Δ* mutant (strain YJI052) (11%) versus 22% for corresponding wild type cells (strain YJI051) (Fig. 14A, left panels). As expected, a very low rate of dead cells was detected in wild type cells carrying empty vector as a control (1%). A slightly stronger suppression effect was seen in WCG4a background. Here, after 40h of *stm1-9* induction *yca1Δ* cells (strain YJI071) showed a remarkably lower amount of dead cells (23%) as compared to corresponding wild type cells (69%) (8% of dead cells were found in cells carrying empty vector as a control) (Fig. 14A, right panels).

Simultaneously, the *yca1Δ* cells overexpressing *stm1-9* were analyzed for FITC-VAD-FMK staining. In this assay, *yca1Δ* mutant cells with BY4741a background that overexpressed *stm1-9* revealed a significant lower amount of FITC-VAD-FMK positive cells (7%) as compared to the corresponding wild type cells (22% of FITC-positive cells) (Fig. 14B, left panels). Thus, the amount of FITC-positive cells of *yca1Δ* mutant corresponded to the rate of FITC-positive cells found in cells containing empty vector as a control (7%). Similarly, after *stm1-9* overexpression, only a minor amount of FITC-VAD-FMK positive cells were scored for the WCG4a derived *yca1Δ* mutant strain (15%) as compared to wild type cells (52%) (Fig. 14B, right panels). Here, control showed a slightly lower rate than the *yca1Δ* mutant (6% FITC-positive cells).

In addition, *yca1* null mutant with WCG4a and BY4741a background that overexpressed *stm1-9* were analyzed for the rate of ROS production. For this purpose, the cells were stained with the drug DHR123 and analyzed by flow cytometry. After 40h of *stm1-9* overexpression, ROS production was significantly reduced in WCG4a derived *yca1Δ* cells: 5% ROS positive cells versus 28% for the corresponding wild type control (strain YJI069) by this approaching the value of cells that did not express *stm1-9* (1%) (Fig. 14C). Similar results were obtained for BY4741 derived *yca1Δ* cells (data not shown).

Taken together, the results prove that apoptotic cell death induced by *stm1-9* overexpression is a process that requires the caspase-like protease Yca1.

A

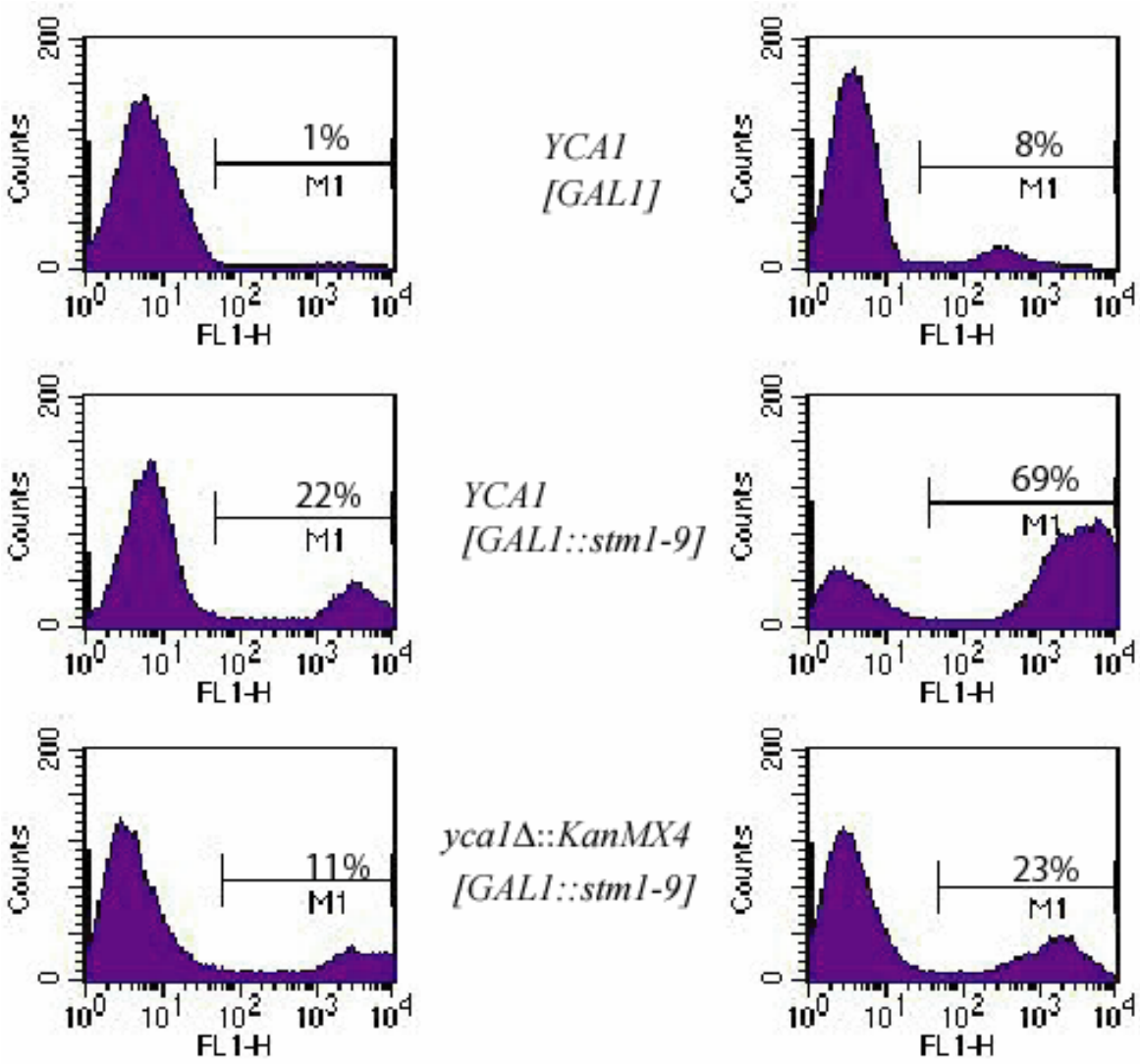


Figure 14: Apoptotic phenotypes induced by *stm1-9* overexpression depend on the presence of Yca1.

A. Lethality induced by *stm1-9* overexpression is reduced after deletion of caspase Yca1. BY4741a (left panels) and WCG4a (right panels) wild type and corresponding *yca1Δ* mutant cells were induced to overexpress *stm1-9* for 26h (left panels) or 40h (right panels) in liquid SC-URA$^-$ galactose medium. For control, corresponding wild type cells bearing empty vector were treated in the same manner. The cells were stained with the DiBaC4 reagent and counted using flow cytometry. M1 denotes the fraction of dead cells showing fluorescence.

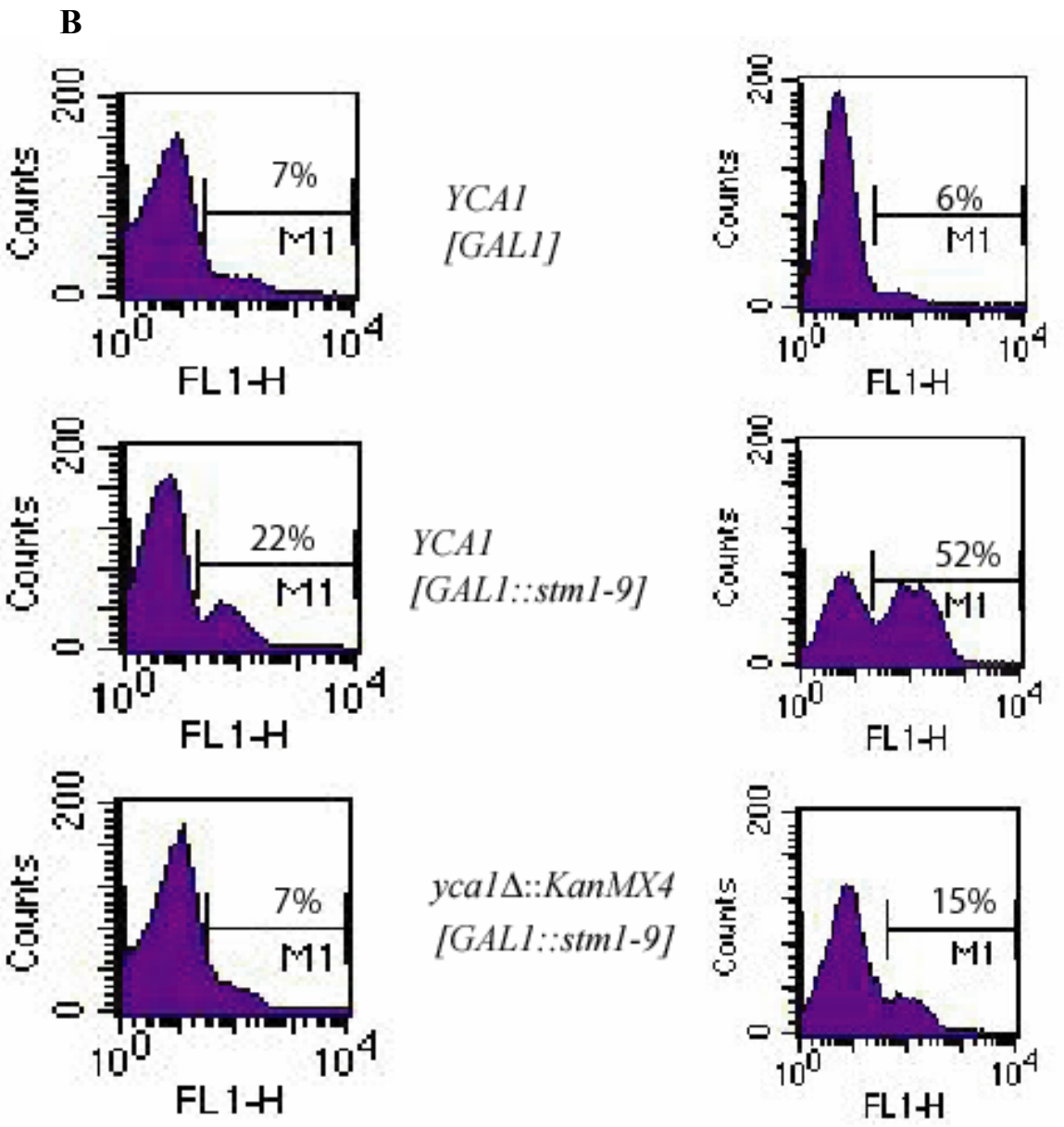


Figure 14: Apoptotic phenotypes induced by *stm1-9* overexpression depend on the presence of Yca1.

B. Deletion of *yca1* in wild type cells expressing *stm1-9* leads to reduced staining with FITC-VAD-FMK.
Histograms showing the level of FITC-VAD-FMK staining in BY4741a (left panels) and WCG4a (right panels) wild type and the corresponding *yca1Δ* mutant cells expressing *stm1-9* for 26h (left panels) or 40h (right panels) in liquid SC-URA$^-$ galactose medium. For control, corresponding wild type cells bearing empty vector were treated in the same manner. The cells were treated with FITC-VAD-FMK and analyzed by flow cytometry. The rate of FITC-positive cells is represented by the fraction of cells with the fluorescent signal due to accumulation of FITC-VAD-FMK dye in the cells in the region M1.

C

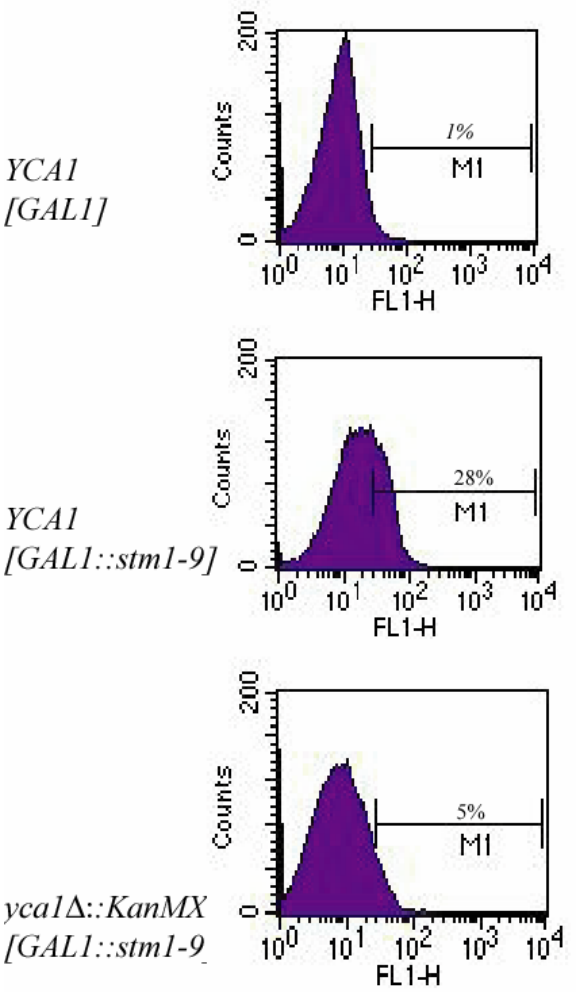


Figure 14: Apoptotic phenotypes induced by *stm1-9* overexpression depend on the presence of Yca1.

C. Deletion of *yca1* in wild type cells expressing *stm1-9* leads to the reduced amount of ROS producing cells. Histograms representing the accumulation of ROS producing cells in *yca1Δ* mutant and wild type cells (WCG4a) induced by *stm1-9* overexpression for 40h. Wild type cells carrying empty vector were used as a negative control. The cells were incubated with dihydrorhodamine123 for 2h at 30°C and analyzed using flow cytometry (filter FL1). M1 denotes the fraction of ROS positive cells. Growth conditions were, as described in legend to Fig. 11.

3.9 Apoptosis-inducing factor 1 (Aif1) is involved in *stm1-9* induced cell death

The apoptosis-inducing factor 1 (Aif1), encoded by *YNR074* gene, was uncovered to contribute to regulation of apoptotic cell death in yeast (Madeo, 2004; Wissing et al., 2004). To check whether *stm1-9* induced cell death is Aif1 dependent, the *AIF1* encoding region was disrupted in both strains BY4741a and WCG4a bearing *stm1-9* allele (YJI053 and YJI073, respectively) and the growth on solid SC-URA$^-$ galactose medium compared with corresponding wild type cells after *stm1-9* overexpression. Wild

type strains bearing *STM1* wild type or the empty vector were taken as controls. Notably, the growth defect induced by overexpression of *stm1-9* was suppressed in both *aif1Δ* mutant cells with BY4741a (strain YJI053) or WCG4a (strain YJI073) background (Fig. 15A / B).

To check whether this suppression effect was accompanied with the reduced expression of apoptotic markers, assays for cell viability, FITC-VAD-FMK staining and ROS production were performed. To examine viability, cells expressing *stm1-9* in *aif1Δ* or *AIF1* wild type background were stained with the DiBaC4 reagent and analyzed by flow cytometry. As expected, *aif1Δ* cells with BY4741a background that overexpressed *stm1-9* for 26h (YJI053) showed reduced rate of lethality (10% of dead cells) as compared to the corresponding wild type cells (strain YJI051) (22% of dead cells) (Fig. 16A, left panels). Cells carrying empty vector as a control showed very low amount of lethality (1%). Similarly, after 40h of *stm1-9* induction in *aif1Δ* cells with WCG4a background (strain YJI073) a significantly lower amount of dead cells were scored (24%) as compared to the corresponding wild type cells (strain YJI069) (69%) (Fig. 16A, right panels). Cells carrying empty vector as a control exhibited low rates of lethality (8%).

In addition, FITC-VAD-FMK staining was examined for *aif1Δ* null mutant overexpressing *stm1-9*. For this purpose, cells were incubated *in vivo* with the FITC-labeled VAD-FMK substrate and analyzed by flow cytometry. BY4741a derived *aif1Δ* mutant (strain YJI053) that overexpressed *stm1*-9 for 26h showed a very small amount of FITC-VAD-FMK positive cells (5%), whereas under same conditions its wild type counterpart (strain YJI051) exhibited 22% of FITC-VAD-FMK positive cells (Fig. 16B, left panels). Cells containing empty vector as a control resembled the rate of FITC-positive cells found in *stm1-9* expressing *aif1Δ* mutant (7%). Similarly, after 40h of *stm1-9* induction in the WCG4a *aif1Δ* strain (YJI073), a significantly reduced amount of cells stained for FITC-VAD-FMK were detected (14%) as compared to the corresponding wild type cells (strain YJI069) (52% of FITC-VAD-FMK positive cells) (Fig. 16B, right panels). Cells with this genetic background carrying empty vector as a control showed only 6% FITC-positive staining.

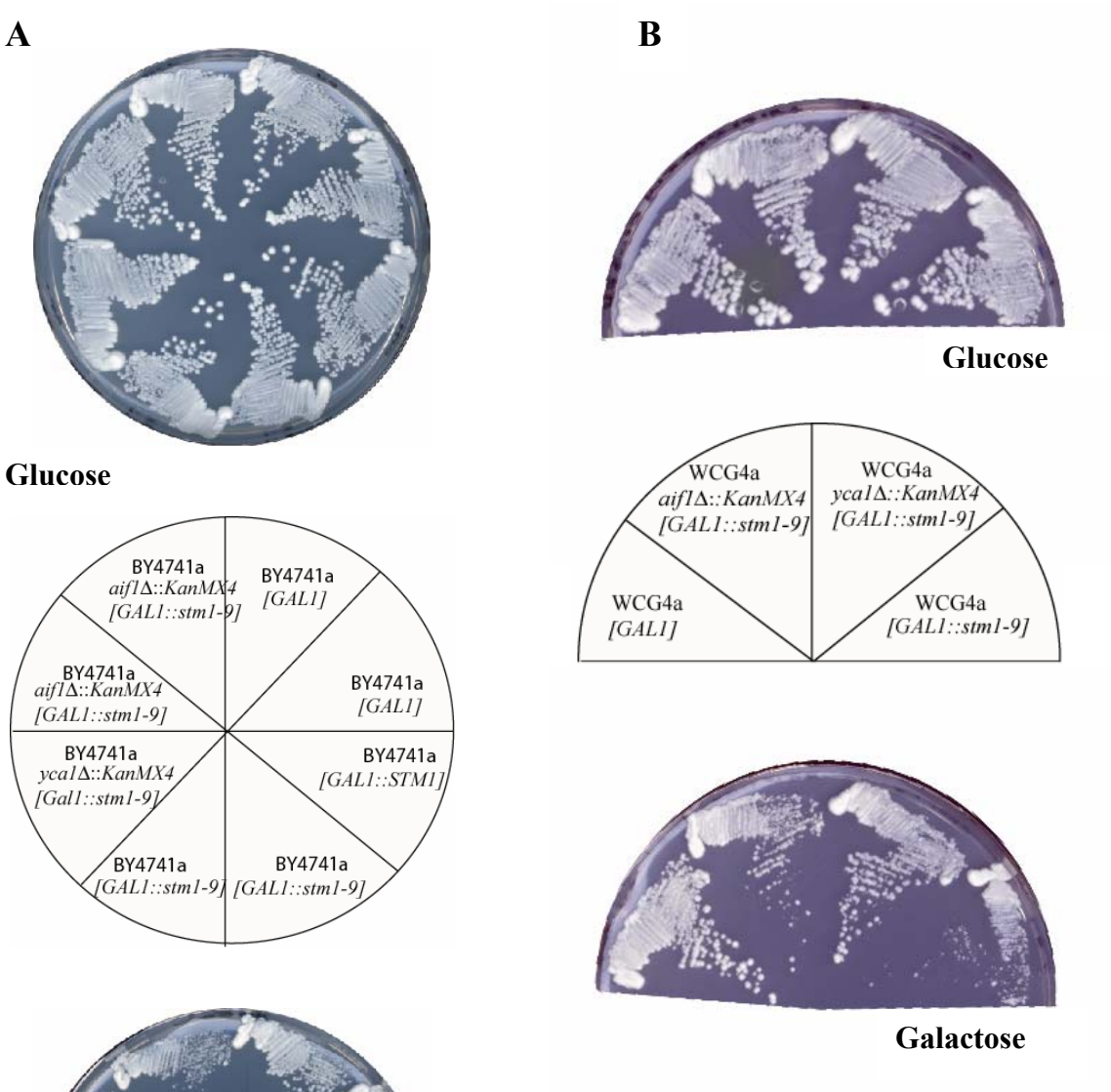


Figure 15: Aif1 is required for induction of the *stm1-9* mediated growth defect.

A. BY4741a wild type cells and corresponding *aif1Δ* mutant expressing *GAL1* driven *stm1-9* were streaked on SC-URA$^-$ medium containing either glucose (repressive conditions, left plate) or galactose (induction of *stm1-9*, right plate). Wild type cells containing either *STM1* wild type or empty vector, as well BY4741a derived *yca1Δ* mutant expressing *stm1-9* were used as controls. The plates were incubated at 30ºC for 3 days.

B. WCG4a wild type cells and corresponding *aif1Δ* mutant expressing *GAL1* driven *stm1-9* were streaked on SC-URA$^-$ medium containing either glucose (repressive conditions, left plate) or galactose (induction of *stm1-9*, right plate). Wild type cells containing empty vector, as well WCG4a derived *yca1Δ* mutant expressing *stm1-9* were used as controls. The plates were incubated at 30ºC for 3 days.

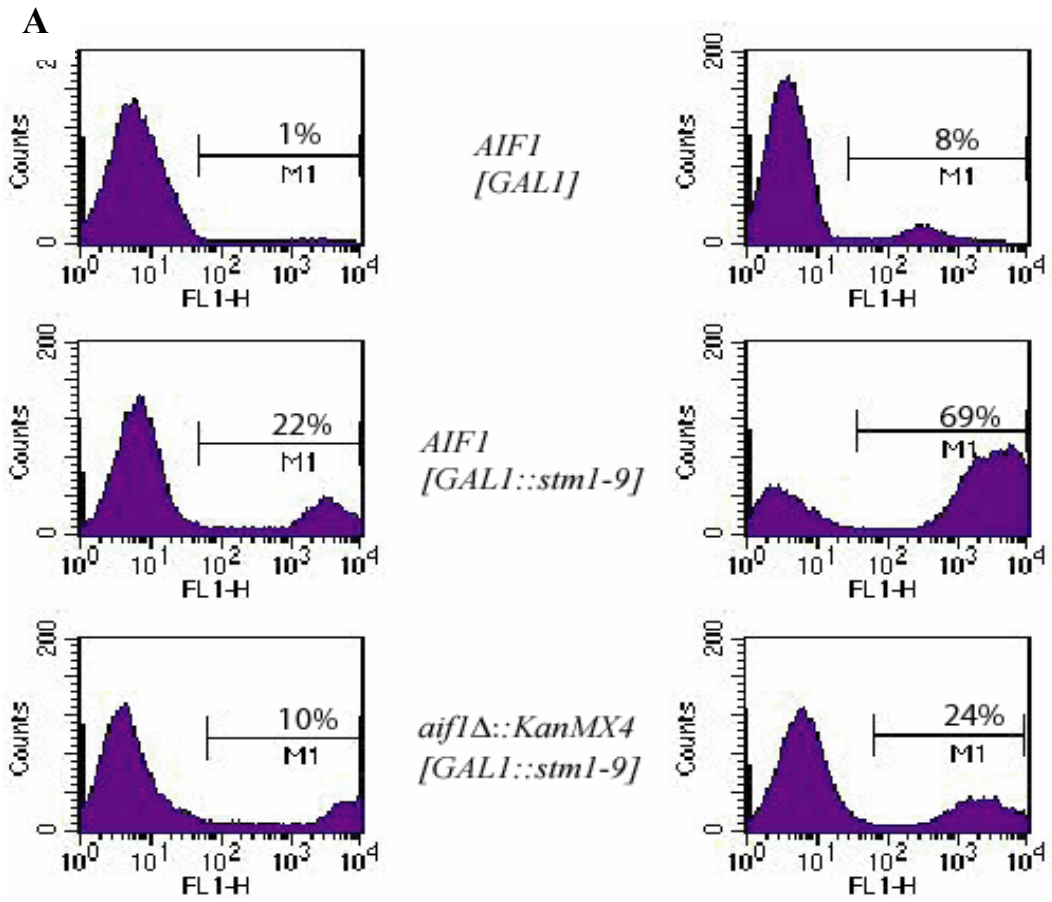


Figure 16: The presence of Aif1 is required for the occurrence of apoptotic phenotypes induced after overexpression of the *stm1-9* allele.

A. Deletion of *aif1* reduces lethality induced by *stm1-9* overexpression.
Histograms representing the rate of cell viability after overexpression of *stm1-9* in *aif1* null mutant with BY4741a (left panels) and WCG4a (right panels) background. The cells were induced to express *stm1-9* in liquid SC-URA$^-$ galactose for 26h (left panels) or 40h (right panels). Cell viability was determined by treating the cells with the DiBaC4 dye followed by flow cytometric analysis. Viability of wild type cells with no or *stm1-9* overexpression was determined as negative or positive control. M1 denotes the fraction of dead cells showing positive staining.

B

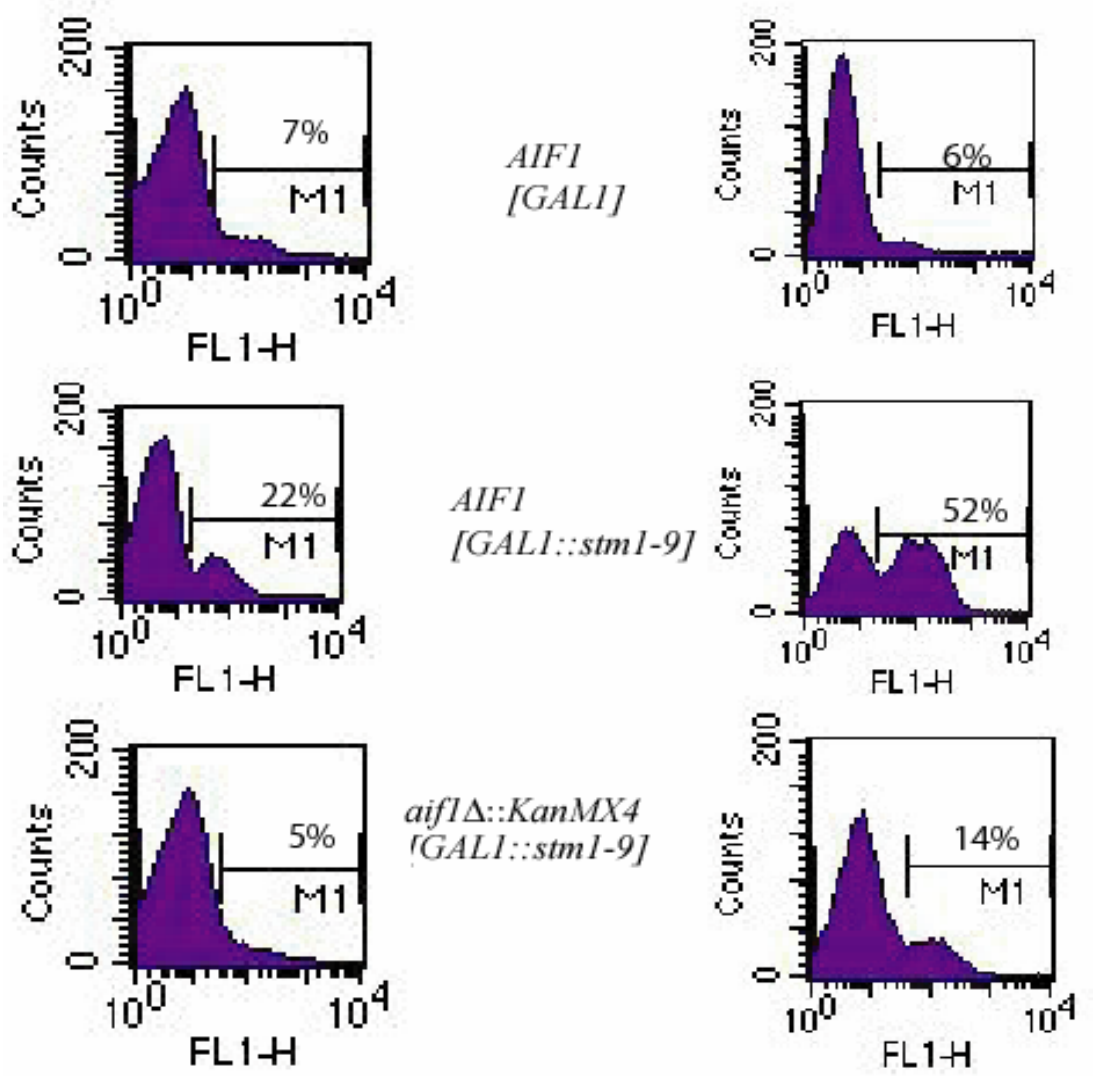


Figure 16: The presence of Aif1 is required for the occurrence of apoptotic phenotypes induced after overexpression of the *stm1-9* allele.

B. *aif1Δ* mutant overexpressing *stm1-9* shows reduced rate of FITC-VAD-FMK staining. The rate of FITC-stained cells of BY4741a derived *aif1Δ* (left panels) or WCG4a derived *aif1Δ* (right panels) mutant cells overexpressing *stm1-9* on galactose for 26h (left panels) or 40h (right panels) is visualized by histograms. Wild type cells containing empty vector (negative control) or *stm1-9* (positive control) were measured for comparison. Cells were labeled with FITC-VAD-FMK and examined by FACS analysis. M1 denotes the fraction of FITC-positive cells showing fluorescence due to accumulation of the dye in the dead cells.

C

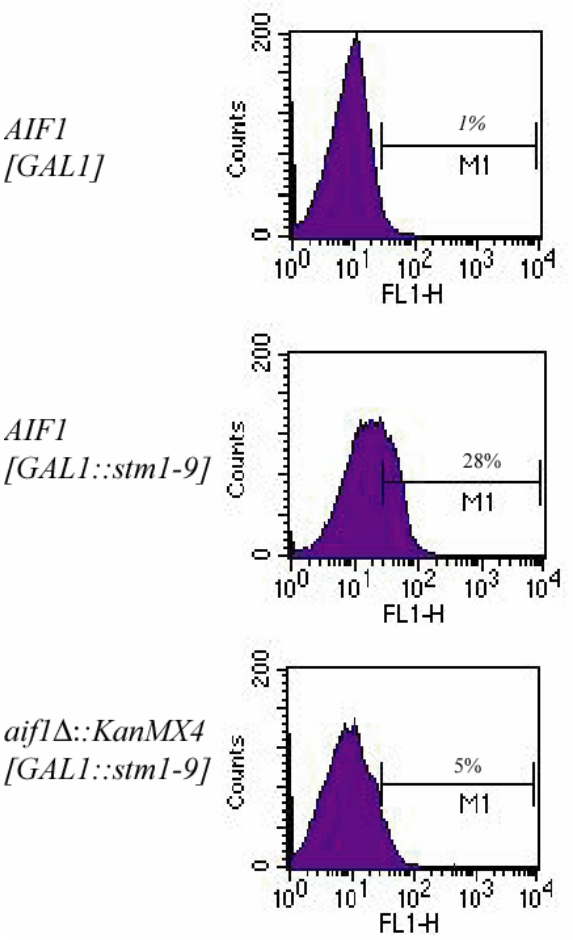


Figure 16: The presence of Aif1 is required for the occurrence of apoptotic phenotypes induced after overexpression of the *stm1-9* allele.

C. Deletion of *aif1* in wild type cells overexpressing *stm1-9* leads to reduced amount of ROS producing cells.
Histograms exhibiting the accumulation of ROS producing cells in *aif1Δ* mutant and the corresponding wild type cells (WCG4a) after overexpression of *stm1-9* in liquid SC-URA$^-$ galactose medium for 40h. Wild type cells carrying empty vector were used as a negative control. The cells were incubated with dihydrorhodamine123 for 2h and analysed by flow cytometry. M1 denotes the fraction of ROS-positive cells.

Moreover, WCG4a derived *aif1Δ* mutant cells (strain YJI073) overexpressing *stm1-9* for 40h were analyzed for generation of ROS. In these mutants ROS production was significantly reduced: 5% ROS positive cells versus 28% for the corresponding wild type control (strain YJI069) by this approaching the value of cells expressing no *stm1-9* (1%) (Fig. 16C). Taken together, the data suggest that significant markers of apoptotic cell death induced by *stm1-9* overexpression are suppressed by deletion of Aif1. These results indicate that is Aif1 is a down-stream element required for *stm1-9* overexpression induced apoptosis in yeast.

3.10 Yca1 and Aif1 have an epistatic role in *stm1-9* induced apoptosis

Previous data showed that cell death induced by *stm1-9* overexpression requires the presence of Yca1 or Aif1 (see above). Moreover, Wissing et al. showed that Aif1-triggered cell death is partially Yca1-dependent. Therefore, the interplay of Aif1 and Yca1 in cell death induced by *stm1-9* overexpression was examined. BY4741a and WCG4a derived *yca1Δ aif1Δ* double mutants harbouring the *stm1-9* encoding overexpression plasmid (strains YJI054 and YJI074, respectively) were created by crossing of corresponding *yca1Δ* and *aif1Δ* single mutants. After induction of *stm1-9* the growth of *yca1Δ aif1Δ* cells on solid SC-URA$^-$ galactose medium was compared with the growth of *yca1Δ* and *aif1Δ* single mutants. Wild type strains expressing *stm1-9* or containing *STM1* wild type or empty vector were inspected for control. Notably, the growth defect induced by overexpression of *stm1-9* was suppressed in *yca1Δ aif1Δ* double mutant cells with BY4741a (strain YJI053) or WCG4a (strain YJI073) background to the same extent as found in *yca1Δ* and *aif1Δ* single mutants (Fig. 17A / B). This behaviour of epistasis suggests that in cells with these genetic backgrounds both, Yca1 and Aif1, may act down-stream to Stm1 in an identical cell death pathway.

Moreover, BY4741a derived *yca1Δ aif1Δ* double mutants (strain YJI054) overexpressing *stm1-9* for 26h in liquid SC-URA$^-$ galactose medium were analyzed for double FITC-VAD-FMK and PI staining using flow cytometry. In parallel, the amount of cells showing fluorescence either after single FITC-VAD-FMK or single PI staining were determined. BY4741a wild type or *yca1Δ* and *aif1Δ* single mutant cells overexpressing pYES2 derived *stm1-9* together with wild type cells containing the empty pYES2 vector were used as controls. Notably, single FITC-VAD-FMK staining yielded very minor amount of FITC-positive stained cells in BY4741a derived *yca1Δ aif1Δ* double mutant overexpressing *stm1-9* (0.5%) resembling the rate of single FITC-positive cells in *yca1Δ* and *aif1Δ* single mutants (0.5%), in contrast to wild type cells bearing either *stm1-9* or the empty vector (21% both upper quadrants or 5%, respectively) (Fig. 18 / B). Similarly, staining of *yca1Δ aif1Δ* double mutant cells with PI detecting dead cells revealed a nearly identical population of PI-positive cells (7%) as found for single

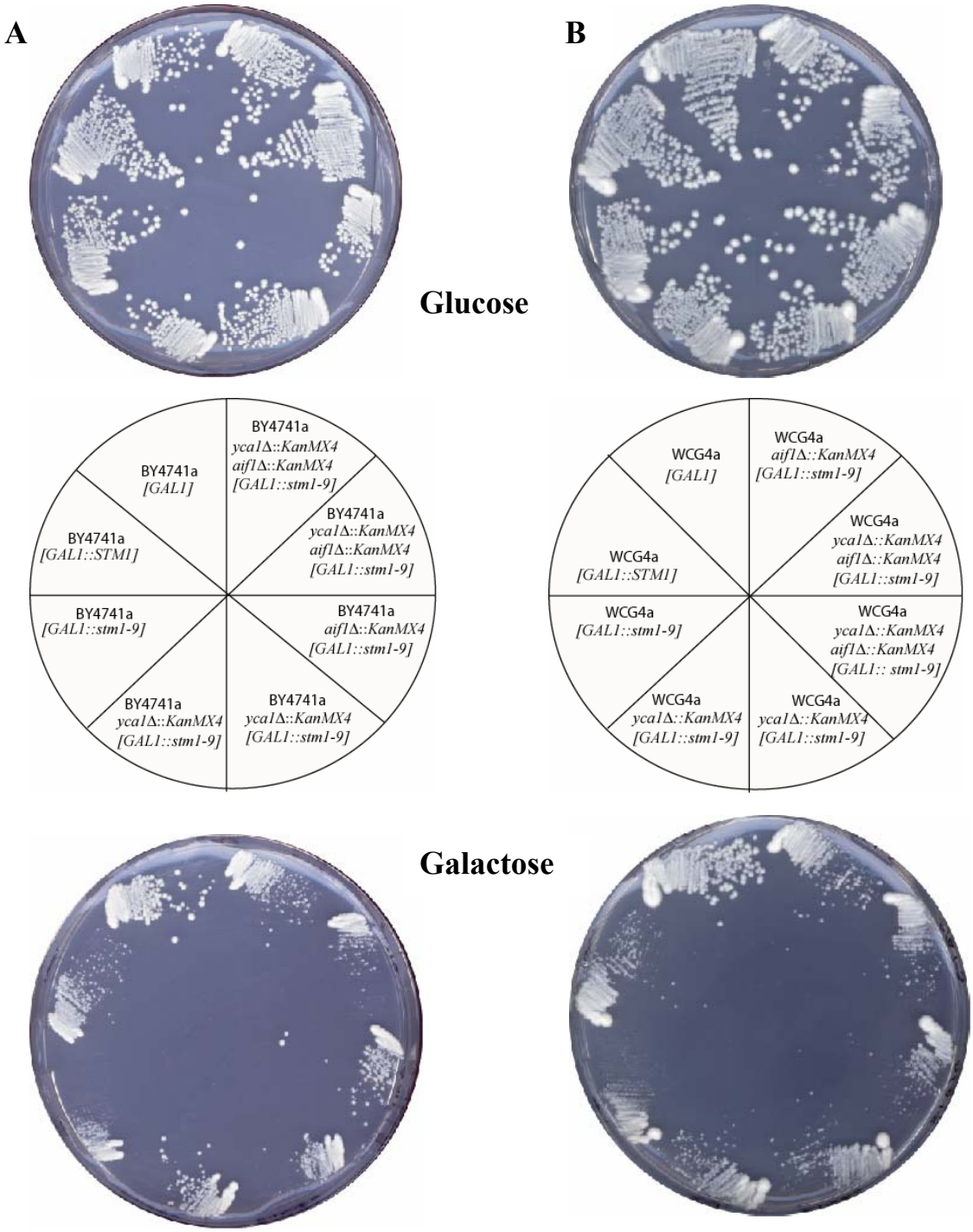


Figure 17: The growth defect caused by *stm1-9* overexpression is suppressed in *yca1Δ aif1Δ* double mutant in the same way as in *yca1Δ* and *aif1Δ* single mutants.

BY4741a derived *yca1Δ aif1Δ* (A) or WCG4a derived *yca1Δ aif1Δ* (B) double mutants and corresponding single mutants expressing *stm1-9* were streaked on solid SC-URA$^-$ medium containing either glucose (repressive conditions, left plates) or galactose (expression of *stm1-9*, right plates). Wild type cells with the respective background carrying *stm1-9*, *STM1* wild type or empty vector were used as controls. The plates were incubated at 30ºC for 3 days.

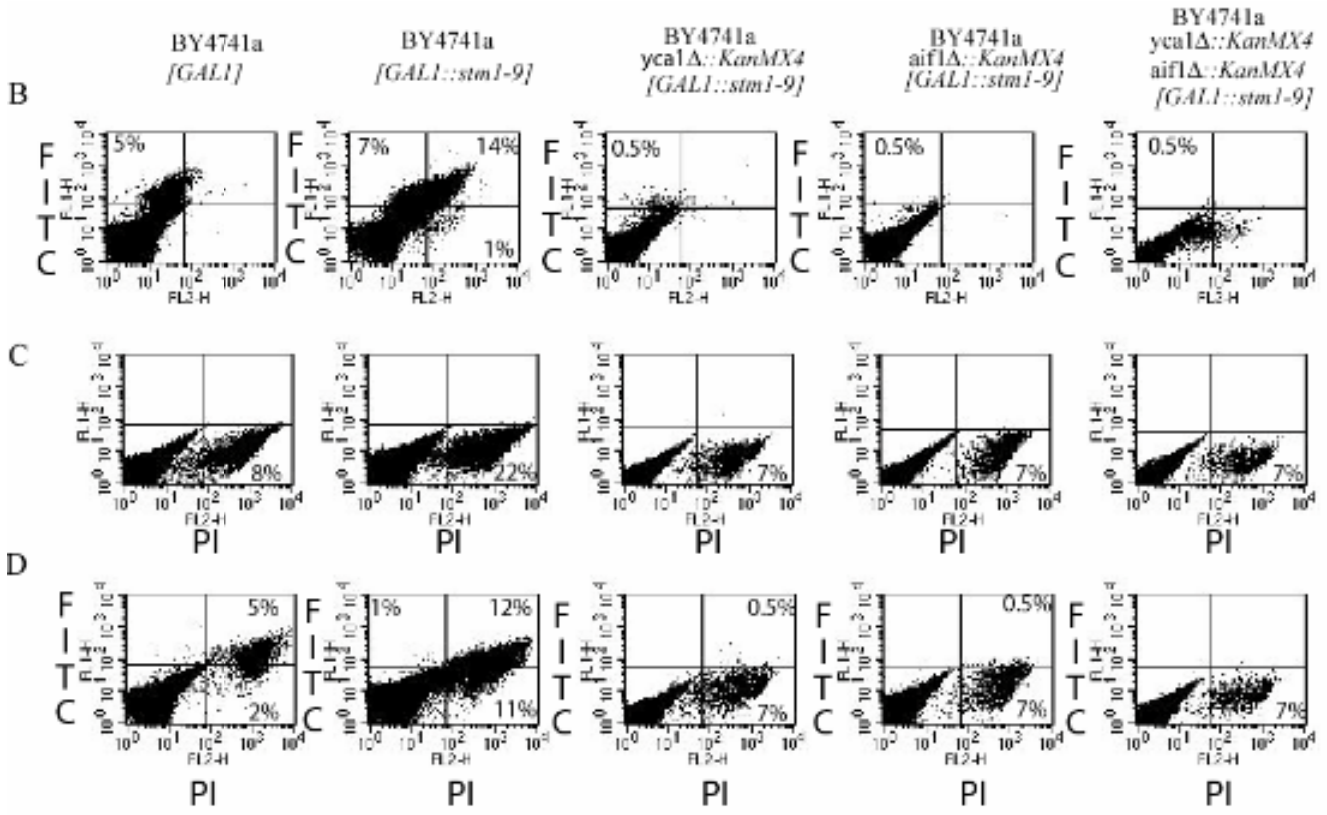


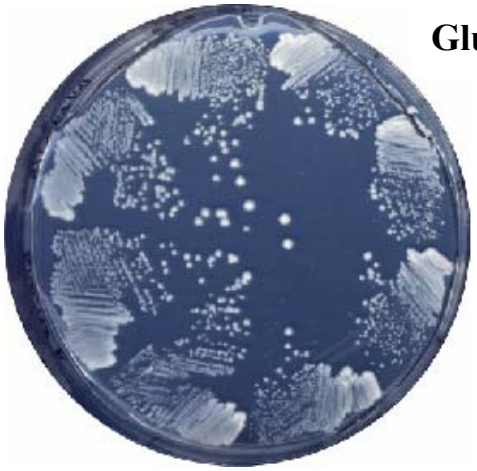
Figure 18: Flow cytometry of double FITC-VAD-FMK and PI stained BY4741a derived *yca1Δ aif1Δ* double mutant uncovers a similar distribution of FITC-and PI-positive cells as compared to the corresponding *yca1Δ* and *aif1Δ* single mutants.

BY4741a derived *yca1Δ aif1Δ* double mutants together with corresponding *yca1Δ* and *aif1Δ* single mutant cells were induced to express *stm1-9* for 26h in liquid SC-URA$^-$ galactose medium. For control, corresponding wild type strains containing pYES2 encoded *stm1-9*, *STM1* wild type or the empty pYES2 vector were treated in the same manner. The cells were single stained with FITC-VAD-FMK (B) or propidium iodide (C) or double stained with both dyes (D) and analysed using flow cytometry.

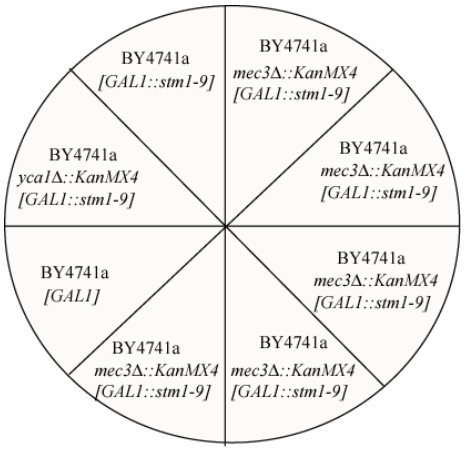
mutants and empty vector control cells (7% and 8%, respectively). In contrast, as expected, wild type cells expressing *stm1-9* showed a remarkable amount of PI-positive cells (22%) (Fig. 18 / C). Also double FITC-VAD-FMK and PI staining of *yca1Δ aif1Δ* double mutant cells yielded a small fraction of PI-positive cells (7%) corresponding to the values of single mutants (7%) versus 11% or 2% for wild type control cells bearing *stm1-9* or the empty vector (Fig. 18 / D). Thus, these data confirm the observations made by inspecting growth of *yca1Δ aif1Δ* null mutants. Double knock-out of both genes leads to the same reversion of *stm1-9* induced growth defects and phenotypic alterations as found in the corresponding *yca1Δ* and *aif1Δ* single mutants. Absence of either *AIF1* or *YCA1* seems to neutralize *stm1-9* induced effect to the same extent. This suppression effect cannot be increased by combination of both deletions. Thus, taken together, the results suggest that both Yca1 and Aif1 have an essential role in the same *stm1-9* triggered cell death pathway.

3.11 Stm1 physically interacts with Mec3

Database search reveals a two-hybrid interaction between the DNA damage checkpoint protein Mec3 and Stm1 (Ito et al., 2001). Mec3 is a DNA-binding protein, which is involved in DNA-repair, telomere silencing, and telomere length maintainance, suggesting that Stm1-Mec3 complexes might function in DNA repair. To prove the possible interaction between Stm1 and Mec3 by genetic means, a *mec3* null mutant was checked for suppression of growth defects caused by *stm1-9* overexpression. For this purpose, BY4741a derived *mec3Δ* mutant cells (YJI030) (obtained by dissection of the diploid strain YLR288c *mec3Δ* / *mec3Δ*) containing the pYES2 derived *stm1-9* overexpression plasmid were streaked on solid SC-URA$^-$ galactose medium and the growth compared with corresponding wild type cells. Notably, the growth defect induced by *stm1-9* overexpression was significantly suppressed in *mec3Δ* mutant cells (Fig. 19). These results suggest that Mec3 acts as a down-stream element in the *stm1-9* induced cell death pathway.



Glucose



Galactose

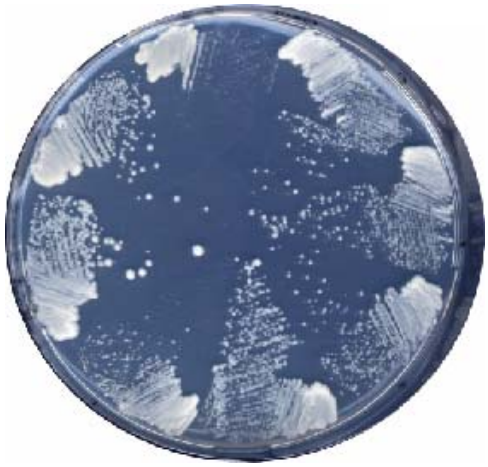


Figure 19: Deletion of *mec3* leads to suppression of the growth defect caused by *stm1-9* overexpression.

BY4741a derived *mec3Δ* mutants and corresponding wild type cells were induced to express *stm1-9* on solid SC-URA$^-$ galactose medium (induction conditions, lower plate). For repression, the cells were grown on solid SC-URA$^-$ glucose medium (upper plate). *yca1Δ* mutant cells expressing *stm1-9* and wild type cells containing empty vector were used as controls. The plates were incubated at 30ºC for 3 days.

To analyze whether Stm1 interacts physically with Mec3, co-immunoprecipitation experiments were done using a strain carrying immuno-epitope-marked Stm1-HA_3 and Mec3-MYC_9 (YJI027). The double tagged strain was created by crossing a strain

carrying Stm1-HA_3 (YJI017) with a strain bearing Mec3-MYC_9 (YMMF21/36) (kindly provided by M. Muzi-Falconi). Anti-HA and anti-MYC antibodies were used to pull down either Stm1-HA_3 or Mec3-MYC_9 together with attached proteins. Immunoblotting with anti-MYC antibodies recognized a protein species with the same electrophoretic mobility as Mec3-MYC_9 (Fig. 20, lane 1, upper panel). In addition, the same blot was reprobed to visualize Stm1-HA_3 (Fig. 20, lane 1, lower panel). In the same way, Stm1-HA_3 was detected by anti-HA blotting in Mec3-MYC_9 immunoprecipitates together with the immunoprecipitated Mec3-MYC_9 (Fig. 20, lane 3). The results prove that Stm1 interacts physically with Mec3 forming a stable complex, which due to the known function of Mec3 is suggested to be involved in DNA repair.

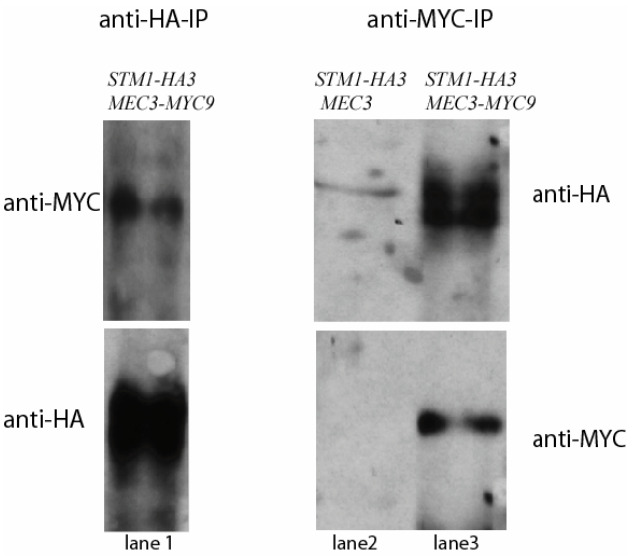


Figure 20: Stm1 *in vivo* physically interacts with Mec3.

Immunoblot showing Stm1-HA and Mec3-MYC immunoprecipitates. Extracts of exponential cells expressing Mec3-MYC_9 and Stm1-HA_3 in liquid YPD medium were subjected to immunoprecipitation using either anti-HA (lane 3) or anti-MYC (lane1) antibodies, respectively. Stm1 and Mec3 were detected in anti-MYC and anti-HA immunoprecipitates by Western blot analysis using anti-HA and anti-MYC antibodies, respectively. Lane 2 represents a negative control for the specificity of anti-HA and anti-MYC antibodies (immunoprecipitation with anti-MYC antibodies from extracts of cells containing un-tagged Mec3 protein).

3.12 *stm1Δ yca1Δ* double mutants show enhanced sensitivity to UV-induced mutagenesis

Previous data showed that Yca1 acts as a down-stream element to Stm1 in the cell death induced by *stm1-9* overexpression (see above). To test whether Stm1 / Yca1 induced cell

death may be required to erase mutated cells, WCG4a derived *stm1Δ yca1Δ* double mutant cells (strain YJI040) were subjected to UV-induced mutagenesis assay. In this assay, aliquots of cultures of *stm1Δ yca1Δ* double mutant (YJI040) and its corresponding wild type (WCG4a) were spreaded on YPD plates, exposed to UV light and grown at the permissive temperature 30ºC for 3 days. To analyze the colonies for a temperature sensitive (ts) mutant phenotype, the cells were replica plated onto YPD or minimal medium supplemented with amino acids required for growth and grown at the restrictive temperature 37°C for several days. Here, approximately 7% of colonies from the whole population of double *stm1Δ yca1Δ* colonies (YJI040) showed ts-phenotype on YPD plates. In contrast, only 1.5% of ts-mutants were determined in cells with wild type

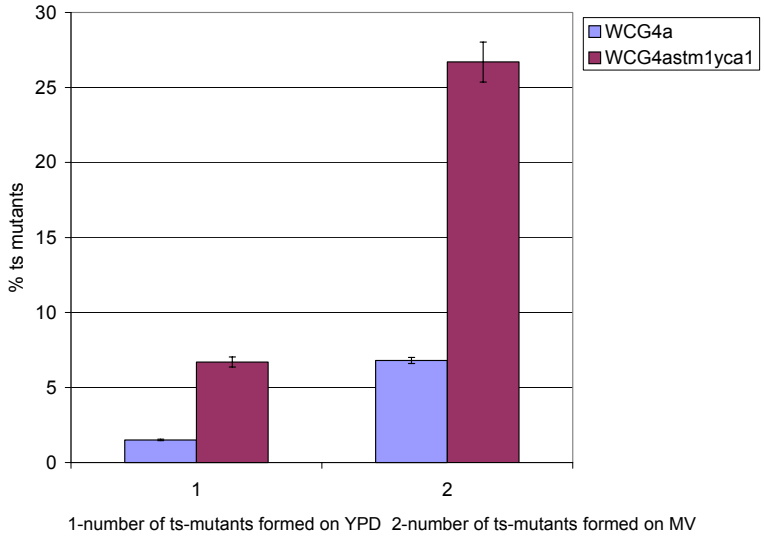


Figure 21: *stm1Δyca1Δ* double mutants are sensitive to UV-induced mutagenesis.

The ratio of ts-mutants was determined in wild type (WCG4a) and *stm1Δ yca1Δ* double mutant cells. Equal amount of cells from both strains were spreaded on YPD plates, exposed to UV light for 30s and incubated in the dark at 30ºC for 2 days. The colonies formed on those plates were replicaplated onto solid YPD medium or solid MV medium containing amino acids necessary for growth and incubated at 30°C and 37°C for 2-3 days. The relative number of ts-mutants was determined by comparing growth of colonies at both conditions.

background (Fig. 21). On minimal medium, an overall higher rate of temperature-sensitive colonies were detected. Nevertheless, cells carrying the *stm1Δ yca1Δ* double mutation (YJI040) showed a significant higher rate of ts-colonies than wild type (27%

versus 7%). These data clearly show that under UV-induced mutagenic conditions double *stm1Δ yca1Δ* mutants are more sensitive as wild type cells. This effect may be the result of the deficiency of *stm1Δ yca1Δ* to undergo apoptosis after DNA damaging.

3.13 Stm1 does not contribute to the cell death triggered by overexpression of high expression lethality (*HEL*) genes in the proteasomal *pre1-1 pre4-1* mutant

Using an overexpression screen, it was shown that Stm1 triggers cell death when overexpressed in proteasomal *pre1-1 pre4-1* mutants (Ligr et al., 2001). These studies showed additionally that overexpression of several other genes such as *NSR1*, *PPA1*, *SAR1*, *YNL208w* or *YOR309c* causes similar apoptotic phenotypes in *pre1-1 pre4-1* mutants. To check whether Stm1 is involved in cell death triggered by these distinct *HEL* genes, a *stm1* null mutant carrying *pre1-1 pre4-1* mutations (strain YJI095) was created by crossing *stm1Δ* strain YIV002 with the proteasomal *pre1-1 pre4-1* mutant strain YHi30/14. The triple mutant YJI095 was transformed with *pYES2* driven plasmids carrying the respective *HEL* gene under the control of the *GAL1* promoter. *NSR1*, *PPA1*, *SAR1*, *YNL208w* or *YOR309c* were then overexpressed in *pre1-1 pre 4-1 stm1Δ* cells and *pre1-1 pre4-1* mutant containing the *STM1* wild type gene (YHi30/14) on solid SC-URA$^-$ galactose medium. Surprisingly, with the exception of *NSR1* the overexpression of the distinct *HEL* genes in the proteasomal *pre1-1 pre4-1* mutant bearing *stm1Δ* (YJI095) leads to even stronger growth defects as compared to *pre1-1 pre4-1* cells containing functionally active *STM1* (strain YHi30/14) (Fig. 22). The results revealed that the cell death caused by overexpression of these *HEL* genes in the proteasomal *pre1-1 pre4-1* mutant is not Stm1 dependent but that their susceptibility to blocking of growth by this means is enhanced when *STM1* is absent.

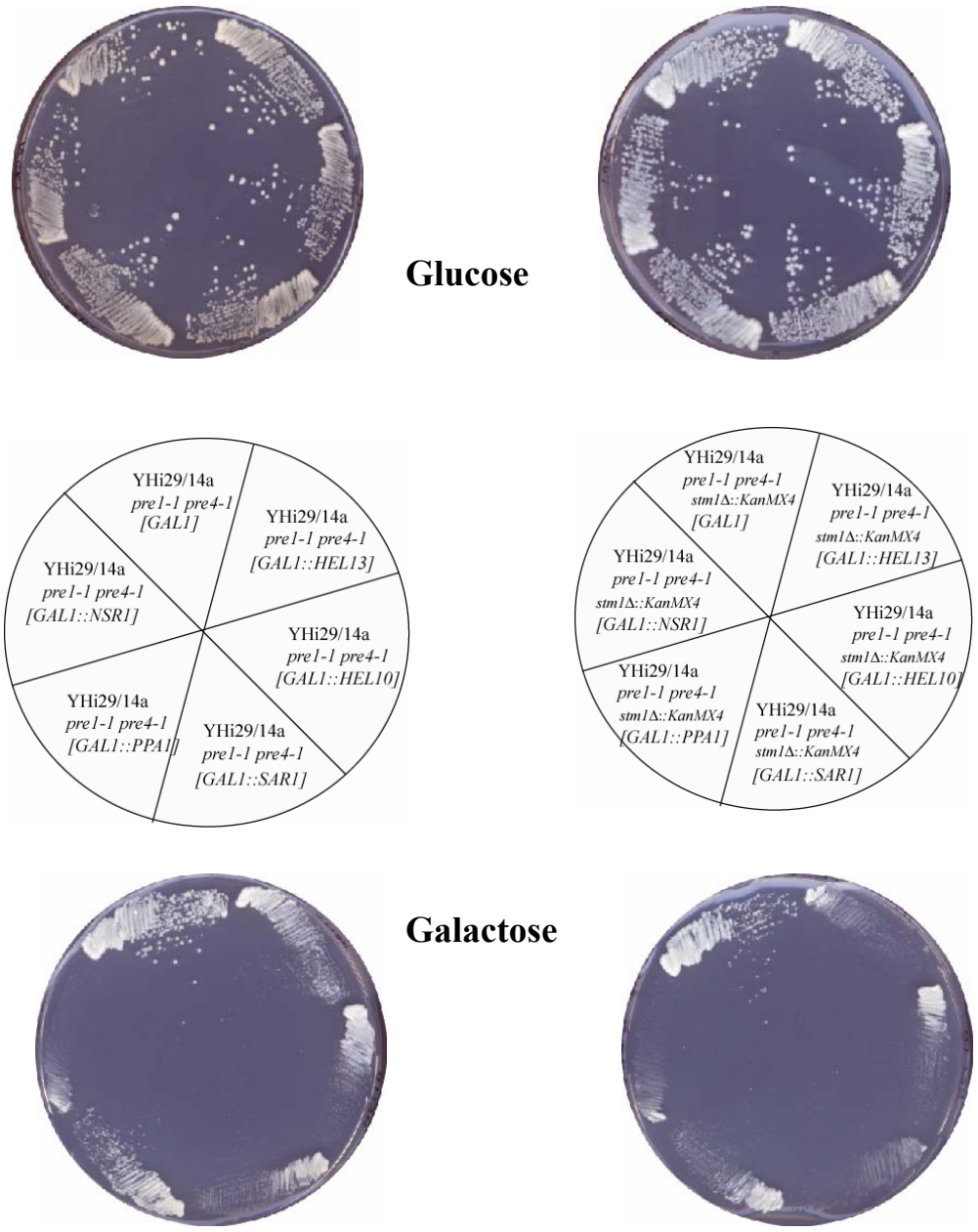


Figure 22: Stm1 does not contribute to the cell death induced by overexpression of distinct high expression lethality (*HEL*) genes in proteasomal deficient cells.

pre1-1 pre4-1 mutant cells containing *STM1* wild type or the *stm1Δ* allele and pYES2-encoded cDNAs of distinct *HEL* genes (*NSR1*, *PPA1*, *SAR1*, *YNL208w* or *YOR309c*) under the *GAL1* inducible promoter were streaked on solid SC-URA$^-$ medium containing either glucose (repressive conditions) or galactose (induction of distinct *HEL* genes). *pre1-1 pre4-1* containing empty vector were used as a control. The plates were incubated at 30ºC for 3 days.

4 Discussion

Selective proteolysis is an essential cellular process, which contributes to balance between synthesis and destruction of cellular proteins at requiring time points. Major pathway of intracellular proteolysis is degradation via the ubiquitin proteasome system. In such manner, the level of the newly identified protein Stm1 is surveyed by the ubiquitin proteasome system. Using an overexpression screen, Stm1 was identified as a new proteasomal substrate (Ligr et al., 2001). Stm1 induces apoptosis-like cell death when overexpressed in yeast mutants with defective proteasomal activity. The particular interest of the work presented here was to further explore the involvement of Stm1 in diverse cellular functions, particularly in apoptotic cell death.

4.1 Stm1 functions neither in telomeric silencing nor in the NHEJ repair of double strand breaks but most probably in maintainance of chromosomal stability

Stm1 is known to bind quanine-rich quadruplex and purine motif triplex DNA representing chromosome ends *in vitro* (Frantz and Gilbert, 1995; Nelson et al., 2000). Moreover, Stm1 was localized at the nuclear periphery, suggesting direct interaction of Stm1 with DNA, especially telomeric regions of the chromosomes (Ligr et al., 2001). Therefore, in the present work, Stm1 was analyzed for its possible involvement in telomere specific functions. In yeast, perinuclear chromatin domains constitute areas where transcriptional repression, so called “silencing” occurs. The “silent” domains are controlled by nuclear pore protein complexes such as Yku70/Yku80, Sir2-4, Mlp1/Mlp2 (Galy et al., 2000). This feature of nuclear pore complexes had been proven by the findings, that deletion of genes coding for pore complex subunits results in the release of telomeres from their perinuclear localization and transriptional de-repression of telomeric genes. Using a system that allows measuring of de-repression of silent telomeric genes, it could be showed that Stm1, however, is not involved in the transcriptional repression of auxotrophic marker genes (*URA3* or *ADE2*) resided at telomeric sites. Hence, these findings indicate that Stm1, in contrast to e.g. Yku70 or Mlp protein complexes, has no

direct role in telomeric silencing. However, one might not exclude the involvement of Stm1 in some other telomeric functions (e.g. regulation of telomere length and structure).

Stm1 was shown to genetically and physically interact with the telomere-binding protein Cdc13, which regulates telomere replication. These results suggest that such Stm1-Cdc13 interaction might be involved in maintainance of telomere structure (Hayashi and Murakami, 2002). Based on these findings, a function of Stm1 in the regulation of telomere elongation was elucidated. For this aim, an *in vivo* telomere elongation assay was applied. In this assay, double strand breaks were induced *in vivo* by HO endonuclease cleavage. Telomerase mediated extension of newly formed DNA telomeric ends was then followed by Southern blotting. This experiment clearly showed that the telomere elongation process was independent of Stm1. Surprisingly, in the assay, HO endonuclease mediated *in vivo* cleavage was accelerated in the absence of Stm1 (Fig.2). This finding leads to the idea that under such conditions, chromosomal structure becomes more accessible to endonuclease cleavage suggesting a possible role of Stm1 in chromosomal stability. Alternatively, one may suggest that under these conditions Stm1 functions in regulation of expression of HO endonuclease.

Ligr et al. found that *stm1* knock-out mutants are sensitive to DNA damaging reagents. The highest sensitivity was found for the drug bleomycin producing double strand breaks. In contrast, *stm1Δ* cells were not sensitive to the alkylating agent MMS, suggesting that Stm1 might function in a specific aspect of DNA repair, e.g. repair of double strand breaks. Non-homologous end-joining (NHEJ) is the most prominent mechanism for repair of double strand breaks in haploid cells. Using an assay for NHEJ established by Boulton et al., it was found that Stm1, nevertheless, does not contribute to the NHEJ repair process. These data, although, do not exclude a possible role of Stm1 in some other DNA repair mechanisms.

One may suggest that Stm1 functions rather indirectly either as a sole player or as a complex with some other proteins.

4.2 Overexpression of mutant allele *stm1-9* induces apoptotic cell death in wild type cells

Stm1, as an *in vivo* substrate of the proteasome, was found to cause apoptosis-like cell death when overexpressed in mutant cells with impaired proteasomal activity (Ligr et al., 2001). However, defects in proteasomal function may lead to the alteration of concentration or activity of other substrates (Hilt and Wolf, 1996). Stabilization of such substrates might have side effects on Stm1 function or be a prerequisite for induction of apoptosis. Therefore, it was interesting to know whether a mutated version of *stm1* could be generated that causes apoptosis in cells harboring a fully active proteasomal system. For this reason, in this work a *stm1* mutant version under the *GAL1* inducible promoter that showed the required effects was created by random mutagenesis and screened by checking its proposed ability to block the growth of yeast wild type cells. Overproduction of mutated Stm1-9 protein indeed resulted in cell lethality accompanied by apoptosis-like cytological and morphological alterations (Fig. 4B). Thus, *stm1-9* overexpression in wild type cells led to cytochrome c release and the appearance of TUNEL-positive nuclei indicating apoptosis induced DNA cleavage.

Recently, a protein with caspase-like activity was found in yeast and named Yca1 (Madeo et al., 2002b). Moreover, in this study, Madeo et al. showed that Yca1 activity might be inhibited by FITC labeled VAD-FMK, a tester substrate used for mammalian caspase, thereby allowing measurement of yeast caspase activity. However, this finding was challenged by the observation that FITC-VAD-FMK labeling is accompanied by propidium iodide (PI) staining, and, thus, possibly an artefactual event that requires disintegration of the plasma membrane (Wysocki and Kron, 2004). Moreover, FITC-VAD-FMK staining appeared to take place in *yca1Δ* null mutant indicating that FITC staining is not dependent of Yca1. Nevertheless, FITC-VAD-FMK staining might be a sign of apoptosis in yeast. Therefore, wild type cells overexpressing *stm1-9* allele were labeled with the FITC-VAD-FMK substrate and analyzed using flow cytometry. Moreover, the cells were stained in parallel with propidium iodide, which is a reporter of plasma membrane integrity. Bivariate flow cytometry analysis revealed that FITC-VAD-FMK staining of wild type cells overexpressing *stm1-9* allele was accompanied by PI staining: FITC-positive stained cells could not be separated from those stained with PI.

These findings mean that only dead cells, which have lost intact plasma membrane, were able to accumulate the FITC-VAD-FMK dye thereby corresponding to the results found by Wysocky et al. Thus, under such conditions FITC-VAD-FMK staining appears to be some kind of artefact and no measure for caspase activation.

As a further sign of apoptosis the production of reactive oxygen species (ROS) was measured. Indeed, in wild type cells expressing *stm1-9* allele a large fraction of ROS-positive cells was found suggesting that the cell death triggered by *stm1-9* overexpression was accompanied with increased ROS production.

Due to the fact, that the apoptosis inducing effect of overexpression of *STM1* is mainly dependent on proteasome deficiency, it was suggested that this effect was based on the accumulation of the stabilized Stm1 protein (Ligr et al., 2001). Thus, it was reasonable that the pro-apoptotic effect of a hyperactive version of Stm1 could be found due to a defect in its degradation and, therefore, proteolytical stabilization. In agreement with this idea, the hyperactive version of Stm1-9 was indeed proven as a proteolytically stable version. Thus, its pro-apoptotic effect is most probably the result of accumulation of the stabilized protein. However, it may also be possible that the pro-apoptotic effect of Stm1-9 is at least partially based on some gain of activity obtained by insertion of the point mutation.

4.3 The cell death triggered by overexpression of *stm1-9* in wild type cells is Yca1 and Aif1-dependent

In yeast, metacaspase Yca1 was identified, which contributes to apoptosis-like cell death of this microorganism (Madeo et al., 2002). In the absence of Yca1, the growth defect caused by overexpression of *stm1-9* in wild type cells was significantly suppressed evidencing that Yca1 is required for the generation of the *stm1-9* induced growth defect (Fig. 13A / B).

Absence of Yca1 leads to a strong reduction of the *stm1-9* induced apoptotic phenotypes such as cell lethality, FITC-VAD-FMK staining and accumulation of ROS-positive cells. These results suggest that Yca1 is required for the proper occurrence of apoptotic changes.

Although, being no proof for Yca1 activation, FITC-VAD-FMK staining of cells after inducing apoptosis via *stm1-9* overexpression is clearly cured when Yca1 is absent meaning that this staining is Yca1 dependent. Taken together, these findings showed that FITC-VAD-FMK staining occurs during the *stm1-9* induced cell death and is suppressed when this cell death is neutralized. Thus, though being of unclear nature FITC staining can be used (at least in the case of *stm1-9* overexpression) as a sign of apoptosis. May be it reflects take up of the dye during the death process because of breakdown of membrane integrity.

Taken together the results indicate that Yca1 acts as a down-stream element of the apoptotic-like cell death induced by *stm1-9* overexpression.

Recently, an AIF orthologue named apoptosis-inducing factor1 (Aif1) was identified in yeast (Wissing et al., 2004). The Aif1 protein is functionally involved in yeast apoptosis. In this study, it could be shown that the pro-apoptotic effect of wild type cells overexpressing *stm1-9* is suppressed after deletion of the *AIF1* gene (Fig. 15A / B). Additionally, *aif1* null mutants expressing *stm1-9* showed reduced lethality, FITC-VAD-FMK labeling and decreased amounts of ROS-positive cells. Thus, the *stm1-9* induced cell death is clearly Aif1 dependent.

Thus, these findings indicate that Aif1 acts as a down-stream element required for *stm1-9* stimulated apoptotic cell death.

To get information on the functional relationship of metacaspase Yca1 and apoptosis-inducing factor Aif1 in *stm1-9* induced apoptotic cell death, the effect of a double deletion of both, *YCA1* and *AIF1* was investigated. In *yca1Δ aif1Δ* double mutant *stm1-9* mediated apoptotic effects were suppressed to the same extent as found in *yca1Δ* and *aif1Δ* single mutants (Fig. 17A / B). Additionally, *yca1Δ aif1Δ* double mutant cells expressing *stm1-9* were analyzed for double FITC-VAD-FMK and PI staining. The rate of FITC-VAD-FMK and PI positive staining of *yca1Δ aif1Δ* double mutants was nearly identical to the rates of staining of *yca1Δ* and *aif1Δ* single mutants, supporting the observation made by inspecting the growth of *yca1Δ aif1Δ* double mutants (Fig. 18).

Taken together, this epistatic behavior suggests that both Yca1 and Aif1 contribute to the same *stm1-9* triggered cell death pathway. Thus, Yca1 and Aif1 are ordered in a sequential way in the *stm1-9* induced apoptosis pathway. This finding, however, does not

exclude that both, Yca1 and Aif1 may execute redundant function in other type of apoptosis, as for instance cell death triggered by reactive oxygen species, nutrient deficiency or aging.

4.4 Stm1 is linked to DNA damage pathways

Two-hybrid data suggest an interaction between Stm1 and the DNA damage checkpoint protein Mec3 (Ito et al., 2001). Mec3 contributes to signaling of DNA damage/repair. In the present studies, co-immunoprecipitation experiments were done using double tagged strain carrying immuno-epitope-marked Stm1-HA_3 and Mec3-MYC_9. Stm1-HA_3 was detected by anti-HA blotting in Mec3-MYC_9 immunoprecipitates together with the immunoprecipitated Mec3-MYC_9 or vice versa. This finding confirms a physical interaction between Stm1 and Mec3.

In addition, to prove a possible interaction between Stm1 and Mec3 by genetic means, a *mec3* null mutant was checked for suppression of growth defects caused by *stm1-9* overexpression. The growth defect induced by *stm1-9* overexpression was significantly suppressed in *mec3Δ* mutant cells. These data suggest the genetical interaction between Stm1 and Mec3.

Taken together, these findings support the idea that Stm1 is a part of a complex that contributes to signaling of DNA damage. Alternatively, genetic interaction suggests that Stm1 has a role in triggering apoptosis in response to DNA damaging. Thus, Stm1 may contribute to the decision between DNA repair or induction of cell death.

In these studies, to test whether Stm1 / Yca1 induced cell death may be required to erase mutated cells, *stm1Δ yca1Δ* double mutants were subjected to UV-induced mutagenesis. *stm1Δ yca1Δ* double mutants showed enhanced sensitivity under UV induced mutagenic conditions. This finding suggests that Yca1 and Stm1 may be required for the proper occurrence of apoptotic cell death after DNA damage.

4.5 Stm1 and other apoptosis-like pathways

Besides Stm1, five other distinct high expression lethality genes (*NSR1*, *PPA1*, *SAR1*, *YNL208w* or *YOR309c*) have been detected, that induce an apoptotic cell death when overexpressed in mutants with defective proteasomal activity (Ligr et al., 2001). To check whether Stm1 is required for induction of apoptosis induced by expression of these pro-apoptotic *HEL* genes, *NSR1*, *PPA1*, *SAR1*, *YNL208w* or *YOR309c* were overexpressed in proteasomal *pre1-1 pre4-1* mutant carrying *stm1Δ* deletion. As investigated by overexpression analysis in *pre1-1 pre4-1* cells lacking *STM1*, apoptotic cell death in these cases was independent of Stm1. These findings suggest that Stm1 might function in a different apoptotic pathway, which is not stimulated by overproduction of these *HEL* genes. Alternatively, one might imagine that pro-apoptotic signals stimulated by overexpression of these *HEL* genes might be induced in apoptotic pathways that are down-stream to Stm1.

Similar results were obtained by analyzing a role of Stm1 in apoptotic cell death induced in a strain where the *PMR1* gene coding for a Ca2+-ATP-ase (Rudolph et al., 1989), was deleted (data not shown), evidencing that Stm1 is not required for apoptotic cell death induced by absence of Pmr1.

5 References

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Declaration

Hiermit versichere ich, dass ich die vorgelegte Arbeit selbstständig angefertigt und keine anderen als die angegebenen Quellen and Hilfsmittel verwendet wurden.

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