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Rieka Reekmans

Proefschrift ingediend tot het verkrijgen van
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“What doesn’t kill you,
makes you stronger”

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Rieka Reekmans
Gent, 30th June 2004

Abbreviations and acronyms

ACD	active cell death
AFLP	amplified fragment length polymorphism
AHP	alkylhydroperoxide peroxidase
AIF	apoptosis-inducing factor
ANOVA	analysis of variance
ANT	adenine-nucleotide transporter
AP	anchoring primers
APC	adenomateous polyposis coli
APG	autophagy
APX	ascorbate peroxidase
ARE	AP1 recognition element
ARF	ADP-ribosylation factor
aRNA	amplified RNA
ARP	arbitrary primers
ASP	apoptosis-specific protein
BAP	B-cell associated protein
BAR	bifunctional apoptosis regulator
BASS	Bax antagonist selected in *Saccharomyces*
Bax	Bcl2 associated protein x
BCA	bicinchonic acid
BCCM	Belgian coordinated collection of microorganisms
Bcl	B-cell lymphoma/leukaemia
BH	Bcl2 homology domain
BI	Bax inhibitor
BIND	biomolecular interaction network database
BIR	baculovirus inhibitor of apoptosis repeat
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
bZIP	basic region leucine zipper
cAMP	cyclic AMP
CAS	cellular apoptosis susceptibility
CCD	charge-coupled device
CCP	cyt-c peroxidase
cDNA	complementary DNA
CHIP	chromatin immunoprecipitation
CL	cardiolipin
CoA	coenzyme A
COX	cyt-c oxidase
cpm	counts per minute
CRD	cysteine-rich domain
CSM	complete synthetic medium
C_t	threshold cycle
CTA	catalase-A
CTT	catalase-T
Cys	cysteine
cyt-c	cytochrome-c
DD	differential display
DED	death effector domain
DLD	D-lactate dehydrogenase
DNase	deoxyribonuclease
DNP	dinitrophenol
DNPH	dinitrophenolhydrazine
DT	diphtheria toxin

dXTP	deoxyribonucleoside triphosphate
EBP	ethylene-responsive element binding protein
EC	enzyme classification
EMBL	European molecular biology laboratory
endoG	endonucleaseG
ER	endoplasmic reticulum
FAD	flavin adenine dinucleotide
Gal	galactose
GFP	green fluorescent protein
GlcNac	N-acetylglucosamine
GLR	glutathione reductase
Glu	glucose
GPX	glutathione peroxidase
GRASP	Golgi reassembly and stacking protein
GRX	glutaredoxin
GSH	reduced glutathione
GSSG	oxidised glutathione
GST	glutathione S-transferase
HA	haemagglutinin
His	histidine
HMM	hidden Markov models
HOCl	hypochlorite
HOG	high osmolarity glycerol
HSF	heat shock transcription factor
HSP	heat shock protein
HXT	hexose transporter
IAP	inhibitor of apoptosis
IGFIR	insulin-like growth factor I receptor
IRES	internal ribosome entry site
LMBP	laboratory of molecular biology plasmid collection
Lowess	locally weighted scatterplot smoother
MAP	mitogen-activated pathway
MB	molecular beacon
MBF	MluI-binding factor
MDA	malondialdehyde
Met	methionine
MM	macromolecule
mRNA	messenger RNA
MPSS	massively parallel signature sequencing
MPT	mitochondrial permeability transition
mtDNA	mitochondrial DNA
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	national centre for biotechnology information
nd	no data
NES	nuclear export signal
NMR	nuclear magnetic resonance
$O_2^{\bullet-}$	superoxide radical
O_2^{2-}	peroxide ion
$OH^{\bullet}$	hydroxyl radical
P/P_i	phosphate
PAGE	polyacrylamide gel electrophoresis
PAPS	3’-phosphoadenosine-5’-phosphosulphate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
PKA	protein kinase-A

PKC	protein kinase-C
PNA	peptide nucleic acid
PR	pathogenesis-related
PSI-BLAST	position-specific iterated BLAST
Q-PCR	quantitative PCR
RAGE	rapid analysis of gene expression
RAP-PCR	random arbitrarily primed PCR
RDA	representational difference analysis
READS	restriction endonucleolytic analysis of differentially expressed sequences
RH_2	reductant molecule
RNase	ribonuclease
ROI	reactive oxygen intermediates
ROOH	hydroperoxide
ROS	reactive oxygen species
rpm	rotations per minute
RT	reverse transcription/real-time
SAGE	serial analysis of gene expression
SAM	sterile alpha motif
SBF	Swi4/6 cell cycle box binding factor
SD	standard deviation
SGD	*Saccharomyces* genome database
SH	subtractive hybridisation
SH3	Src-homology 3 domain
SHOM	sequencing by hybridisation with an oligo matrix
SICD	sugar-induced cell death
SOD	superoxide dismutase
SSH	suppression subtractive hybridisation
STRE	stress-responsive element
SUN	*SIM4*, *UTH1*, *NCA3* and *SUN4* gene family
TBA	thiobarbituric acid
tBuOOH	*tert*-butylhydroperoxide
TIFF	tagged image file format
TM	transmembrane
TOF-MS	time-of-flight mass spectrometry
TOGA	total gene expression analysis
TPX	thioredoxin peroxidase
Trp	tryptophane
TRR	thioredoxin reductase
TRX	thioredoxin
TSA	thiol-specific antioxidant
UAS	upstream activating sequence
UDP	uridine diphosphate
UPR	unfolded protein response
UV	ultra violet
VAMP	vesicle-associated membrane protein
VDAC	voltage-dependent anion channel
WT	wild type
YAP	yeast AP1-like protein
YME	yeast metalloprotease
YMGV	yeast microarray global viewer

Rationale – Research outline

Moving beyond the morphological description of apoptosis to the biochemistry of the involved pathways requires a characterisation at the molecular level. In many experimental systems cell death can be prevented by inhibitors of macromolecular synthesis, suggesting that *de novo* transcription and translation are needed to provide activators of the cell death program (Fesus, 1999). However, these data should not be taken as direct evidence for a gene-directed program of cell suicide.

Transcriptional profiling techniques identified apoptosis-responsive genes in development (Bielke *et al.*, 1997), atherosclerosis (Martinet *et al.*, 2002), and drug-induced apoptosis (Baudet *et al.*, 1998; Wang *et al.*, 1999; Sun, 2000; Unami *et al.*, 2003). However, a recurring criticism to the use of mRNA expression profiling is that the transcriptome may not faithfully represent the proteome in higher eukaryotes. Studies comparing the steady-state levels of proteins with those of their corresponding mRNAs have indicated that mRNA abundance provides little predictive value in higher organisms (Anderson and Seilhamer, 1997; Anderson and Anderson, 1998), highlighting the importance of translational and posttranslational control mechanisms. The observation that a number of apoptotic proteins contain an Internal Ribosome Entry Site (IRES) to control their level of expression during cellular stress (Holcik *et al.*, 2000) further contributes to the negative connotation of transcriptome analyses for apoptotic pathway identification in higher eukaryotes.

The application of a genetically tractable organism to answer functional biology questions in mammalian cells has proved an extremely useful strategy in many fields, with success relying on the functional conservation. The yeast *Saccharomyces cerevisiae* has been extensively used to address issues such as cell cycle control, protein secretion and regulation of gene expression (Walworth *et al.*, 1989). Therefore, in addition to experiments in higher cells, the cell death community examines the extent to which *S. cerevisiae* can be used for dissecting the action mechanism of mammalian proteins that are key players in apoptosis (Ameisen, 1996). Certain mammalian pro-apoptotic proteins (e.g. Bax) can induce yeast cells to die in a manner related to their intrinsic biochemical activities in mammalian cells (Xu *et al.*, 1999), permitting the use of yeast for cell death research. Furthermore, a large-scale comparison of mRNA and protein responses in *S. cerevisiae* demonstrated a nice correlation (Futcher *et al.*, 1999; Ideker *et al.*, 2001; Griffin *et al.*, 2002), indicating transcriptome analysis to be suited for the investigation of apoptosis-like cell death in this lower eukaryote.

Bax toxicity is also observed upon ectopic overexpression in *Schizosaccharomyces pombe* (Jurgensmeier *et al.*, 1997), *Pichia pastoris* (Martinet *et al.*, 1999), *Klyveromyces lactis* (Poliakova *et al.*, 2002), *Candida albicans* (De Smet *et al.*, submitted) and even in *Escherichia coli* (Asoh *et al.*, 1998). Keeping the bacterial endosymbiosis in mind, these findings could be seen as a major argument for the evolutionary conservation of a basic pathway. The molecular study of Bax-induced cell death in *S. cerevisiae* may thus unravel ancestral cell death pathways. As both Bax-induced cell

death in *S. cerevisiae* and DED-induced cell death in *E. coli* are accompanied with oxygen stress (Madeo *et al.*, 1999; Lee *et al.*, 2000), the basic concept conserved from bacteria to man may be the accumulation of reactive oxygen species. On the other hand, one could interpret these findings as an argument against an apoptosis-like pathway. Ectopic expression of Bax coul lead to non-specific toxicity in whatever organism it is highly expressed in. In this view, Bax toxicity in yeast may well be the result of an overwhelming cellular stress.

Research Objectives

In this work, we investigated the transcriptional responses of the cell to the expression of the cell death-inducing murine Bax protein, using the yeast *S. cerevisiae* as a model system. The elucidation of the Bax-induced transcriptional responses was regarded to have the potential for extrapolation to mammalian cells, thereby extending the current knowledge concerning Bax functionality. In addition, the identification of cell death-responsive yeast genes *per se* was supposed to identify potential targets for antifungal drugs.

Further, we tried to focus on the mechanism by which oxidative stress is generated following Bax expression, by comparing the transcriptional response upon Bax expression with the changes elicited by a $HBBBBBB_2O_2$ treatment of the same toxicity.

Strategy Outline

At first, the kinetics of the transcriptional responses associated with Bax-induced cell death in yeast were analysed by macroarray technology (Reekmans *et al.*, 1999). This time-course methodology was expected to give a global overview of the cellular pathways affected by the dying process. Late time-points (> 15h) were not included, as apoptosis is known to be associated with the loss of 28S and 18S ribosomal RNA at late stages (Delic *et al.*, 1993; Lafarga *et al.*, 1997). The major drawback of this strategy was the lack of discrimination between the pathways directly (causative) affected and the secondary or homeostatic circumstantial changes.

Second, we focussed on the Bax-specific part of the early Bax-induced transcriptional responses (1 h) via a comparative macroarray and microarray methodology, now taking the transcriptional response to an oxidative treatment as reference. In this case, the genes specifically responsive to Bax expression were seen as the Bax-induced transcriptional changes occurring before oxidative

(mitochondrial) damage. The Bax-specific genes were considered as interesting targets for cell death prevention, whatever executional cell death pathway is initiated (Reekmans *et al.*, 2001).

A schematic representation of our strategy is depicted below (Fig. 1).

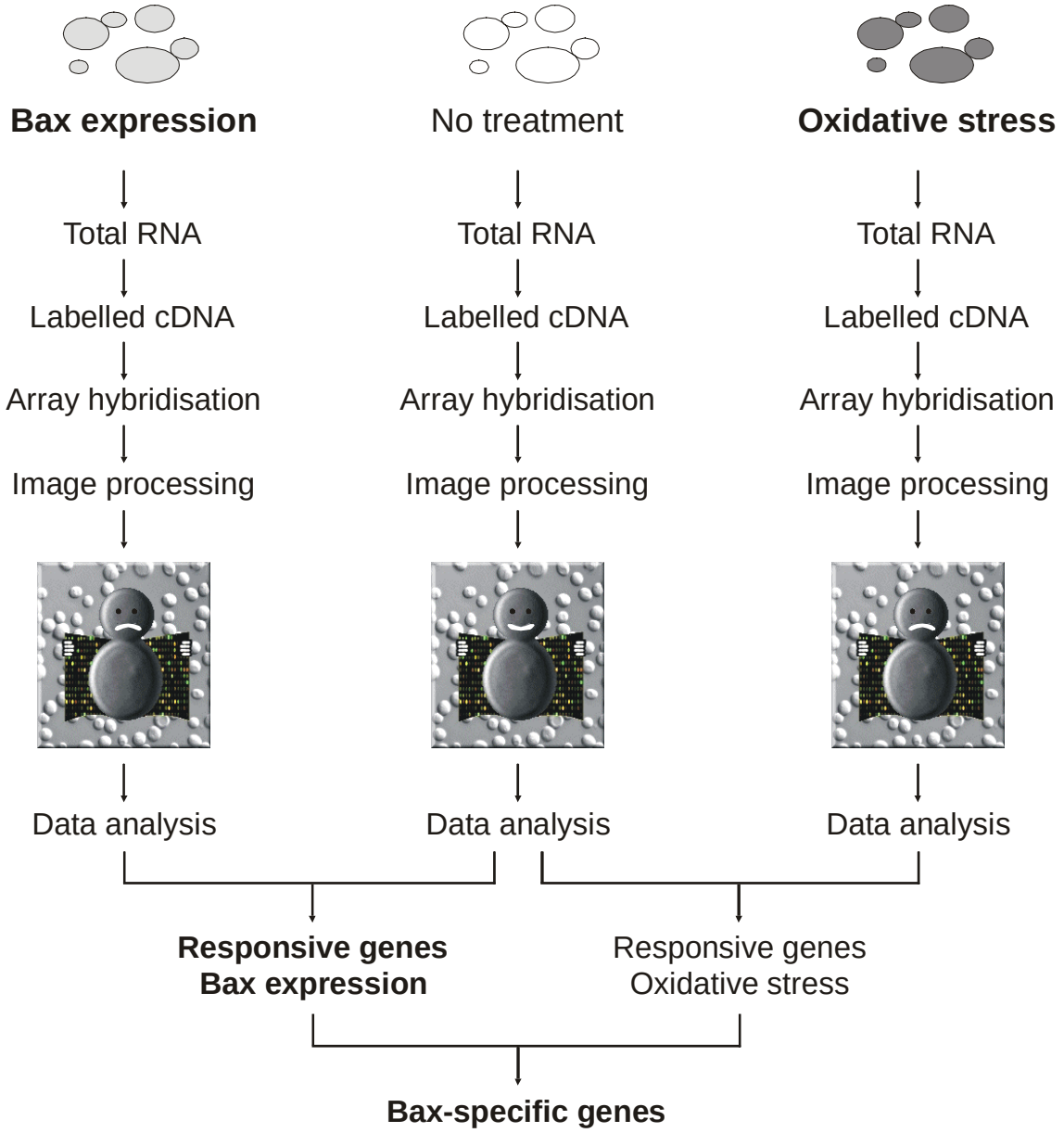

Fig. 1: Schematic representation of the rationale of this work. The kinetics of the Bax-induced transcriptional responses were studied via hybridisation of labelled complementary DNA (cDNA) to the yeast macroarray and comparison with the non-treated control at several time points (30 min, 1 h, 2 h, 3 h, 6 h and 15 h). To focus on Bax-specific transcriptional responses, the effects of an oxidative stress – which is regarded a more or less related treatment – were compared with the Bax-elicited responses (1 h) via macro- and microarray.

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Chapter 1
Introduction to active cell death

This chapter describes the concept of active cell death and the role of the Bcl2 protein family herein. The generality of the Bax toxicity and the multifunctionality of this Bcl2 family member are discussed. Further, the Bax-induced cell death process in *S. cerevisiae* is detailed and a Bax toxicity model is proposed. Finally, active cell death processes in other unicellular organisms are described and used as a reflective platform to comment on evolutionary conservation.

1. Concepts of cell death

Up until twenty years ago, cell death research held much the same status among serious scientists as aromatherapy does among physicians: an interesting and amusing concept, decorative but definitively not mainstream (Fraser and Evan, 1996). Although death may be considered as being in opposite to life, from a biological point of view death appears as life's undissociable companion. Indeed, homeostasis of cell numbers requires a carefully orchestrated balance between proliferation (input) and cell death (output) processes.

In the early days of cell death research, the Greek word *apoptosis* (Kerr *et al.*, 1972) was used to describe the morphology associated with developmental cell death. The concept of *programmed cell death* was first used by these developmental biologists to refer to the predictable onset of cell death during tissue remodelling (Lockshin, 1981). However, the term 'programmed' is often interpreted as meaning that death is a consequence of the activation of a genome-encoded biochemical pathway for cell suicide. As it is evident by now that not all components of the cell death pathway are regulated at the transcriptional level, we prefer to use the term *active cell death* indicating the cell is an active participant in its own demise and including any lethal process required for the active dying process.

2. Active cell death phenotypes in mammalia

2.1 Apoptosis

2.1.1 Morphological description

The distinct morphological changes of cells undergoing apoptosis are sequentially characterised by (i) normotonic cell shrinkage; (ii) cytoplasmic condensation; (iii) crowding of normal-appearing organelles; (iv) nuclear condensation (pyknosis); (v) hypercondensation of the chromatin in tight apposition to the nuclear envelope (margination) into compact and apparently simple geometric figures (crescents, spheres); (vi) oligonucleosomal 'ladder-type' cleavage of the chromosomal DNA (genomic death); (vii) nuclear fragmentation (karyorrhexis); (viii) violent blebbing and ruffling of the plasmamembrane (zeiosis, pop-corn cytolysis); (ix) packaging of the cellular contents into membrane-enclosed vesicles (apoptotic bodies); and (x) elimination of the corpses by phagocytosis (Godman *et al.*, 1975; Wyllie *et al.*, 1980). Fig. 1.1a depicts apoptosis ultrastructurally.

As a curiosity, it were the apoptotic bodies that formed the actual inspiration for naming this cell death 'apoptosis', because this Greek word refers to the shedding of leaves and the falling of petals from flowers (Kerr *et al.*, 1972). Another typical feature of apoptosis includes the exposure of phosphatidylserine on the outer plasmamembrane leaflet (Martin *et al.*, 1995). In addition, changes in membrane fluidity and ionic charge, altered behaviour of sugar chains and conformational changes in membrane proteins are also likely to occur (Hengartner, 2001).

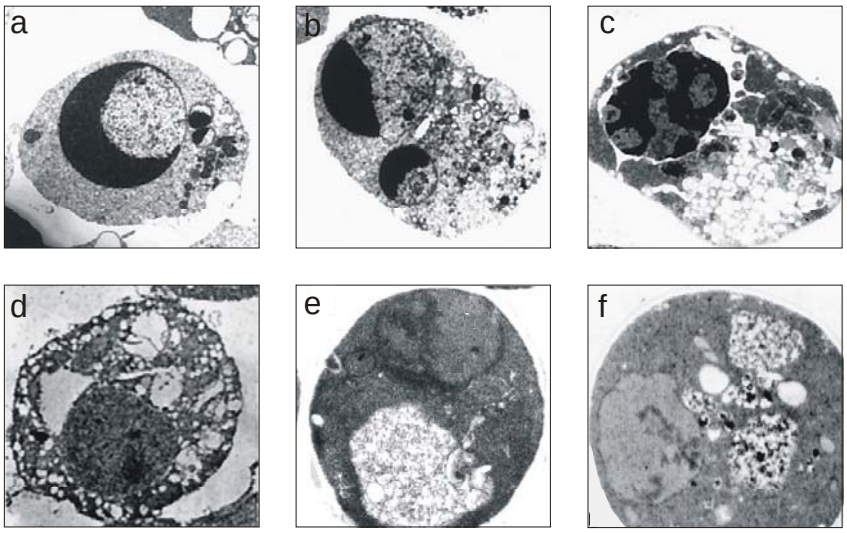

Fig. 1.1: Electron micrographs illustrating active cell death processes in mammalia (a-d) and *S. cerevisiae* (e-f). Panel (a): Morphology of apoptosis triggered by Fas antibody (adapted from Xiang *et al.*, 1996). Cellular ultrastructure of Bax-induced cell death in mammalia without (b) or with (c) caspase-inhibitors added (adapted from Xiang *et al.*, 1996). Panel (d): Cell morphology of ligand-triggered paraptosis (adapted from Sperandio *et al.*, 2000). Ultrastructure of a wild-type yeast cell after Bax protein expression (e) or H_2O_2 treatment (f) Adapted from (Madeo *et al.*, 1999).

2.1.2 Orchestrated dance of death

The process of apoptosis can be subdivided into three different phases: (i) initiation; (ii) the effector stage; and (iii) the degradation phase. The initiation phase can be triggered by external signals (environmental, physical or chemical stresses) or internal processes (mitotic catastrophe, replication failure, developmental cell death), activating a complex regulatory network of signal transduction cascades and damage pathways (Frohlich and Madeo, 2000). Whereas the initiation phase depends on the type of apoptosis-inducing stimulus, the effector phase and degradation stage are common to all apoptotic processes. During the effector phase, the membrane integrity of the mitochondria is lost and the resulting mitochondrial dysfunction is the 'decision to die'. Mitochondria are known to function as 'signal integrators' and disruption of its homeostasis is like opening Pandora's box. Although the effector phase takes place at the mitochondrial level, the contributions of other organelles are substantial (Gross *et al.*, 1999). Mitochondria are thought to streamline all organelle-specific responses into a common pathway (Ferri and Kroemer, 2001).

Many of the morphological features of apoptosis are now thought to be signs of the degradation phase (Kroemer, 1997a). The degradation phase is in fact beyond the 'point of no return' during which soluble factors released by mitochondria activate caspases, catabolic cysteine proteases responsible for the apoptotic degradation of substrates vital for cellular homeostasis (Zamzami *et al.*, 1998). The activation of caspases is the *sine qua non* for the complete manifestation of the apoptotic morphology (Cohen, 1997; Earnshaw *et al.*, 1999), yet is dispensable for cell death to occur in many

systems (Xiang *et al.*, 1996; McCarthy *et al.*, 1997; Goping *et al.*, 1998). The blockade of apoptosis by caspase inhibitors is known not to permit long-term survival (Fletcher *et al.*, 2000; Xue *et al.*, 2001) and even accelerated cell death in some settings (Vercammen *et al.*, 1998).

Though caspases have been the best-known proteases involved in apoptosis, studies revealed that also the ubiquitin-proteasome pathway has an important role (Drexler, 1997; Orlowski, 1999). Heat Shock Proteins (HSPs) also influence the apoptotic process (Samali and Orrenius, 1998), for instance through direct physical interaction with key components of the apoptotic machinery (Beere *et al.*, 2000; Xanthoudakis and Nicholson, 2000; Beere and Green, 2001). Thus, it seems likely that the interplay between apoptosis and stress responsive signalling pathways regulates cellular survival in response to damage (Ravagnan *et al.*, 2001).

2.1.2.1 Mitochondria

Mitochondria appear to play a central role in the induction of apoptosis (at least in the so-called intrinsic systems), which can be controlled by a number of interrelated events. These include (i) disruption of electron transport, oxidative phosphorylation and ATP synthesis; (ii) the release of cytochrome-c, triggering the activation of the caspase family of proteases (Kluck *et al.*, 1997), together with other mitochondrial proteins (Single *et al.*, 1998; Patterson *et al.*, 2000; Van Loo *et al.*, 2002), such as Apoptosis-Inducing Factor (AIF) (Susin *et al.*, 1996) and endonucleaseG (endoG) which induce nucleosomal DNA fragmentation independent of caspase activity (Li *et al.*, 2001; van Loo *et al.*, 2001); (iii) the alteration of the cellular reduction-oxidation potential, which results in the release of Reactive Oxygen Species (ROS) into the cytosol (Zamzami *et al.*, 1996; Wang, 2001); and (iv) disintegration of mitochondrial reticulum leading to mitochondrial fragmentation (Karbowski and Youle, 2003), due to a blockade in mitochondrial fusion (Karbowski *et al.*, 2004).

One of the first biochemical changes known to occur in the mitochondria of apoptotic cells was the release of cytochrome-c (cyt-c), the only water-soluble component of the electron transport chain. This mitochondrial cyt-c release is thought to be preceded by the import of cytosolic Ca^{2+} into the electronegative matrix via the calcium uniporter or by a simple diffusion (Gunter and Pfeiffer, 1990; Loew *et al.*, 1994). High mitochondrial Ca^{2+} load is known to alter the lipid organisation of the inner mitochondrial membrane via interaction with the anionic head of CardioLipin (CL), a phospholipid mainly found in the inner mitochondrial membrane (Grijalba *et al.*, 1999). This Ca^{2+}-cardiolipin complexation changes the lipid packing of the membrane and these extensive membrane rearrangements are thought to initiate increased ROS production (Grijalba *et al.*, 1999). As cardiolipin itself is particularly rich in unsaturated fatty acids, it is very susceptible to ROS attack, generating cardiolipin hydroperoxide (CL-OOH). Because cyt-c does not bind to CL-OOH and CL-OOH is generated in mitochondria before cyt-c release, it was suggested that the generation of CL-OOH triggers the dissociation of cyt-c from the electron transport chain (Grijalba *et al.*, 1999; Shidoji *et al.*, 1999; Petrosillo *et al.*, 2001), the primary event needed for cyt-c to be released

(Gottlieb, 2000; Ott *et al.*, 2002). In addition, the fusion of individual cristae and the opening of the junctions between cristae and the intermembrane space enhance cyt-c mobilisation (Scorrano *et al.*, 2002). The actual release of cyt-c through the outer membrane was observed to be rapid, complete and kinetically invariant (Goldstein *et al.*, 2000) and normally precedes the specific (proteinaceous pore-mediated) or the unspecific (lipidic pore-mediated) Mitochondrial Permeability Transition (MPT). However, a subset of mitochondria maintains a functional electron transport chain and this mitochondrial heterogeneity provides the cell with the ATP needed for the active cell death process (D'Herde *et al.*, 2000). The release of cytochrome-c itself confers an additional blockade of the electron flow (Wang, 2001) and further increases ROS production, promoting the oxidation of mitochondrial membrane lipids and proteins whose damage contributes to or is responsible for the large increase in membrane permeability.

2.1.2.2 Endoplasmic reticulum

The Endoplasmic Reticulum (ER) plays a key role in (i) the post-translational modification, folding and sorting of the newly synthesized proteins (Booth and Koch, 1989; Lodish *et al.*, 1992); (ii) the maintenance of cellular calcium homeostasis; (iii) synthesis of lipids and sterols; and (iv) the cellular responses to stress (Brostrom and Brostrom, 1990; Li *et al.*, 1993). The high Ca^{2+} content of the ER lumen appears to be necessary for maintaining the structural and functional integrity of the ER as well as for a variety of other cellular functions, including translation, cell cycle progression and cell division (Short *et al.*, 1993; Lam *et al.*, 1994). The nuclear envelope, in continuity with the ER lumen, also requires high Ca^{2+} levels for nuclear transport. This was demonstrated by the fact that depletion of Ca^{2+} results in a blockade of the nuclear pore complexes (Greber and Gerace, 1995). Evidence is accumulating that the ER plays an important role not only in cell proliferation but also in apoptosis (Rao *et al.*, 2001). Many apoptotic stimuli are known to empty the ER Ca^{2+} store (Baffy *et al.*, 1993; Lam *et al.*, 1993) and this depletion precedes mitochondrial cyt-c release (Nakamura *et al.*, 2000; Nutt *et al.*, 2002b). The molecular basis for the ER-mitochondria cross talk is not fully known at present, but the integral B-cell Associated membrane Protein BAP31 is certainly involved (Ng *et al.*, 1997; Ng and Shore, 1998; Nguyen *et al.*, 2000). Although BAP31 is an inhibitor of apoptosis (Wang *et al.*, 2003), the caspase cleavage product formed in response to ER stress (Zuppini *et al.*, 2002) induces mitochondrial fission and cyt-c release in the cytosol (Breckenridge *et al.*, 2003).
In addition, close contacts between some mitochondria and the sites of ER Ca^{2+} release are known to exist (Rizzuto *et al.*, 1993; Csordas *et al.*, 1999), facilitating rapid Ca^{2+} accumulation (Rizzuto *et al.*, 1998) and signal propagation by mitochondrial calcium waves (Pacher and Hajnoczky, 2001).

2.1.2.3 Golgi complex

Morphological studies have shown the Golgi complex, with its essential role in membrane traffic, becomes fragmented and scattered during apoptosis (Philpott *et al.*, 1996; Sesso *et al.*, 1999). The

molecular mechanisms underlying this fragmentation are unknown, but the molecules that appear to be involved are Golgin-160 (Mancini *et al.*, 2000) and GRASP65 (Golgi Reassembly And Stacking Protein of 65 kDa), a protein required for Golgi stack formation (Lane *et al.*, 2002). The Golgi fragmentation in apoptotic cells is, at least in part, due to Golgin-160 and GRASP65 cleavage by caspases (Lane *et al.*, 2002).

2.1.2.4 Lysosomes

Lysosomes are known for their involvement in non-specific waste disposal and are essentially 'suicide bags' containing large numbers of catabolic enzymes capable of hydrolysing virtually every type of biological macromolecule. For many years, lysosomes have been thought to be solely involved in necrotic and autophagic cell death, with their role in apoptosis limited to the digestion of engulfed apoptotic bodies.

These 'old-fashioned' lysosomal proteins became 'fashionable' again at the moment the lysosomal cysteine proteases cathepsin B, cathepsin D, and cathepsin L became implicated in apoptosis (Roberg and Ollinger, 1998; Roberts *et al.*, 1999; Guicciardi *et al.*, 2000). Lysosomal membrane permeabilisation induces cell death in a mitochondria-dependent fashion, promoting mitochondrial oxidant production, cyt c release, chromatin condensation, and apoptosis (Boya *et al.*, 2003; Zhao *et al.*, 2003).

2.2 Paraptosis

2.2.1 Morphological description

Paraptosis was first observed after overexpression of the intracellular domain of IGFIR (Insulin-like Growth Factor I Receptor) as an alternative, non-apoptotic form of active cell death. Ultrastructurally, this cell death process lacks typical features of apoptosis like nuclear fragmentation, apoptotic body formation, chromatin condensation and cell blebbing. Instead, cytoplasmic vacuolation is the prominent characteristic (Fig. 1.1d). However, these vacuoles are non-autophagic and constituted by dilated ER or swollen mitochondria (Sperandio *et al.*, 2000).

2.3 Autophagy

2.3.1 Morphological description

Under adverse conditions, cytoplasmic self-components can be degraded and recycled without any steric constraint via autophagy, an active and non-selective process mediated by autophagic vacuoles and lysosomes (Baba *et al.*, 1994).

Primarily, autophagic vacuolation reflects an adaptive survival response, as an attempt to maintain the functional status of the cell under stress (Henics and Wheatley, 1999). Additionally, organelles

(e.g. mitochondria) can be specifically degraded via the process of pexophagy when they become damaged, non-functional or are no longer needed (Campbell and Thomas, 1998; Kim and Klionsky, 2000; Xue *et al.*, 2001). This organelle-specific 'housecleaning' overlaps with autophagy but occurs with faster kinetics (Kim and Klionsky, 2000). Finally, when the process of vacuolisation is no longer reversible, the capacity of the cell to compromise or sacrifice further cytoplasmic substances, presumably becomes exhausted. Where damage limitation fails, cells die due to the progressive degradation of cytoplasmic components. Nuclear vacuolisation is rare, but generally the nucleus becomes destructed during autophagic cell death (Bursch *et al.*, 2000).

2.3.2 Orchestrated dance of death

Autophagy is a tightly regulated process and 14 *APG* (AutoPhaGy) genes have been identified in *S. cerevisiae*, acting in a conjugating cascade and being required for the autophagic process (Tsukada and Ohsumi, 1993). Mammalian homologues of some of the yeast autophagy proteins have been characterised.

Yeast Apg5 was initially suggested to be a homologue of the human Apoptosis-Specific Protein (ASP), a protein known to accumulate in the cytoplasm of cultured mammalian cells following induction of apoptosis (Hammond *et al.*, 1998). Although this observation suggested the early stages of autophagy or pexophagy may play a role in apoptosis, findings indicate ASP and Apg5 are unrelated proteins (Yung *et al.*, 2002).

Beclin1, the human homologue of the yeast Apg6 protein, is known to promote autophagic cell death (Yue *et al.*, 2002) and decreases in its protein level have been correlated with the development or progression of breast tumours (Liang *et al.*, 1999). This finding is indicative for the process of autophagy being implicated in autophagic cell death.

2.4 Continuum of phenotypes

Apoptotic morphology results from one of the most elaborate forms of Active Cell Death (ACD) and it may be viewed as a far end of a continuum of death modes, with varying contributions of the cellular machinery (Leist and Jaattela, 2001). For example, most of the cells in which caspases are blocked or knocked-out will die, even if they do not acquire apoptotic morphology. They look more like cells with elaborately developed autophagy in which the chromatin has condensed in a less compact and lumpier shape (Xiang *et al.*, 1996; Xue *et al.*, 2001).

We propose a signal causing mitochondrial dysfunction activates multiple pathways simultaneously (Fig 1.2). The cell fate is then determined by the relative speed of each process in a given model system and by the antagonists of the individual pathways that are differentially expressed in different cell types. In addition, the availability of ATP (Eguchi *et al.*, 1997; Leist *et al.*, 1997) has the potential to influence the cell death phenotype.

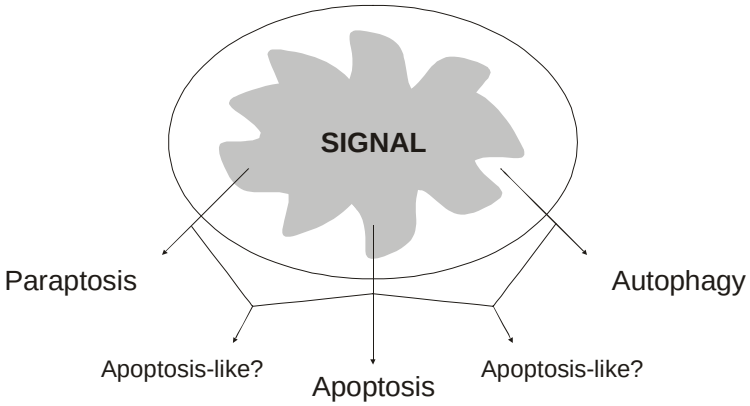

Fig. 1.2: The active cell death phenotype may vary along a continuum of death modes, based on the relative speed by which the initially independent apoptotic, paraptotic or autophagic pathways are emanated from the mitochondria. The strength of the toxic trigger, cellular physiology and/or mechanical constraint is thought to affect this continuum. The faster a pathway progresses, the more its characteristics will influence the classification of the death phenotype.

3. Bax-mediated active cell death

3.1 Bax is a Bcl2 family member

3.1.1 Bcl2: the founding member

3.1.1.1 Bcl2 as a proto-oncogene

The *bcl2* gene was first isolated and analysed due to its consistent involvement in t(14;18) chromosomal translocations commonly observed in human B-cell malignancies (Bakhshi *et al.*, 1985). In relation herewith, 'bcl2' is the acronym for 'B-Cell Lymphoma/Leukaemia-2' (Chen-Levy and Cleary, 1990). In the observed translocations, the *bcl2* is moved from its normal chromosomal position at 18q21 into juxtaposition of powerful enhancer elements in the immunoglobulin heavy-chain locus at 14q32, resulting in its transcriptional deregulation and the accumulation of abnormally high levels of *bcl2* mRNA and protein (Reed, 1994).

3.1.1.2 Intracellular localisation of Bcl2

The Bcl2 protein was demonstrated to be non-uniformly localised at (i) the outer mitochondrial membrane (Krajewski *et al.*, 1993); (ii) the inner mitochondrial membrane (Hockenbery *et al.*, 1990); (iii) the perinuclear ER (Givol *et al.*, 1994); (iv) the inner and outer membranes of the nuclear envelope (Wang *et al.*, 1999; Hoetelmans *et al.*, 2000); and (v) to a lesser extent the inner surface of the plasmamembrane (Chen-Levy and Cleary, 1990; de Jong *et al.*, 1994; Lithgow *et al.*, 1994). About ten years ago, Krajewski and colleagues argued that the speckled distribution could indicate Bcl2 participating in protein complexes involved in some aspect of transport (Krajewski *et al.*, 1993). As will be outlined below (See 3.2.2), this initial suggestion was not that far beyond.

3.1.1.3 Bcl2 is an anti-apoptotic protein

Overexpression of the Bcl2 protein contributes to neoplastic cell expansion (Vaux *et al.*, 1988), due to the inhibition of apoptosis, rather than by accelerating the rate of proliferation (Hockenbery *et al.*,

1990; Mazel *et al.*, 1996a). Conversely, anti-sense mediated suppression of Bcl2 expression was demonstrated to induce or accelerate cell death (Reed *et al.*, 1990). Bcl2 is known to block apoptotic cell death in multiple contexts (Korsmeyer, 1992) and its almost all-round protective capacity has been interpreted as the result of an enormous variety of anti-apoptotic effects. Activities include (i) regulation of cell cycle progression (Mazel *et al.*, 1996b); (ii) stabilising lysosomal membranes (Zhao *et al.*, 2000); (iii) enhancing the proton efflux from mitochondria (Shimizu *et al.*, 1999); (iv) preventing mitochondrial cyt-c release (Yang *et al.*, 1997); (v) regulating ER-associated Ca^{2+} fluxes (Lam *et al.*, 1994); (vi) modulating mitochondrial Ca^{2+} homeostasis (Murphy *et al.*, 1996); (vii) stabilising the mitochondrial membrane integrity (Susin *et al.*, 1996); (viii) reducing ROS levels (Hockenbery *et al.*, 1993); (ix) maintaining B cell memory (Nunez *et al.*, 1991); (x) stimulation of morphogenesis (Lu *et al.*, 1995); (xi) acceleration of senescence (Vairo *et al.*, 1996; Tombor *et al.*, 2003), and (xii) relocalisation of reduced glutathione into the nucleus (Voehringer *et al.*, 1998). However, it cannot be ruled out that some of its known anti-apoptotic effects are in fact inter-connected and merely represent the multi-step way of the dying process.

3.1.2 A quick picture of the Bcl2 family

Bcl2 was the founding member of a progressively expanding protein family (Fig. 1.3), comprising several subfamilies consisting of cell death promoters or cell death preventors.

The Bcl2 subfamily consists of Bcl2 (Hockenbery *et al.*, 1990), $BclX_L$ (Boise *et al.*, 1993), Mcl1 (Zhou *et al.*, 1997), Bfl1/A1 (Lin *et al.*, 1993), Bclw (Gibson *et al.*, 1996) and BclB (Ke *et al.*, 2001) and directly inhibits active cell death. The Bax subfamily, containing Bax-α (Oltvai *et al.*, 1993), Bax-ε (Shi *et al.*, 1999), Bax-σ (Schmitt *et al.*, 2000), Bax-ω (Zhou *et al.*, 1998), Bax-ψ (Cartron *et al.*, 2002), Bak (Chittenden *et al.*, 1995), and Bok (Hsu *et al.*, 1997; Inohara *et al.*, 1998) has direct death-promoting activity. The Bik subfamily, comprising Bad (Yang *et al.*, 1995), Bim (O'Connor *et al.*, 1998), Bid (Wang *et al.*, 1996), Bik (Boyd *et al.*, 1995), Hrk (Inohara *et al.*, 1997), Blk (Hegde *et al.*, 1998), $BclX_S$ (Minn *et al.*, 1996), $BclX_{ES}$ (Schmitt *et al.*, 2004), and others, is functionally inactive in terms of direct killing. Bid, Bim and Bad are known to activate Bax and/or Bak proteins, thereby being indirectly death promoting (Wang *et al.*, 1996; Cheng *et al.*, 2001). $BclX_S$ acts as a dominant competitor for Bcl2 (Boise *et al.*, 1993) while Bik and Blk are known to bind and inactivate the anti-apoptotic subfamily (Boyd *et al.*, 1995; Hegde *et al.*, 1998). In general, members of the Bik subfamily are pro-apoptotic due to (i) the direct inactivation of anti-apoptotic Bcl2 family members, (ii) the release the pro-apoptotic proteins from their anti-apoptotic counterparts, or (iii) direct activation of pro-apoptotic proteins (Huang and Strasser, 2000; Moreau *et al.*, 2003).

Sequence alignment of the Bcl2 family proteins have identified several conserved regions, denoted as the Bcl2 Homology domains BH1, BH2, BH3 and BH4. Members of the Bcl2 subfamily contain all four conserved BH domains but the Bax subfamily generally lacks the BH4 domain. The Bik

subfamily contains 'BH3-only' proteins, displaying sequence homology only within the BH3 domain (exception: $BclX_S$). Thus, the minimum requirement for a death-promoting protein seems to be the presence of a BH3 domain.

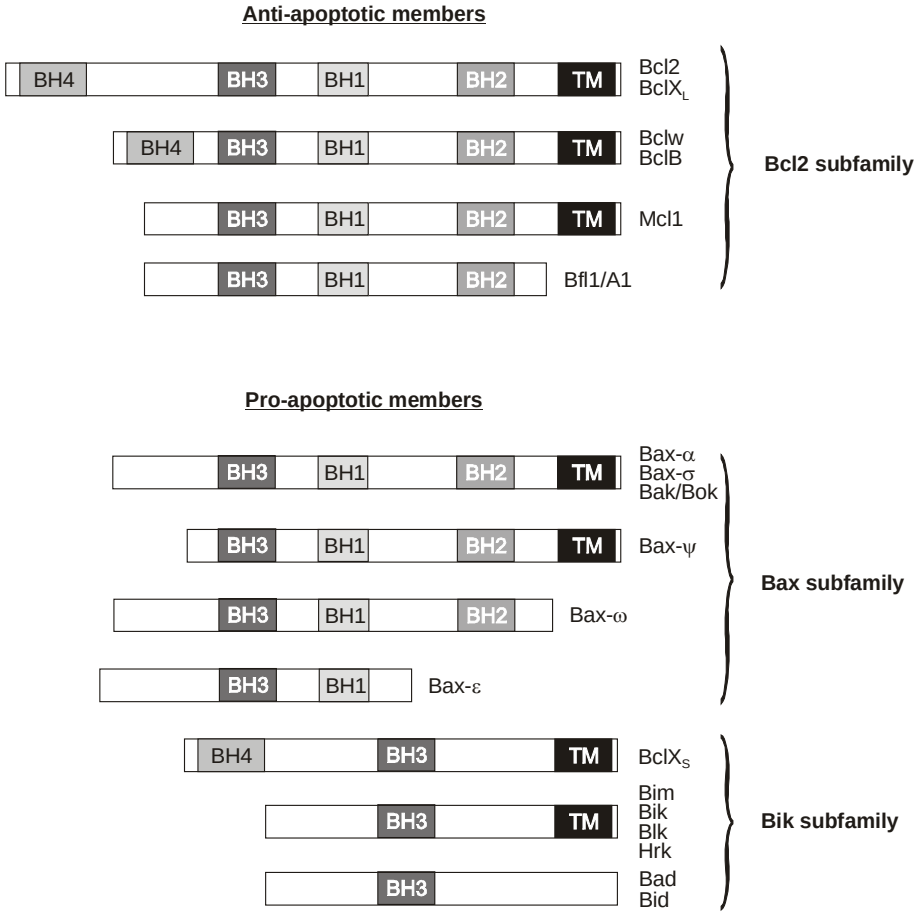

Fig. 1.3: Classification of the Bcl2 family based on their domain organisation. General domain architecture of the proteins is shown (not to scale) and the Bcl2 homology (BH) and transmembrane domains (TM) are indicated. Adapted from (Cory and Adams, 2002; Borner, 2003) with minor modifications.

Most members of the Bcl2 family possess a hydrophobic carboxy-terminal fragment, formerly proposed to function as a TransMembrane (TM) domain facilitating the interaction with intracellular membranes. Indeed, the carboxy-terminal hydrophobic sequence of Bcl2 was shown to serve as a signal anchor segment responsible for mitochondrial targeting (Nguyen *et al.*, 1994). Contradictory, the corresponding domain of the Bax protein, although having an essential role in the control of its pro-apoptotic activity by affecting Bax conformation (Suzuki *et al.*, 2000) does not appear to be a mitochondrial addressing signal (Tremblais *et al.*, 1999; Oliver *et al.*, 2000). It was found that in fact another domain (more specifically: the pore-forming domain) regulates mitochondrial localisation of the Bax protein (Nouraini *et al.*, 2000).

3.2 Multifunctionality: the family spirit

The Bcl2 family proteins appear to defy the dictum ‘one protein, one function’. Their multi-functionality comprises (i) dimerisation with other Bcl2 family members; (ii) pore or ion channel activity; and (iii) binding to non-homologous proteins (Zamzami *et al.*, 1998). According to the latter, Bcl2 is known to bind at least eleven non-homologous proteins, including Beclin-1 (Liang *et al.*, 1998) and BI-1 (Bax Inhibitor) (Xu and Reed, 1998), while Bax was demonstrated to interact with the outer mitochondrial membrane Voltage-Dependent Anion Channel (VDAC) (Narita *et al.*, 1998; Shimizu *et al.*, 1999), the inner mitochondrial membrane Adenine-Nucleotide Transporter (ANT) (Marzo *et al.*, 1998a; Brenner *et al.*, 2000) and the SH3 (Src-Homology 3)-domain containing protein Bif1 (Cuddeback *et al.*, 2001). However, associations between Bax and VDAC or ANT could not be demonstrated by others (Mikhailov *et al.*, 2001) and should be regarded as controversial at the moment.

3.2.1 Heterodimerisation-dependent functionality

One of the most notable characteristics of the Bcl2 family is their ability to form heterodimers. Such interactions were demonstrated by (i) co-immunoprecipitation (Oltvai *et al.*, 1993); (ii) yeast two-hybrid (Sato *et al.*, 1994; Sedlak *et al.*, 1995); and (iii) *in vitro* binding assays (Zha *et al.*, 1996a). In addition, these interactions were also observed in intact mitochondria via (iv) protein cross-linking (Gross *et al.*, 1998) and (v) fluorescent resonance energy transfer (Mahajan *et al.*, 1998). Ottilie and colleagues observed that some mutations, abrogating the death-inhibiting function of Bcl2, also prevented this protein to heterodimerise with Bax (Bcl2-Associated protein X) (Ottilie *et al.*, 1997). Based on these results, it was suggested that the death-inhibiting function of Bcl2 was dependent on its ability to form Bax heterodimers. Additionally, some mutants of Bax are known to lack both the apoptotic activity and the capacity to heterodimerise with death inhibitors (Chittenden *et al.*, 1995; Zha *et al.*, 1996a).

3.2.1.1 The rheostat model: from simple to complex

Based on the finding that the ratio of Bcl2 to Bax predetermines the cell’s susceptibility to a given apoptotic stimulus (Oltvai and Korsmeyer, 1994), it was suggested that Bax and Bcl2 regulate cell death by a duelling dimer concept, providing a rheostat switch mediating the extent of the response to a given apoptotic trigger (Oltvai *et al.*, 1993). In this simple rheostat model, Bcl2 promotes cell survival by complexing with Bax, thereby antagonising the death-promoting activity of the latter (Oltvai *et al.*, 1993; Yin *et al.*, 1994). This would implicate the Bcl2 family proteins to exist in two conformations: one in which the protein creates a binding pocket and the other in which an adapter module is exposed, so it can burry this site into the binding pocket of partner proteins (Sattler *et al.*, 1997).

In the meantime, the Bcl2 family of proteins has expanded significantly and includes no longer only Bax and Bcl2, but many more anti- and pro-apoptotic molecules. The physical interactions among these Bcl2 family proteins allow pro- and anti-apoptotic proteins to engage in hand-to-hand combat with each other, vying for supremacy over cell life and death decisions and thus, their relative ratios are likely to determine the ultimate sensitivity of the cell to an apoptotic stimulus (Oltvai *et al.*, 1993; Sato *et al.*, 1994; Sedlak *et al.*, 1995). In this way, the rheostat model was upgraded as being controlled by several pairs of pro- and anti-apoptotic Bcl2 proteins to make the decision step of apoptosis.

Cheng and colleagues demonstrated that some members of the Bik subfamily (BH3-domain only) are able to bind both anti- and pro-apoptotic Bcl2 family proteins (Cheng *et al.*, 2001), thereby neutralising the former and activating the latter. Based on these findings, the receptor-to-adaptor transition is no longer favoured in that some BH3-only subfamily members could function as adaptor for the receptor-like Bcl2 family members. In addition, the competition for BH3-only molecules between the Bcl2 and Bax subfamilies could determine a rheostat switch influencing cellular susceptibility.

3.2.2 Heterodimerisation-independent functionality

Further studies revealed $BclX_L$ mutants being capable of suppressing Bax-induced cell death, while having no Bax-binding activity (Cheng *et al.*, 1996; Tao *et al.*, 1997; Minn *et al.*, 1999), suggesting that inhibition of cell death does not require heterodimerisation. In addition, Bcl2 was found to be capable of prolonging cell survival even in the absence of Bax (Knudson and Korsmeyer, 1997). Conversely, Bax was reported to promote cell death irrespective of heterodimerisation with Bcl2 (Simonian *et al.*, 1996; Mikhailov *et al.*, 2001) and was even apoptotic without Bcl2 present (Zha and Reed, 1997). This indicates that Bcl2 and Bax may be capable of functioning independently. However, these results do not exclude the possibility that interaction with the BH3-subgroup activators may either assist or be required for the cell death-inducing mechanism of the Bax protein.

3.2.2.1 Three-dimensional structure of Bcl2 family members

The human $BclX_L$ protein was the first Bcl2 family member for which the X-ray and NMR (Nuclear Magnetic Resonance) structure became known (Muchmore *et al.*, 1996). The 3D-structure of this protein (Fig 1.4) shows two central hydrophobic α-helices surrounded by five amphipathic α-helices in which three functionally important Bcl2 homology regions (BH1-3) are in close spatial proximity, forming an elongated hydrophobic pocket that represents the binding site for the BH3 domain of other Bcl2 family members (Muchmore *et al.*, 1996; Aritomi *et al.*, 1997). The overall fold of $BclX_L$ closely resembles that of Bax (Suzuki *et al.*, 2000), Bcl2 (Petros *et al.*, 2001; Huang *et al.*, 2002), and Bclw (Hinds *et al.*, 2003). The remarkable structural similarity contrasts with the rather low percentage of their amino acid similarity (20%), mainly concentrated in the three BH regions.

Although the overall fold of the Bax protein (Suzuki *et al.*, 2000) is similar to Bcl2 and $BclX_L$, it's crystal structure indicated differences in structural topology and charge variability in some regions, including (i) a low level of interhelical polar interactions; (ii) a more extensive exposition of the central α-helices indicating the Bax protein is loosely packed; (iii) the presence of many more hydrophobic patches; (iv) a concentration of positive charges at the bottom of the molecule, suggesting electrostatic interactions with negative membrane charges; (v) the presence of the carboxy-terminal domain in the BH3-binding hydrophobic pocket which contributes to the solubility of Bax by effectively reducing the exposed hydrophobic surface; and (vi) a buried amino-terminal domain. In relation herewith, the structural changes in the Bax protein associated with cell death should displace the carboxy-terminal helix from the pocket, the resulting conformational change allowing mitochondrial Bax docking and exposing the amino-terminal helix.

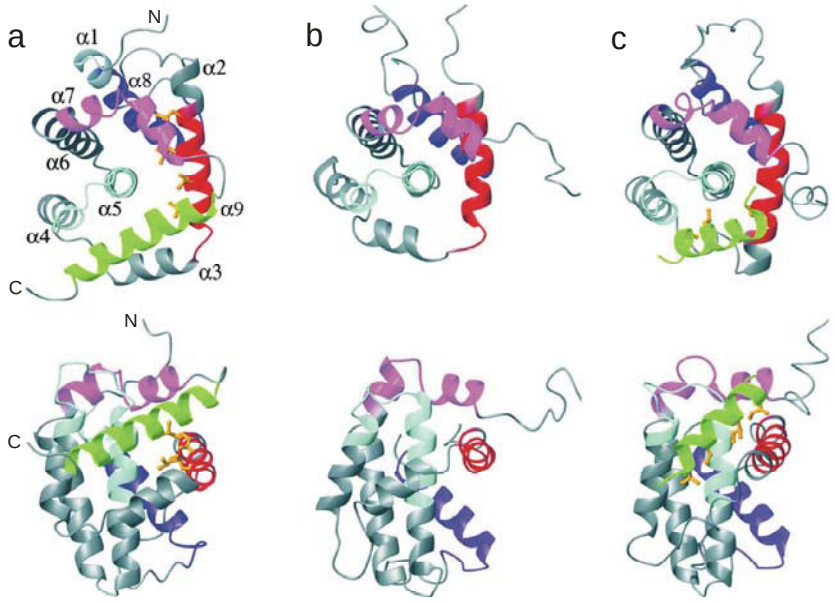

Fig. 1.4: Structural comparison between Bax and Bcl-X_L based on two different views of (a) Bax, (b) Bcl-X_L and (c) Bcl-X_L bound to the BH3 peptide. Views straight down the central helix (top panels) and views on the protein side (lower panels). Adapted from Suzuki *et al.*, (2000).

3.2.2.2 Look-alike: the ion channel model

Comparison with other proteins for which the structures are available, revealed striking structural similarity of Bcl2 family proteins to the pore-forming domains of certain bacterial toxins (Parker and Pattus, 1993; Elkins *et al.*, 1997), including colicins which kill sensitive *Escherichia coli* cells via the formation of a high-conductance, non-specific ion channel in the target cell's cytoplasmic membrane. The structure of these bacterial toxins possesses a hydrophobic 'dagger', shielded by a 'cloak' of amphipathic α-helices, allowing these proteins to lead 'dual lives' in that they may exist in

a soluble state, but under certain conditions will insert into membranes to form a channel (Donovan *et al.*, 1982; Shin *et al.*, 1993; Oh *et al.*, 1996). If function follows form, the obvious prediction is that Bcl2 family members can function as ion channels. By analogy to the bacterial toxins, the two central hydrophobic helices presumably insert perpendicular across the lipid bilayer, with the protein undergoing profound conformational changes, perhaps like opening an umbrella, with the surrounding amphipathic helices folding upwards and resting on top of the membrane (Reed, 1997).
However, though structurally similar to bacterial colicins, this does not necessarily imply functional similarity. For example, the membrane-insertion domain of colicin closely resembles the globin fold, a helical sandwich prototype used by proteins (e.g. myoglobins and phycocyanins) in various contexts (Holm and Sander, 1993). Thus, it can be argued that the 3D-structure of Bcl2 family proteins could merely represent a common fold for soluble packing of hydrophobic and amphipathic helices.
Several studies demonstrated the ability of Bcl2 and $BclX_L$ to form ion channels in membranes composed of 30-40% acidic lipids (Antonsson *et al.*, 1997; Minn *et al.*, 1997; Schendel *et al.*, 1997; Schlesinger *et al.*, 1997). However, this channel forming capacity is most prominent at acidic pH, and Bcl2 channels are even known to close at physiological pH. In addition, Bcl2 and $BclX_L$ lost their channel-forming activity in membranes composed solely of neutral lipids, this mimicking intracellular membranes (Schendel *et al.*, 1998). Moreover, the observation that no novel channel activities could be linked to Bcl2 overexpression, suggests that Bcl2 does not form channels in native mitochondrial membranes (Murphy *et al.*, 2001).
In contrast, the protein Bax does form ion channels when incorporated into membranes containing neutral lipids only, supporting the notion that pore-forming activity is an intrinsic capacity of Bax (Antonsson *et al.*, 1997). Findings suggest that Bcl2 does not form a pore at physiological pH but merely prevents Bax from doing so (Antonsson *et al.*, 1997). Although the Bax protein readily forms ion channels in a synthetic bilayer (Kuwana *et al.*, 2002), it does not form its own channels in liposomes incorporated with VDAC proteins, the latter better representing the outer mitochondrial membrane. Bax might thus be involved in forming a Bax/VDAC hybrid channel (Shimizu *et al.*, 1999; Shimizu *et al.*, 2000) or might induce VDAC conformational changes (Tsujimoto and Shimizu, 2000). It was demonstrated that Bax proteins are capable to cause membrane instability by decreasing the linear tension of phospholipid bilayers (Basanez *et al.*, 1999). This destabilising effect could facilitate its insertion within membranes, leading to the formation of lipidic pores or lipid-protein complexes.

3.2.2.3 Look-alike: the protein translocator model

The arrangement of the α-helices in Bcl2, $BclX_L$ and Bax is also reminiscent of the membrane-translocation domain of the Diphtheria Toxin (DT) (Choe *et al.*, 1992; Parker and Pattus, 1993). The DT, produced by *Corynebacterium diphtheria* is composed of two parts, the A- and B-fragments

respectively possessing ADP-ribosylating and pore-forming activity (London, 1992). The DT gains access to the cell via receptor-mediated endocytosis and upon encountering the acidic pH inside the endosome, the B-fragment forms a channel for transporting the A-moiety (20kDa) across the membrane into the cytosol (Kagan *et al.*, 1981; Donovan *et al.*, 1985). In addition, an intriguing feature of the B moiety is its capacity to translocate proteins, other than the A-fragment (Lazebnik, 2001). In contrast to pores formed by colicins, channels formed by the DT B-fragment are not sufficient to kill the cell, suggesting its main function to be a chaperone, translocating proteins through the channel. Therefore, based on the structural similarity between Bcl2 family proteins and the translocation-domain of DT, it was suggested the former could be able to translocate other molecules or themselves through membranes. In accordance herewith, Bcl2 has been localised at both the outer and the inner mitochondrial membrane (Hockenbery *et al.*, 1990) and immuno-electron-microscopy studies indicated Bax to be able to redistribute from the outer to the inner mitochondrial membrane (Marzo *et al.*, 1998b). More particular, it was reported that in pure liposomes Bax could form a tetrameric channel with an estimated pore size of about 22Å, being able of transporting cytochrome-c, which has a Stokes diameter of 17Å (Saito *et al.*, 2000). Atomic force microscopic imaging of the interaction of apoptotic Bax with membrane bilayers also showed the presence of toroidal-shaped pores sufficiently large to allow passage of proteins (Epand *et al.*, 2002b). These findings suggest a Bax oligomer to constitute the structural entity of the cytochrome-c translocating channel.

3.3 Generality of Bax toxicity

Considering the evolutionary distance between the species in which (heterologous) expression of the Bax protein was shown to be lethal, one can propose that (i) Bax is interacting with a conserved cellular target that is part of an ancenstral cell death pathway; or (ii) Bax is abrogating a conserved cellular function via its pore-forming capacity; or (iii) Bax is eliciting a general cellular stress, which is followed by a cell-type specific cell death pathway.

3.3.1 In mammalia

Bax expression at physiological levels is not lethal *per se* (Deckwerth *et al.*, 1996), but over-expression of the Bax protein has been demonstrated to accelerate cell death following a death signal (Oltvai *et al.*, 1993). In addition, hyperexpression of Bax without another death stimulus was sufficient to induce apoptosis (Xiang *et al.*, 1996) (Fig. 1.1b) and this Bax-induced cell death did not even require the activation of caspases (Fig. 1.1c). Indeed, the presence of caspase inhibitors – although preventing DNA fragmentation and degradation of nuclear and cytosolic substrates – still allowed cyt-c release, ROS production, DNA condensation and vacuolisation (Fig. 1.1c) to occur, finally leading to cell death (Xiang *et al.*, 1996). It is postulated that the energy depletion and ROS

generation, accompanying the interrupted oxidative phosphorylation, will ultimately be toxic to most cells. Moreover, if endoG would be released in response to Bax translocation, this caspase-independent endonuclease might contribute to the cellular demise, hereby accounting for the observed apoptosis-like chromatin condensation (Xiang *et al.*, 1996). Apoptosis is associated with the redistribution of the Bax protein from cytosol to mitochondria, ER, Golgi, and nuclear membranes (Wolter *et al.*, 1997; Mandal *et al.*, 1998; Gajkowska *et al.*, 2001; Pan *et al.*, 2001b; Zong *et al.*, 2003). It has been suggested that Bax is present in the cytosol of normal cells because it lacks a functional anchoring signal (Tremblais *et al.*, 1999) and that apoptosis-induced conformational changes allow the protein to translocate to membranes (Epand *et al.*, 2002a; Guo *et al.*, 2003). In mitochondrial membranes, Bax was shown to multimerise at discrete foci (Karbowski *et al.*, 2002). This Bax redistribution is important to promote cell death (Wolter *et al.*, 1997; Nomura *et al.*, 2003; Sawada *et al.*, 2003), and membrane insertion is essential for bioactivity (Nechushtan *et al.*, 1999). The membrane integration could be triggered (Antonsson *et al.*, 2000; Epand *et al.*, 2002a) or accompanied (De Giorgi *et al.*, 2002) by Bax oligomerisation and it is conceivable that ion gradients could locally induce the conformational changes allowing Bax integration (Tremblais *et al.*, 1999). Referring to the data obtained by Antonsson and colleagues, the behaviour of Bax could be reminiscent of the bacterial toxin aerolysin for which oligomerisation is a prerequisite for membrane integration and channel formation (Antonsson *et al.*, 2001). The mitochondrial membrane insertion of Bax is sufficient for releasing cyt-c (Antonsson *et al.*, 2000), but the translocation of larger proteins to the cytosol should require further multimerisation. This would not be that surprising, keeping in mind the many examples of small bacterial toxins multimerising to form huge channels (Gilbert *et al.*, 1999). For example, pneumolysin, a toxin produced by *Streptococcus pneumoniae* oligomerises after binding to cholesterol in cell membranes, forming a ring of 30-50 subunits with a size of about 400 Å, allowing the passage of large proteins. In addition, Nechustan and colleagues (2001) demonstrated a novel step in the Bax pro-apoptotic mechanism, immediately subsequent to mitochondrial translocation. Via confocal and electronmicroscopy, they observed Bax resides in the mitochondria only during a brief period, after which the Bax proteins actually leave the mitochondrial membranes. Adjacent to mitochondria, thousands of Bax molecules were seen coalescing into large clusters and this cluster formation was observed to be caspase-independent and essential for Bax cytotoxicity (Nechushtan *et al.*, 2001; Capano and Crompton, 2002).

The (over)expression of Bax in cultured cells is known to have a variety of pro-apoptotic effects including (i) modulation of cell cycle-controlling protein levels (Brady *et al.*, 1996), (ii) depletion of the ER Ca^{2+} pool (Pan *et al.*, 2001b), (iii) mitochondrial Ca^{2+} accumulation (Nutt *et al.*, 2002a), (iv) accumulation of proteins in ER and mitochondria (Nutt *et al.*, 2002b); (v) increase of mitochondrial intermembrane contact sites (He *et al.*, 2003); (vi) sensitisation of the mitochondria to cyt-c release (Wei *et al.*, 2001; Nutt *et al.*, 2002b), (vii) mitochondrial fragmentation (Karbowski *et al.*, 2002; Karbowski and Youle, 2003), (viii) aggregation of mitochondria around the nucleus (Eskes *et al.*,

1998); (ix) increase in ROS production (Xiang *et al.*, 1996); (x) reduction/loss of the mitochondrial membrane potential (Adams and Cory, 1998; Finucane *et al.*, 1999); and (xi) decline of total glutathione levels (Bojes *et al.*, 1997). Referring to the anti-apoptotic activities of the Bcl2 protein (See 3.1.1.3), it is striking to observe Bcl2's potential to neutralise these effects triggered by the pro-apoptotic protein Bax. As this is unlikely to happen by chance, it brings further evidence to the rheostate model (See 3.2.1.1) to determine cellular fate.

3.3.2 In fruit flies

Ectopic expression of mammalian Bax protein in *Drosophila* induced embryonic cell death and deregulated the normal cell death pattern in the developing eyes and wing discs. In addition, the functionality of the mammalian Bcl2 protein is also conserved, as demonstrated by its capacity to reduce developmental cell death and to antagonise Bax functionality (Gaumer *et al.*, 2000).

3.3.3 In plants

Infection of *Nicotiniana benthaminiana* (tobacco) plants with a tobacco mosaic virus vector, carrying the mammalian *bax* gene, caused cell death in lead cells (Lacomme and Santa Cruz, 1999). This localised tissue collapse resembled somehow the hypersensitivity response, a defence system upon pathogen recognition resulting in localised cell death at the site of infection (Greenberg, 1996). Although the Bax-induced cell death morphology has not been analysed, the accumulation of the defence-associated Pathogenesis-Related PR1 protein and the capacity of okadaic acid, a protein phosphatase inhibitor, to block cytotoxicity suggested the Bax-induced cell death in tobacco to be an active process (Lacomme and Santa Cruz, 1999). Bax-induced cell death was also studied at the whole plant level in transgenic *Arabidopsis thaliana* plants (Kawai-Yamada *et al.*, 2001) and was found to be associated with leaf discoloration, cytoplasmic shrinkage and DNA fragmentation.

3.3.4 In bacteria

The mammalian Bax protein induces cell death in *E. coli* accompanied with (i) an elongated cell morphology; (ii) accumulation of monounsaturated fatty acids; (iii) increased ROS levels; (iv) the generation of nicked DNA; and (v) an increase in DNA mutation frequency (Asoh *et al.*, 1998).

It was suggested Bax could potentially cause these effects by forming ion channels in the cell membrane. To compensate, the respiratory chain may become activated to enhance the proton-pumping activity and the accelerated oxygen consumption could trigger the generation of ROS. However, as none of these effects has been demonstrated directly, this should be regarded illustrative only. A bacterial mutant, resistant to the lethal effects of Bax and the superoxide generator paraquat, has been identified, indicating Bax-induced cell death in *E. coli* to be ROS-mediated (Nanbu-Wakao *et al.*, 2000).

3.4 Bax toxicity in *S. cerevisiae*

3.4.1 Heterologous expression of pro-apoptotic protein Bax

3.4.1.1 Bax overexpression strategy

Bax expression in wild-type yeast has been demonstrated to invariably arrest proliferation, followed or not by lethality, depending on Bax expression levels and cellular metabolism (Greenhalf *et al.*, 1996). In Bax-overexpressing cells, the Bax protein colocalises to yeast mitochondria (Zha *et al.*, 1996b) and yeast killing is actually dependent on Bax translocation to this organelle. Keeping in mind ATP strongly increases the amount of Bax present in mitochondrial membranes (Priault *et al.*, 1999b), it is perhaps not surprising that Bax kills respiring cells more efficiently and that the addition of oligomycin, a specific F_0F_1-ATPase inhibitor, partially prevents Bax effects on yeast (Priault *et al.*, 1999b).

Mutational studies demonstrated the two hydrophobic central helices of the Bax protein to be critical for conferring a lethal phenotype in yeast (Matsuyama *et al.*, 1998a), indicating the Bax lethality to be correlated with its ability to form pores in membranes.

Further, data reported by Saito and colleagues, suggesting a sufficient density of Bax molecules to be required for an oligomeric pore to form in the mitochondrial outer membrane (Saito *et al.*, 2000), may explain the high expression levels needed to actually kill *S. cerevisiae*.

3.4.1.2 Morphology of Bax toxicity

Ectopic hyperexpression of Bax under conditions favouring respiratory metabolism has been reported to induce cell death associated with a variety of morphological abnormalities (Fig. 1.1e), including (i) cytoplasmic shrinkage (Abudugupur *et al.*, 2002), (ii) vacuolisation (Zha *et al.*, 1996b); (iii) nuclear dissolution (Zha *et al.*, 1996b); (iv) mitochondrial swelling (Zha *et al.*, 1996b); (v) lumpy chromatin condensation (Ligr *et al.*, 1998); (vi) DNA strand breakage (Ligr *et al.*, 1998); (vii) plasmamembrane blebbing (Ligr *et al.*, 1998); and phosphatidyl serine exposure (Ligr *et al.*, 1998). In their terminal stages of cell death, cells had undergone a nearly complete autophagy with absence of the nucleus and most cytosolic organelles, although the plasmamembrane remained intact (Zha *et al.*, 1996b). There was no evidence of compact chromatin condensation or oligonucleosomal DNA degradation (Zha *et al.*, 1996b), the latter probably due to the lack of linker DNA between the yeast nucleosomes (Lowary and Widom, 1989).

Cells recovering from Bax-induced sublethality were demonstrated to be enriched for *petite*s (Harris *et al.*, 2000), defined as cells lacking functional mitochondria. The autophagic process triggered by Bax expression could be involved herein, in that mitochondria which have lost their functionality upon Bax activity, may have been removed by pexophagy.

3.4.1.3 Biochemical effects

Bax expression in yeast cells induces cyt-c release (Manon *et al.*, 1997), thus paralleling the result obtained in mammalian cells. This Bax-induced cyt-c release does not require cardiolipin (Iverson *et al.*, 2004) and is known to underlie the decrease of the cyt-c oxidase enzyme activity (Manon *et al.*, 2001). It was demonstrated that this effect on the terminal enzyme of the respiratory chain was mediated at least in part by the mitochondrial Yeast MetalloproteasE Yme1. However, cyt-c release is not essential for cell death to occur (Roucou *et al.*, 2000), and thus Bax may kill yeast cells by a mechanism independent of cyt-c release.

In addition to cyt-c release, a number of biochemical alterations are associated with Bax-induced cytotoxicity, including (i) chromosomal instability (Greenhalf *et al.*, 1999); (ii) mitochondrial matrix alkalinisation (Matsuyama *et al.*, 2000), concomitant with cytosolic acidification; (iii) mitochondrial hyperpolarisation (Minn *et al.*, 1999); (iv) reduction of swollen mitochondria (Dimitrova *et al.*, 2004), (v) permeabilisation of mitochondrial outer membranes (Priault *et al.*, 1999b); (vi) decreased oxygen consumption (Harris *et al.*, 2000); (vii) intracellular accumulation of ethanol (Harris *et al.*, 2000); (viii) reduction of total glutathione level (Kampranis *et al.*, 2000); (ix) increased ROS levels (Madeo *et al.*, 1999; Gross *et al.*, 2000); (x) decrease of total fatty acid level suggesting an inhibition of fatty acid biosynthesis (Kampranis *et al.*, 2000); (xi) mitochondrial lipid peroxidation (Priault *et al.*, 2002); (xii) protection against plasma membrane permeabilisation (Marza *et al.*, 2002); (xiii) loss of the plasmamembrane phosphatidylserine asymmetry (Ligr *et al.*, 1998); (xiv) disruption of the vacuolar integrity and intracellular protein traffic (Dimitrova *et al.*, 2004); and (xv) tendency towards a reduction of the mitochondrial membrane potential (Kampranis *et al.*, 2000), although not generally observed (Minn *et al.*, 1999; Gross *et al.*, 2000).

In addition, the DNA content profile of Bax expressing cells appeared to be similar to the profile of normal cells (Kampranis *et al.*, 2000), suggesting Bax expression froze cells at any stage of the cell cycle while they were replicating.

3.4.1.4 Physiological relevance

Ectopic expression of mammalian proteins can sometimes be non-specifically toxic in yeast. A first argument for the lethal effects of Bax being specific, referred to the capacity of Bcl2 to partially abrogate Bax cytotoxicity in yeast (Sato *et al.*, 1994). From our point of view, this argument should be considered with caution in that the ability of Bcl2 to prevent non-physiological Bax activity, by being its preferred binding partner, cannot be ruled out. Zha and colleagues (Zha *et al.*, 1996b) provided a more convincing argument by demonstrating that Bax mutants, incapable of inducing apoptosis in mammalian cells, were also not cytotoxic in yeast, excluding this foreign protein interfering non-specifically with yeast physiology.

Cell death processes induced by a variety of stressors, including (i) ectopic Bax expression (Zha *et al.*, 1996a; Ligr *et al.*, 1998); (ii) low extracellular levels of H_2O_2 (Madeo *et al.*, 1999) or acetic acid

(Ludovico *et al.*, 2001); (iii) absence of the essential protein Asf1 (Yamaki *et al.*, 2001); or (iv) the presence of a particular *cdc48* allele (Madeo *et al.*, 1997) in *S. cerevisiae*, were all demonstrated to culminate in a similar morphology (e.g. compare Fig. 1.1e with Fig. 1.1f). Particularly, some of the biochemical changes accompanying Bax-induced cell death like DNA nicking, phosphatidylserine exposure and ROS accumulation, were observed in old yeast cells as a consequence of aging (Laun *et al.*, 2001), suggesting the observed Bax-induced changes in yeast physiology are the consequences of yeast dying. In this way, a conditional Bax expression system in yeast can be seen as a tool to induce yeast cell death on demand.

The morphology of Bax-induced cell death in yeast resembles that of cells dying in the presence of caspase inhibitors (e.g. compare Fig. 1.1c with Fig. 1.1e) (Xiang *et al.*, 1996). However, caspases are believed not to be present in yeast, and an optimistic interpretation of these data would be that an aspect of apoptosis could be found in dying yeast (Greenhalf *et al.*, 1999). In accordance with the latter, a primordial cell death pathway would be activated, this being a framework upon which the caspase-dependent pathway has developed. If present, this primordial pathway could also constitute an evolutionary old back-up pathway, implicating that the similarity in final death morphology not necessarily includes conceptual similarity. In accordance herewith, the yeast cell's active dying process may not resemble downstream events of apoptosis at the molecular level (Torgler *et al.*, 2000).

The observation that a yeast caspase-like protein interferes with cell death induced upon H_2O_2 treatment or aging (not tested for Bax-induced cell death) (Madeo *et al.*, 2002; Herker *et al.*, 2004), indicates that some conceptual resemblance may be present.

We currently favour the hypothesis that the ability of Bcl2 family members to regulate cell death and survival at the mitochondrial level (and perhaps other organelles as well) is conserved from yeast to man, implying the power of yeast genetics may be useful for mapping upstream components.

3.4.2 Mutational analysis

3.4.2.1 The F_0F_1-proton pump

The F_0F_1-proton pump synthesises ATP form ADP and P_i by using the proton electrochemical gradient generated by the respiratory chain. The peripheral, hydrophobic F_1-portion is required for enzyme catalysis (Ryrie and Gallagher, 1979), whereas the hydrophobic F_0-sector is involved in proton translocation (Kagawa and Racker, 1966). When F_0 dissociates from F_1, the F_1-portion actually hydrolyses ATP.

Genetic analysis of yeast indicated that a certain subunit of the F_0F_1-proton pump (Atp4) is required for murine LexA-Bax-induced cell death (Matsuyama *et al.*, 1998b) and disruption of *ATP4* is known to promote a mutant with the F_1-part loosely linked to the partially assembled F_0-sector (Paul *et al.*, 1992). The reason why the functionality of the F_0F_1-proton pump is required for the cytotoxic

actions of Bax is presently unknown, but possibilities include that (i) F_0F_1 is needed to create a local pH environment for oligomerisation of Bax proteins; or (ii) F_0F_1 is a downstream effector, contributing to cytosolic acidification and matrix alkalinisation via backwards proton pumping (Matsuyama and Reed, 2000). It has been suggested that Bax potentially triggers the reverse operation of this proton pump by blocking the exchange of ATP for ADP via ANT, hereby rising the matrix ATP/ADP ratio (Matsuyama *et al.*, 2000). However, the reverse operation of the F_0F_1-pump would eventually cause a steady-state condition in which H^+-efflux and -influx are equilibrated. Therefore, it is conceivable that Bax channels render the outer membrane more porous, causing a faster dissipation of the proton gradient through leakage to the cytosol. In this case, the F_0F_1-proton pump would run in reverse as long as ATP is available in the mitochondrial matrix. Although this backwards proton-pumping hypothesis is becoming increasingly popular, we do not rule out the possibility that Bax actually impairs the H^+-flow through F_0, thereby contributing to cytosolic acidification and matrix alkalinisation via an increase in mitochondrial outer membrane porosity, concomitant with an ongoing electron transport chain. Alternatively, the F_0F_1-proton pump may not be required at all for Bax-induced cell death, but its absence may have protective effects. For instance, the more acidic environment within the local intermembrane space could somehow preclude Bax from performing its functions (Reed *et al.*, 1998). Referring to results obtained by Priault and colleagues (Priault *et al.*, 1999b), indicating ATP to have a direct effect on Bax mitochondrial localisation and cytotoxicity, the requirement of F_0F_1-proton pump components for Bax functionality may be interpreted in terms of sufficiently high intracellular ATP levels needed for Bax effects.

3.4.2.2 ADP-ribosylation factor-like protein 1

ADP-Ribosylation Factors (ARF) and ADP-ribosylation factor-like proteins form a family of highly conserved guanine nucleotide-binding proteins, thought to participate in vesicular transport in both exocytotic and endocytotic pathways (Boman and Kahn, 1995; Moss and Vaughan, 1995).

Genetic analysis of yeast indicated that Arl1, the ADP-ribosylation factor-like protein 1, is required for Bax-induced cell death (Abudugupur *et al.*, 2002). Mutated *ARL1* resulted in a defective central vacuole formation and a complete inhibition of the cell death induced by Bax, suggesting that *ARL1* plays an important role in the progression of Bax-induced cell death via autophagocytosis.

3.4.2.3 The life-span increasing protein Uth1

The protein Uth1 belongs to the 'SUN' family of homologous proteins (Sim1, Uth1, Nca3) (Camougrand and Rigoulet, 2001), and is involved in mitochondrial biogenesis and stress response (Camougrand *et al.*, 2000). The absence of Uth1 delayed human c-myc-Bax-induced cell death (Camougrand *et al.*, 2003) by preventing mitochondrial lipid oxidation and ROS production. *UTH1* was also required for rapamycin to exert is autophagy-promoting effect under respiratory conditions,

suggesting Bax may interact with an autophagy-related cell death pathway involving mitochondria. As *UTH1* deletion also delays aging (Kennedy *et al.*, 1995), this is the first gene providing a link between stress response, aging and cell death.

3.4.3 Overexpression screenings

A large variety of screenings have been performed, selecting for mammalian or plant apoptosis-inhibitors able to suppress Bax lethality in yeast.

On the other hand, strategies to look for yeast proteins, overcoming Bax-induced cell death upon overexpression or able to interact with pro-apoptotic mammalian proteins, are underrepresented in the published literature to date.

3.4.3.1 The Bax-inhibiting BI-1 proteins

The first mouse LexA-Bax-induced cell death suppressor identified by a functional screening in *S. cerevisiae* was the human Bax Inhibitor-1 (BI-1) protein (Xu and Reed, 1998). BI-1, an integral membrane protein containing multiple membrane-spanning segments, is predominantly localised to the nuclear envelope and the ER, with minor mitochondrial association (Xu and Reed, 1998). In addition, the overexpression of BI-1 in mammalian cells suppressed apoptosis induced by a variety of stimuli (e.g. Bax expression) and conversely, BI-1 antisense spontaneously induced apoptotic cell death; thus confirming and extending the result obtained in yeast and indicating BI-1 to be an apoptosis-inhibitor (Xu and Reed, 1998). Similarly, *Brassica napus* BI-1, BnBI-1, and *Nicotinana tabacum* BI-1, NtBI-1, also suppress Bax-induced apoptosis in mammalian cells (Bolduc *et al.*, 2003), with the *Arabidopsis thaliana* homologue of BI-1, AtBI-1, being an exception by actually inducing apoptosis (Yu *et al.*, 2002).

Additionally, plant BI-1 proteins were demonstrated to become rapidly upregulated *in planta* during wounding or pathogen challenge, pointing to a physiological relevant suppressive effect (Kawai-Yamada *et al.*, 2001; Huckelhoven *et al.*, 2003; Matsumura *et al.*, 2003).

Further, both AtBI-1 and the *Oryza sativa* homologue of BI-1, OsBI-1, were able to suppress mouse LexA-Bax lethality in yeast (Kawai *et al.*, 1999; Sanchez *et al.*, 2000). In addition, a yeast BI-1 homologue, yBI-1, was cloned and demonstrated to protect yeast against oxidative stress and murine LexA-Bax-induced lethality (Chae *et al.*, 2003).

3.4.3.2 Antioxidant defence proteins

A novel plant Glutathione S-Transferase/Peroxidase (GST/GPX) is known (Kampranis *et al.*, 2000) to suppress murine LexA-Bax cytotoxicity in yeast. As GST/GPX expression in yeast was found to significantly enhance resistance to H_2O_2 stress, this result underscores the relationship between oxidative stress and Bax-induced cell death in yeast. More particular, expression of this antioxidant

defence gene was demonstrated to revert the Bax-induced effects on intracellular glutathione levels and redox potential.

Moon and colleagues (Moon *et al.*, 2002) demonstrated soybean Ascorbate PeroXidase (APX) to suppress murine LexA-Bax-induced cell death, also by functioning as an antioxidant in yeast.

3.4.3.3 The AtVAMP protein

The *A. thaliana* Vesicle-Associated Membrane Protein AtVAMP was shown to suppress Bax-induced cell death in yeast (Levine *et al.*, 2001), by reducing the amount of lipid hydroperoxides and the degree of plasmamembrane protein oxidation. However, substantial ROS accumulation was still observed in these cells, indicating AtVAMP protection to be downstream of the oxidative burst. AtVAMP was demonstrated to be involved in intracellular vesicle traffic, suggesting that membrane recycling and membrane repair could be the protective mechanism (Levine *et al.*, 2001).

3.4.3.4 The AtEBP protein

Pan and colleagues identified the *A. thaliana* Ethylene-responsive element Binding Protein (At-EBP) to function as a dominant suppressor of murine LexA-Bax-induced cell death in yeast, in which its nuclear localisation was essential for protection. Although no yeast sequence homologous to AtEBP could be found, they argued the analysis of the AtEBP transcriptional activity to be very promising (Pan *et al.*, 2001a).

3.4.3.5 The bifunctional apoptosis regulator BAR

A screen for cDNAs encoding suppressors of murine LexA-Bax-induced cell death in *S. cerevisiae* identified an unknown protein, nicknamed as BAR (Bifunctional Apoptosis Regulator) (Zhang *et al.*, 2000). The BAR protein was shown to contain a SAM (Sterile Alpha Motif) domain, required for its interactions with Bcl2 and $BclX_L$ and for suppression of Bax-induced cell death in yeast and mammalian cells. Further, BAR also contained a domain homologous to DED (Death Effector Domain) for interacting with DED-containing caspases. At present, it remains unclear why BAR suppressed Bax-induced cell death in yeast, given the absence of a BAR-Bax interaction.

3.4.3.6 Proteins of unknown function

Greenhalf and colleagues (Greenhalf *et al.*, 1999) screened for genes from a human cerebellum cDNA library that conferred resistance towards human c-myc-Bax lethality in yeast. Two BASS genes (Bax Antagonist Selected in *Saccharomyes*), designated as BASS1 and BASS2, were selected and encoded proteins of unknown function. In addition, both BASS1 and BASS2 demonstrated anti-apoptotic activity in mammalian cells.

3.4.4 Functional studies

3.4.4.1 The adenine nucleotide transporter (ANT)

Marzo and colleagues (Marzo *et al.*, 1998a) demonstrated the absence of the adenine nucleotide transporter to fully inhibit murine HA-Bax-induced cell killing in yeast. This result was interpreted, in terms of a functional interaction between Bax and ANT to be needed for Bax to kill the cells. This interaction was proposed to block mitochondrial ATP/ADP exchange, causing substrate lack and leading to mitochondrial hyperpolarisation and ROS generation. The decreasing ATP levels were predicted to stimulate glycolysis, resulting in lactate generation and thus cytosolic acidification (Vander Heiden and Thompson, 1999). Contradictory, other studies indicated that the cytotoxic action of human c-myc-Bax in yeast does not require ANT at all (Priault *et al.*, 1999a) and that the absence of ANT does not modify the kinetics of the murine Bax-induced lethality (Kissova *et al.*, 2000).

Although mammalian ANT was shown to physically interact with Bax (Marzo *et al.*, 1998a), we observed the bindingsite sequence not to be conserved in yeast ANT. This indicates the functional interaction – if any – between these two proteins in yeast may not comprise direct binding.

3.4.4.2 The voltage-dependent anion channel (VDAC)

Shimizu and colleagues (Shimizu *et al.*, 1999) demonstrated that VDAC-deficient mitochondria from yeast do not release cyt-c upon addition of human recombinant Bax *in vitro*, suggesting the requirement of the VDAC protein for Bax-induced killing in yeast. However, studies indicated VDAC not to be involved in cyt-c release upon human Bax-c-myc expression (Priault *et al.*, 1999b), or in human/mouse HA-Bax or mouse Bax lethality in yeast (Gross *et al.*, 2000; Kissova *et al.*, 2000; Polcic and Forte, 2003).

3.4.4.3 Mitochondrial functionality

Bax-mediated cytotoxicity in yeast was first indicated to depend on the presence of the mitochondrial genome, based on the observation *petite* cells were not killed at all by human c-myc-Bax (Greenhalf *et al.*, 1996), although a growth-arrest phenotype was still present. However, Matsuyama and colleagues (Matsuyama *et al.*, 1998b) observed *petite* cells to have just a reduced sensitivity to murine LexA-Bax-mediated killing and others could not even observe any effect of the mitochondrial genome on the ability of murine Bax to kill cells (Kissova *et al.*, 2000).

The human c-myc-Bax toxicity was demonstrated to be reduced in yeast strains lacking the ability to perform oxidative phosphorylation (Priault *et al.*, 1999a) and the respiratory capability was seen as an important determinant of human Bax toxicity (Harris *et al.*, 2000). In contradiction herewith, Kissova and colleagues (2000) reported anaerobically grown wild-type yeast cells to be affected by murine Bax in the same way as respiratory-competent cells.

Thus, due to conflicting results concerning mitochondrial functionality to be required for Bax killing in yeast or not, this topic remains open for further investigation.

3.4.4.4 Discrepancy: why such a mess?

Whether mitochondrial function is crucial for Bax toxicity or not is still controversial, but the consensus appears to be that Bax can kill yeast independently of functional mitochondria, though the kinetics and efficiency of cell death will be superior in respiring yeast. This implies that Bax may kill *any* yeast cell as long as enough Bax protein is mitochondrially translocated.

It was proposed that Bax expression levels may, at a certain concentration, modulate cell death by interacting with other proteins, but at higher concentrations Bax could oligomerise, exerting an autonomous effect involving channel formation and/or destabilisation of intracellular membranes (Zamzami and Kroemer, 2001). As expression levels are likely to vary among experimental settings, discrepancies are probably not that surprising. For instance, amino-terminal Bax fusions with Green Fluorescent Protein (GFP-Bax) (Nechushtan *et al.*, 1999), HaemAgglutinin (HA-Bax) (Tremblais *et al.*, 1999) or LexA (LexA-Bax) (Xu *et al.*, 2000) were demonstrated to stabilise the protein compared to the native protein, hereby increasing its protein level. Further, the optimisation of the *bax* codon usage to yeast preferences was performed in several studies (Greenhalf *et al.*, 1996; Priault *et al.*, 1999a; Camougrand *et al.*, 2003) and may also increase the actual protein level via enhanced translation. Thus, differences in experimental set-ups may well be the reason why comparisons between several, seemingly similar analyses result in conflicting data.

Strikingly, upon reviewing literature, we found a relationship between the strength of the Bax expression system and the observed phenotype of the yeast strain lacking *ANT*: the weaker the expression system, the higher the chance to observe Bax resistance. Results of Manon and co-workers are in accordance herewith, the protective effect of the loss of *YME1* being more clearly visible in a weaker expression system (Manon *et al.*, 2001).

Further, while screening the phenotype of knock-outs for Bax-induced cell death, the potentially reduced fitness of that particular mutant could favour Bax survival due to a reduced Bax functionality (slow metabolism, low ATP level); and thus one should be aware the side-effects not explaining the results. Particularly, yeast strains with impaired mitochondrial functionality are known to have the enzymes involved in galactose metabolism repressed and consequently the *GAL1*, *GAL10* or *GALL* promoter (Mumberg *et al.*, 1994), used for ectopic expression of the Bax protein, does not seem to function properly in these cells (Algeri *et al.*, 1981; Donnini *et al.*, 1992). Therefore, data concerning the necessity of mitochondrial functionality for Bax toxicity reporting the use of *GAL* driven Bax expression should be regarded with caution.

In addition, laboratory strains can have different genetic backgrounds, associated with important molecular differences (Rogowska-Wrzesinska *et al.*, 2001). Thus, strain differences may contribute

to heterogeneous results. In accordance herewith, the efficacy of Bax killing was shown to be strain dependent (Xu *et al.*, 2000).

We and others alike (Priault *et al.*, 1999a) currently favour the use of an expression system in which the mitochondrial status does not affect the inducability of the promoter, for instance the tet-on (Belli *et al.*, 1998) or tet-off (Gari *et al.*, 1997) TETracycline-regulatable systems. Additionally, the use of one common reference yeast strain for the investigation of Bax-induced cell death in *S. cerevisiae* (or yeast cell death in general) is very recommended. This should allow one feeling confident when comparing the results of Bax toxicity phenotypes of mutants affected in different cellular functions obtained by various research groups.

3.4.5 Bax toxicity model

In cells lacking endogenous Bcl2 homologues, such as yeast cells, Bax cytotoxicity is deprived of its dimerisation-dependent mechanism (See 3.2.1), limited to whatever intrinsic cytotoxic function it possesses. Based on the observation that (i) the deletion of the putative pore-forming domain of Bax abolishes its cytotoxic activity in yeast (Matsuyama *et al.*, 1998a); and (ii) the presence of Bax at the mitochondrial outer membrane is associated with the formation of a novel, high conductance channel (Pavlov *et al.*, 2001), the phenotype of Bax expression in yeast may be a reflection of its ability to form channels either by itself or in association with one or more outer mitochondrial membrane components. To the best of our knowledge, we are not aware of any attempts to force targeting of Bax to other organelles and thus, it cannot be concluded that Bax's only site of action in yeast are the mitochondria.

Expression of Bax in yeast was shown to increase ROS levels (Madeo *et al.*, 1999) and several additional data supporting ROS as an endogenous regulator of yeast cell death include (i) H_2O_2 can induce active cell death in yeast (Madeo *et al.*, 1999); (ii) aged mother cells show markers of oxidative stress and cell death (Laun *et al.*, 2001; Herker *et al.*, 2004); (iii) Bcl2 expression in non-dividing yeast cells lacking SuperOxide Dismutase (SOD) extend their life span (Longo *et al.*, 1997); and (iv) ROS levels increase during cell death induced by heat shock (Davidson *et al.*, 1996) or in a *cdc48* (S565G) yeast strain (Madeo *et al.*, 1997). Based on these data ROS may thus be an important regulator of active cell death by amplifying suicide signals from mitochondria.

We propose that ROS are not the only endogenous regulator of yeast cell death, and perhaps also Ca^{2+}signalling (Fig. 1.5) is involved, this being suggested by data presently known in mammalian cells (See 3.3.1). Further, Bax-induced cell death in yeast may be orchestrated not only by mitochondria, but ER, nucleus and vacuoles (lysosomes) (Dimitrova *et al.*, 2004) (See 2.1.2) may also be involved. However, which organelle is first touched on is hard to predict and maybe there is stochasticity around.

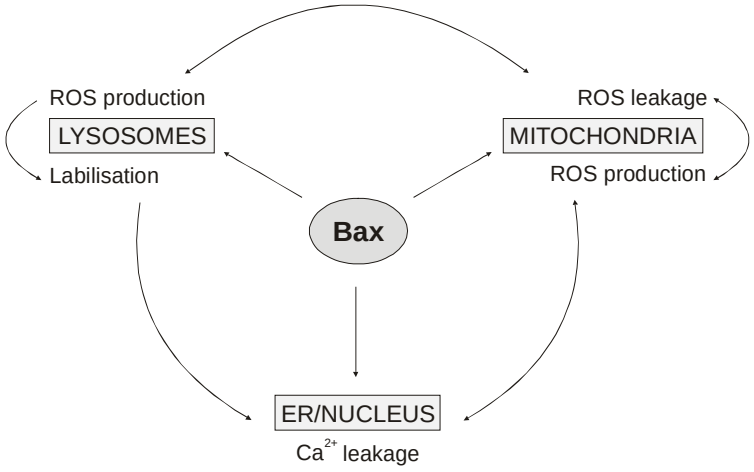

Fig. 1.5: Bax-orchestrated organelle interplay may be responsible for its cytotoxicity in *S. cerevisiae*. The mitochondrial translocation of the Bax protein is known (Zha *et al.*, 1996b) and this localisation may amplify the ROS production. The Bax translocation to any of the other organelles is not known, but interaction may be no requirement at all, and perhaps ROS diffusing throughout the cell actually impairs organelle functionality. On the other hand, Bax proteins may translocate temporarily to the ER and/or other organelles, possibly rather early and/or in tiny amounts, with great chance to be overlooked.

4. The origin of active cell death

4.1 Active cell death in lower eukaryotes

It is a tenet in current thinking that active cell death evolved to meet the particular needs of multicellular life. This idea is not based on experimental evidence, showing that all unicellulars lack an active dying process, but rather is a reflection of the current excitement about the now obvious benefits of active cell death for multicellular life forms in particular.

Implicit to the specific association of ACD with multicellularity is the idea that bacteria, lower unicellular eukaryotes and protozoa would have no need for an orderly process of cell death.

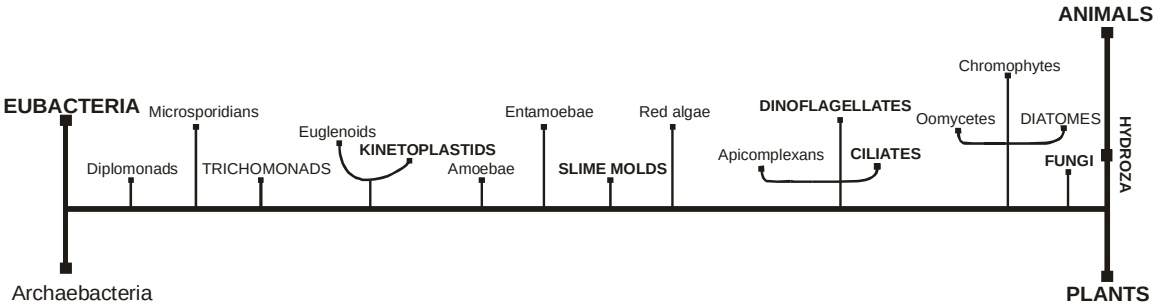

Fig. 1.6: Phylogenetic view. Branches represented by small capitals indicate active cell death is described, at least for one member. Adapted from Ameisen, 1996.

The boundaries defining regulated ACD pathways as a strictly multicellular phenomenon have been significantly altered (Fig. 1.6) with the observation that ACD can be induced in lower eukaryotic organisms phylogenetically distant from vertebrates including (i) the amitochondriate protist *Trichomonas vaginalis* (Chose *et al.*, 2002); (ii) the kinetoplastids *Trypanosoma cruzi* (Ameisen *et al.*, 1995), *T. brucei rhodensiense* (Welburn *et al.*, 1997), *T. brucei brucei* (Ridgley *et al.*, 1999),

Leishmania major (Arnoult *et al.*, 2002), *L. donovani* (Das *et al.*, 2001; Lee *et al.*, 2002; Sen *et al.*, 2004), *L. amazonensis* (Moreira *et al.*, 1996; Holzmuller *et al.*, 2002) and *L. mexicana* (Zangger *et al.*, 2002); (iii) the slime mold *Dictyostelium discoideum* (Cornillon *et al.*, 1994; Arnoult *et al.*, 2001; Tatischeff *et al.*, 2001); (iv) the dinoflagellate *Peridinium gatunese* (Vardi *et al.*, 1999); (v) the ciliates *Tetrahymena theromophila* (Davis *et al.*, 1992; Christensen *et al.*, 1995; Mpoke and Wolfe, 1996) and *Stylonychia lemnae* (Maercker *et al.*, 1999); (vi) the diatom *Thalassiosira weisflogii* (Berges and Falkowski, 1998), (vii) the fungi *Candida albicans* (Helmerhorst *et al.*, 1999; Gyurko *et al.*, 2000; Helmerhorst *et al.*, 2001; Ruissen *et al.*, 2001; Phillips *et al.*, 2003; Coyle *et al.*, 2004), *Zygosaccharomyces bailii* (Ludovico *et al.*, 2003), *Aspergillus nidulans* (Cheng *et al.*, 2003), *Mucor racemosus* (Roze and Linz, 1998), *Podospora anserina* (Saupe, 2000), and *Klyveromyces lactis* (Mazzoni *et al.*, 2003b); and (viii) the hydrozoa *Hydra vulgaris* (Cikala *et al.*, 1999; Technau *et al.*, 2003) and *Hydractina echinata* (Seipp *et al.*, 2001).

Additionally, overexpression of pro-apoptotic proteins in (i) *S. cerevisiae* (Greenhalf *et al.*, 1996; Tao *et al.*, 1999), (ii) *C. albicans* (Baev *et al.*, 2001); (iii) *Schizosaccharomyces pombe* (Ottilie *et al.*, 1992; Zhao *et al.*, 1996; Ink *et al.*, 1997; Jurgensmeier *et al.*, 1997; Zhao *et al.*, 1998), (iv) *Pichia pastoris* (Martinet *et al.*, 1999); and *Klyveromyces lactis* (Poliakova *et al.*, 2002), are known to initiate ACD.

In *S. cerevisiae*, apoptosis-like cell death is also observed in cells with a defective cell cycle (Madeo *et al.*, 1997), upon inactivation of Asf1 (Yamaki *et al.*, 2001), Cdc13 (Qi *et al.*, 2003) or Stm1 (Ligr *et al.*, 2001), after replicative and chronological aging (Laun *et al.*, 2001; Herker *et al.*, 2004; Leslie, 2004), in the absence of mRNA decapping (Mazzoni *et al.*, 2003a), upon nutrient imbalance (Granot and Dai, 1997; Granot *et al.*, 2003), and after cell treatment with H_2O_2 (Madeo *et al.*, 1999), acetic acid (Ludovico *et al.*, 2001; Ludovico *et al.*, 2002), high salt (Huh *et al.*, 2002), osmotin (Narasimhan *et al.*, 2001), α-factor mating pheromone (Severin and Hyman, 2002), UV irradiation (Del Carratore *et al.*, 2002; Markkanen *et al.*, 2004), synthetic compounds (Hiramoto *et al.*, 2003; Balzan *et al.*, 2004; Coyle *et al.*, 2004), and hypochlorite (HOCl) (King *et al.*, 2004).

The observation that ACD have been described for several branches along the phylogenetic tree, strongly indicate ACD is ubiquitously an essential attribute of life (Ameisen, 2002). The question is, whether the morphological similarities (Table 1.1, Table 1.2) indicate the presence of an activated endogenous cell death program comparable to the cell death machinery of higher eukaryotes.

We suggest this remains to be seen, because the final death morphology could be regarded as a *post mortem* consequence of entropic processes not implicating parallel pathways (See 3.4.1.4). The only way to address this question is to identify the genes that allow the execution and regulation of ACD in these unicellular eukaryotes.

Trigger / Model organism / Characteristics	Bax mammalia	Bax + ZVAD mammalia	IGFIR mammalia	Bax *S. cerevisiae*	Bax *Sch. pombe*	H_2O_2 *S. cerevisiae*	CH_3COOH *S. cerevisiae*	*cdc48* (S565G) *S. cerevisiae*	Δ*ASF1* *S. cerevisiae*	Autophagy *S. cerevisiae*
Nuclear fragmentation	+	–	–	+	–	+	–	–	+	–
Chromatin condensation	+§	+	–	+	+	+	+	+	+	–
DNA laddering	+	–	–	–	–	–	?	–	–	–
Vacuolisation	–	+	+°	+	+	+	–	–	+	+
Cell membrane integrity	+	+	+	+	+	+	+	+	+	+

Table 1.1: Characteristics of active cell death. The effect of Bax expression in mammalian cells, with or without caspase inhibitors, is compared to its cytotoxic effects in yeast. The effect of Bax expression in yeast clearly resembles the cell death phenotype of mammalian cells in which caspases were blocked. However, it is not comparable to paraptosis (IGFIR). In addition, death morphology due to Bax expression in yeast is similar to other toxic insults, like H_2O_2 addition or absence of an essential protein. (?) Not known to date; (+)/(–) presence/absence; (°) Non-autophagic vacuoles. Chromatin is loosely condensed (+), unless indicated (+§).

Model organism / Characteristics	Leishmania	Dictyostelium	Peridinium	Hydra	Mammalia
Nuclear fragmentation	+	+	?	?	+
Chromatin condensation	+	+	?	+	+
DNA laddering	–	–	–	+	+
Vacuolisation	–	+	–	–	–
Cell membrane integrity	+	+	+	+	+

Table 1.2: Comparison of the active cell death characteristics of some lower eukaryotes with mammalian apoptosis. The unicellulars are arranged according to phylogeny. Nuclear fragmentation and chromatin condensation may be very 'old' characteristics, as they are observed in Leishmania's. On the other hand, DNA laddering is more 'recent'.

4.2 Why active cell death in unicellulars?

4.2.1 Once upon a time: the beginning of an end

The central position of mitochondria in apoptosis has provided a theory for the evolutionary origin of ACD, suggesting it could have arisen as a by-product of endosymbiosis and may be linked to the origin of mitochondria. According to the serial endosymbiont theory, eukaryotic cells started out as anaerobic organisms before establishing a stable endosymbiotic relationship with aerobic bacteria, whose oxidative phosphorylation system they subverted for their own use (Taylor, 1974). Thus, the origin of eukaryotic ACD could be interpreted in terms of molecular cross talk (Fig. 1.7) that took place at the endosymbiont/host interface, hereby providing the basis for the mitochondrial localisation of killer components (Kroemer, 1997b; Mignotte and Vayssiere, 1998).

In this way, the surprising parallel between bacterial sporulation and mitochondrial fragmentation during programmed cell death of mammalian cells may be seen in an evolutionary perspective (Frank *et al.*, 2001; Frank *et al.*, 2003).

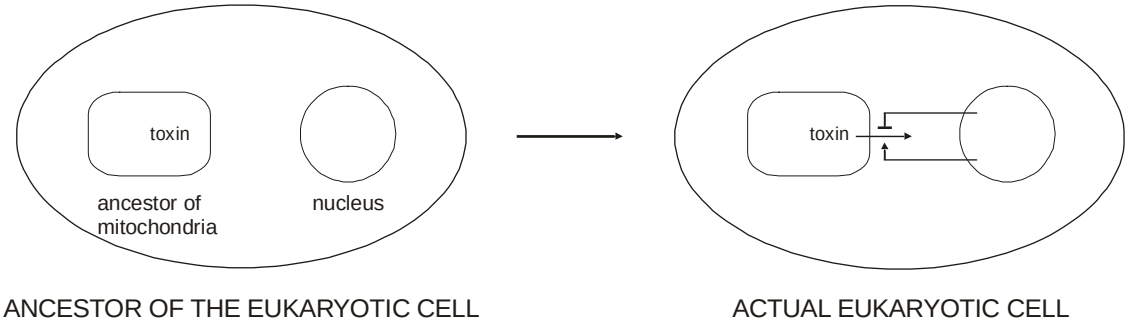

Fig. 1.7: Mechanism giving rise to endosymbiosis and control of active cell death. The endosymbiotic bacterium might have contained molecules lethal for the host in the future mitochondrial matrix or in the intermembrane space. In this context, the host cell would have been constrained to control the exchanges between mitochondria and the cytosol. In unicellular eukaryotes, damage to mitochondrial membranes might provoke the release of these molecules in the cytoplasm. Metazoa might have developed means to control these events. Adapted from Mignotte and Vayssiere, 1998.

A form of cell death with some features resembling apoptosis has been described for the amitochondrial hydrogenosome-containing unicellular organism *Trichomonas vaginalis* (Chose *et al.*, 2002). However, hydrogenosomes and mitochondria were suggested to have a common ancestral origin (Bui *et al.*, 1996; Dyall *et al.*, 2000). Therefore, the analysis of cell death in primitive eukaryotes lacking both mitochondria and hydrogenosomes (e.g. *Giardia intestinalis*, *Giardia lamblia*) (Martin and Muller, 1998), which under the hypothesis described above should be devoid of such features, is of particular importance.

4.2.2 For the good of the state: a happy end

Why should it be useful for unicellular organisms to possess a mechanism for self-eradication? The answer seems likely to come from appreciating that unicellular organisms give rise to colonies or populations of cells, all possessing the same genome derived from a common ancestor. Thus, cellular behaviours that sacrifice some of the members of this population so that others can survive ultimately ensure propagation of the original genome to future generations. In this context, the question becomes whether the lack of genes allowing ACD may result in the counterselection of the whole colony. It is attractive to postulate that ACD evolved as a defence strategy in single-celled organisms for the good of the colony itself (Ameisen, 1996, 2002). Its natural selection might have been achieved by some theoretical evolutionary pressure to (i) facilitate the constant selection of the fittest cell; (ii) optimally adapt cell numbers to the environment, such as fluctuations in nutrient availability; or (iii) to serve as a means for altruistic cell death to prevent the spread of virus during infection; or (iv) to ensure that cells with damaged DNA do not pass on their defective genomes to future generations.

However, ACD in unicellulars also present a serious problem to populations uniformly challenged by a lethal factor, as ACD of all cells would be counterproductive. In this case, it is suspected that, as with bacteria, part of yeast populations consists of persistors with a disabled death program (Lewis, 2000).

4.3 Archaeology of active cell death

We are led to the hypothesis of a single and therefore ancient core molecular mechanism to which successive layers of complexity were added during evolution. Based on this predicted shared ancestry, one may expect some ACD key events and/or components to be conserved.
In addition, host/parasite interactions may have exerted additional selective pressures on the cell death phenotype of parasites (e.g. kinetoplastids), resulting in the more recent emergence of an apoptotic machinery in these unicellular eukaryotes through convergent evolution (Arnoult *et al.*, 2002).

4.3.1 Conserved key events?

ROS have an important role in the regulation of apoptosis (Hockenbery *et al.*, 1993; Zhang *et al.*, 1997). In addition, these molecules are also seen as an important regulator of yeast cell death, based on the observation that they are both necessary and sufficient for ACD in *S. cerevisiae* (Madeo *et al.*, 1999). Moreover, ROS generation is also associated with *P. gatunese* ACD (Vardi *et al.*, 1999), indicating the increase of ROS to be a key event in the ancestral cell death pathway. Further, this may point towards the initial ACD purpose to be an altruistic response to severe oxidative damage.

4.3.2 Conserved components?

On teleological grounds, it can be speculated that the core of cell death control would involve structures, which are essential for both life *and* death (Kroemer, 1997b). Indeed, if cell death control were to be exerted by proteins fulfilling essential metabolic functions, this ‘fail-safe’ mechanism would ensure mutants with a disabled cell death program to be no longer viable. Perhaps, one should focus on the essential proteins of unicellulars to go back to the ‘core business’ of ACD.

4.3.2.1 Mitochondrial effectors

Arnoult and colleagues (2001) demonstrated *D. discoideum* to contain a homologue of mammalian AIF localised to the mitochondria, which upon the onset of ACD translocated to the nucleus resulting in DNA degradation. This finding indicated AIF may be an ancestral and phylogenetically conserved mitochondrial effector of nuclear degradation (Arnoult *et al.*, 2001). Although AIF homologues were found in all primary kingdoms (Bacteria, Archaea, Eucaryota), they appear not to be present in *S. cerevisiae* (Lorenzo *et al.*, 1999; Lorenzo and Susin, 2004).

The mitochondrial release of cyt-c is an important biochemical event in apoptosis and cytosolic cyt-c is known to be an apoptotic signal itself (Kluck *et al.*, 1997). Although mitochondrial cyt-c release was demonstrated to occur in *S. cerevisiae*, yeast cyt-c preparations were not able to initiate apoptosis in mammalian cells implying the need for specific features present in mammalian cyt-c that allow caspase-dependent pathways to become activated (Reed *et al.*, 1998).

4.3.2.2 Conserved domains

The apoptosis machinery is based on several ancient domains that didn't originate for this function, but rather have been recruited from proteins already present in the common ancestor (Aravind *et al.*, 1999; Koonin and Aravind, 2002). For example, Uren and co-workers (Uren *et al.*, 1998) reported the first cell death protein family in single-celled organisms. According to their data, a BIR (Baculovirus Inhibitor of apoptosis Repeat) protein is present in *S. cerevisiae* and this yeast protein (Bir1) seemed to have a role in cell division (Uren *et al.*, 1999; Yoon and Carbon, 1999; Li *et al.*, 2000). However, as the presence of a BIR domain does not strictly imply the protein to be involved in apoptosis inhibition, it would be premature to conclude that yeast uses an ACD mechanism similar to metazoan apoptosis.

The BH3 domain of human hRad9, which promotes apoptosis in the absence of p53 (Komatsu *et al.*, 2000b), is conserved in the *S. pombe* SpRad9. As overexpression of SpRad9 in mammalian cells caused apoptosis, SpRad9 was suggested to be the first member of the Bcl2 protein family identified in yeast (Komatsu *et al.*, 2000a).

Conserved domains have also been identified in zebrafish (*Danio rerio*) (Inohara and Nunez, 2000).

4.3.2.3 Bcl2 family and Bcl2 homology domains

In mammalian cells, an apoptotic stimulus can induce the translocation of cytosolic Bax proteins to mitochondrial membranes, thereby killing the cells, unless opposed by Bcl2 (Goping *et al.*, 1998). This scenario is highly reminiscent of an ancient system in bacteria, involving secreted channel-forming colicins and opposing immunity proteins that, upon binding, prevent channel formation as a means of conferring cytoprotection to resistant bacterial strains (Cramer *et al.*, 1995). Keeping the endosymbiont hypothesis in mind (Taylor, 1974), the colicin-immunity system thus could have provided a framework for the subsequent emergence of the Bcl2 family of proteins; many of which are localised to mitochondrial membranes and, like the colicins, are inextricably involved in the regulation of cell life and death. In-house BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) database searches of the *S. cerevisiae* genome did not detect obvious homologues of Bcl2-like proteins, and even very sensitive database searching, performed by implementing the HMM (Hidden Markov Models) profile (Durbin *et al.*, 1998), could not extend this observation.

Bcl-2 homology domains were cloned form sponges, the phylogenetically oldest and still extant phylum of Metazoa (Wiens *et al.*, 2000) and these sponge Bcl-2 homologues conferred stress resistance to human cells (Wiens *et al.*, 2001).

4.3.2.4 Caspases

Uren and colleagues identified new relatives of caspases, which they termed metacaspases and paracaspases (Uren *et al.*, 2000), via iterative PSI-BLAST (Position-Specific Interated BLAST) searches. Their homology to human caspases is not restricted to the primary sequence, but extends to the secondary structure as well. Metacaspases encompass a member in *S. cerevisiae*, Yca1, and this first Yeast CAspase is known to regulate apoptosis-like cell death in yeast (Madeo *et al.*, 2002), although not all pathways seem to be Yca1-dependent (Wadskog *et al.*, 2004). In addition, some mammalian caspases and a metacaspase of *Trypanosoma brucei* are cytotoxic in yeast (Kang *et al.*, 1999; Ryser *et al.*, 1999; Szallies *et al.*, 2002).

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Chapter 2
Introduction to oxidative stress

Aerobic life takes advantage of the strong oxidising capability of molecular oxygen to obtain energy efficiently. Paradoxically, cells are also exposed to the toxic by-products of aerobic metabolism and there has been strong evolutionary pressure for aerobic organisms to evolve many efficient systems to deal with oxygen-derived oxidant molecules and the types of damage they may cause (Halliwell and Gutteridge, 1984).

The aim of this chapter is to describe the reactive oxygen species, the macromolecular damage associated with oxidative stress and the antioxidant defence systems present in the budding yeast *Saccharomyces cerevisiae*.

1. Reactive oxygen species

Although molecular oxygen contains an even number of electrons, it has two unpaired ones each located in a π* antibonding orbital, and it is said to be in the triplet ground state (Fig. 2.1). These two electrons have the same spin quantum number and therefore, have parallel spin. This makes molecular oxygen relatively unreactive in the context of biological macromolecules, since if it were to oxidise another atom or molecule by accepting a pair of electrons from it, both new electrons must be of parallel spin to fit into the vacant spaces of the orbitals. This spin requirement imposes an important restriction on oxidation by molecular oxygen because an electron pair in an atomic or molecular orbital usually has antiparallel spin. Therefore, molecular oxygen is generally constrained to one-electron transfer reactions at a time (Taube, 1965; Halliwell, 1987).

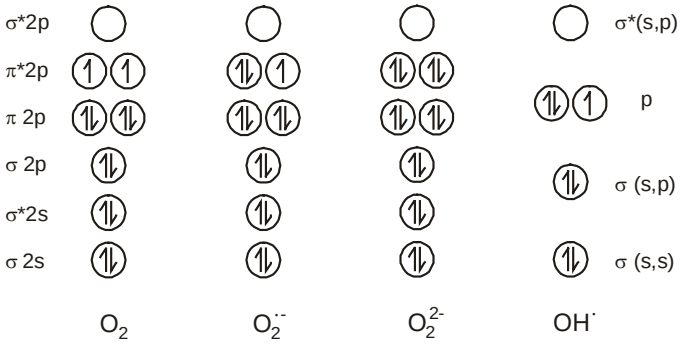

Fig. 2.1: Occupancy of the molecular orbitals for oxygen**,** the superoxide radical and the peroxide ion (adapted from Halliwel and Gutteridge, 1984). Both molecular and atomic orbitals are part of the orbital diagram for the hydroxyl radical (Goeminne, Ghent University, personal communication). The energy level of each orbital is represented by its relative position: more energy is required for filling an upper orbital.

As an example, the mitochondrial electron transport system consists of a series of membrane-associated electron carriers that function in an integrated fashion to carry electrons from the energy source to oxygen molecules, eventually reducing the final electron acceptor to H_2O. During this four-electron reduction, a diatomic oxygen molecule is tightly bound by cytochrome-c oxidase to prevent deleterious effects of the intermediate products (Halliwell and Gutteridge, 1990). Under normoxia it is expected that 1-4% of the electrons dedicated to mitochondrial electron transport are directly transferred to oxygen by ubisemiquinone (complex III) or by the flavin mononucleotide group of NADH dehydrogenase (complex I), resulting in its incomplete reduction and the generation of Reactive Oxygen Species (ROS) (Richter, 1988; Beyer, 1991; Liu *et al.*, 2002). Besides mitochondria, also the endoplasmic reticulum is an intracellular site of ROS production (Fleury *et al.*, 2002), as oxidative protein folding (Tu *et al.*, 2000) and sterol biosynthesis can lead to NAD(P)H dependent electron transport (Winston and Cederbaum, 1983).

Free radicals or Reactive Oxygen Intermediates (ROI) are highly unstable molecules, which have one or more unpaired electrons. As such, these chemical entities may be stabilised through the attack on and removal of electrons from other sources with the concomitant destabilisation of the donor molecule. These free radical species, such as superoxide and hydroxyl radicals, together with other non-radical but also highly oxidant oxygen-derived molecules, such as hydrogen peroxide, are collectively known as ROS. They have high reactivity towards biological macromolecules and can

disrupt the cellular redox homeostasis towards a more oxidised state. This cellular condition, known as oxidative stress, occurs when the load of oxygen-derived free radicals and activated oxygen species increases beyond the antioxidant buffering capacity of the cell. Under normal physiological conditions, antioxidant defence mechanisms are adequate to maintain ROS at a basal unharmful level and to repair cellular damages (Halliwell and Gutteridge, 1984). However, these defence systems are not 100% efficient and, consequently, oxidative lesions are known to accumulate with age (Shigenaga *et al.*, 1994; Laun *et al.*, 2001).

1.1 The superoxide radical

The moderately reactive superoxide radical is formed by the one-electron reduction of oxygen. However, this reaction is thermodynamically unfavourable [E_0 $O_2/O_2^{\bullet-}$: -0.33V] (Ilan *et al.*, 1976). A way to overcome this spin restriction lies in the interaction of O_2 with another paramagnetic centre to participate in exchange coupling. Transition metals, such as Fe or Cu, frequently have unpaired electrons and are excellent catalysts of O_2 reduction (Fig. 2.2).

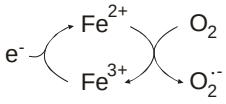

Fig. 2.2: Generation of the superoxide radical.

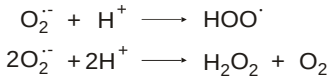

Fig. 2.3: Reactivity of the superoxide radical.

The primary source of the superoxide anion is mitochondrial respiration (Gralla and Kosman, 1992). Although superoxide anions are not strongly reactive, they must be removed efficiently from cells since they can give rise to hydrogen peroxide and other more damaging free radical species. In aqueous neutral pH solutions, the superoxide anion disproportionates to H_2O_2, whereas protonation of $O_2^{\bullet-}$ gives rise to the hydroperoxyl radical, a far more reactive species that can abstract hydrogen atoms from polyunsaturated fatty acids and thus initiates lipid auto-oxidation (Fig. 2.3).

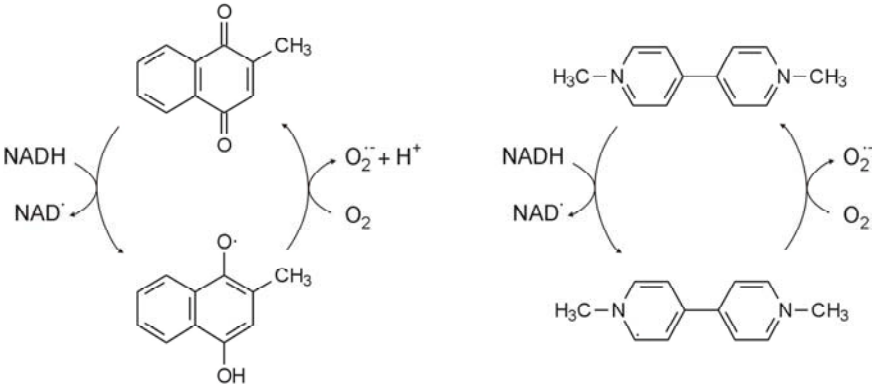

Fig. 2.4: The redox cycling via menadione (left) or via paraquat (right) is known to produce superoxide radicals.

Superoxide radicals can also be generated through redox cycling (Fig. 2.4) via certain compounds, such as paraquat or menadione. These chemical agents cross biological membranes with relative ease and engage in redox chemistry under aerobic conditions (Greenberg *et al.*, 1990).

1.2 Hydrogen peroxide

Hydrogen peroxide is formed in the cell through $O_2^{\bullet -}$ disproportionation by superoxide dismutases (Fig. 2.5), as well as through the action of the enzyme acyl-CoA oxidase. The latter catalyses the first reaction of the fatty acid β-oxidation cycle (Wang *et al.*, 1992), converting acyl-CoA chains to trans-2,3-dehydroacyl-CoA chains and H_2O_2 at the expense of molecular oxygen.

$$2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2$$
$$O_2 + RH_2 \longrightarrow H_2O_2 + R$$

Fig. 2.5: Generation of hydrogen peroxide.

Hydrogen peroxide has no unpaired electrons and as such, it cannot be considered a free radical (Halliwell and Gutteridge, 1984). However, H_2O_2 efficiently crosses biological membranes and serves as a key chemical reactant in the generation of the highly reactive and damaging free hydroxyl radical via several mechanisms.

1.3 The hydroxyl radical

The main toxic effects of both the superoxide anion and hydrogen peroxide result from their ready conversion to the extremely reactive $OH^{\bullet}$. The hydroxyl radical reacts in a diffusion-limited manner, only 5-10 molecular diameters (30Å) away from its site of formation (Pryor, 1986). It is responsible for most biological damage and $OH^{\bullet}$ indiscriminately reacts with sugars, amino acids, phospholipids, nucleotides and organic acids (Lesko *et al.*, 1980; Halliwell and Aruoma, 1991). The spontaneous homolytic decomposition of H_2O_2 can be a source of $OH^{\bullet}$, but the metal-dependent Fenton reaction is a more efficient means of generating hydroxyl radicals from hydrogen peroxide (Fenton, 1894; Halliwell and Gutteridge, 1984). The Fenton chemistry involves Fe^{2+} or Cu^{1+} and the reduced form of either metal can be generated by the coupled Haber-Weis reduction of $O_2^{\bullet -}$ (Haber and Weiss, 1934; Halliwell and Gutteridge, 1984) (Fig. 2.6).

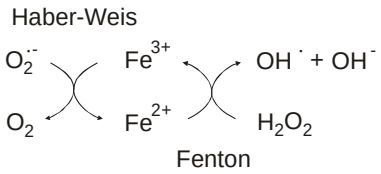

Fig. 2.6: Coupled chemistry of Haber-Weis and Fenton reaction: neutralisation of a superoxide ion at the expense of hydroxyl radical generation.

The instantaneous elimination of the substrates for hydroxyl formation is primordial, as no efficient scavenging systems could be evolved due to their high reactivity. As a matter of fact, cells are provided with a fine-tuned mechanism that prevents the accumulation of copper and iron to toxic levels, yet guarantees sufficient enzyme cofactors for normal biological reactions.

2. Oxidative damage to macromolecules

Oxidants can damage a wide variety of cellular molecules, including lipids, proteins and nucleic acids. Given the wide range of cellular targets, there is little knowledge of the events that lead to the loss of cell viability following severe oxidative damage. Moreover, in a given population the individual cells may lose viability as a result of different types of damage.

2.1 Damage to lipids

Lipid peroxidation is a highly detrimental process for membrane structure and function (Pradhan *et al.*, 1990). It impairs the structural integrity of membranes by generating shorter fatty acyl chains and increasing the membrane fluidity. Unsaturated lipids are main targets of attack by ROS, leading to a process of autocatalytic lipid peroxidation. The free radical initially abstracts a hydrogen atom from a methylene group (Fig. 2.7) and the allylic hydrogens adjacent to double bonds in unsaturated fatty acyl chains are highly susceptible (a). Only certain ROS are able to react in this way, including hydroxyl and peroxyl free radicals, while superoxide anions are not considered to be sufficiently reactive (Halliwell and Gutteridge, 1990). During this process, reactive fatty acyl radicals and peroxyl radicals are formed and these contribute to the autocatalytic propagation (b) of damage to lipids, proteins and DNA MacroMolecules (MM). A coupling between two fatty acyl radicals (c) terminates the transmission of radical injury but results in acyl chain cross-linking. The fatty acyl peroxyl radicals, formed in the presence of oxygen, also give rise to alkoxyl radicals – via the Fenton chemistry – resulting in fatty acyl chain fragmentation (d).

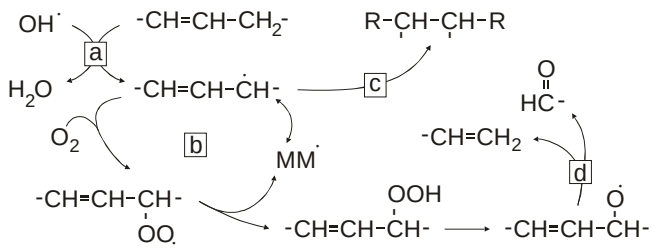

Fig. 2.7: Free radical damage to lipids is initiated by the abstraction of a hydrogen atom (a). Propagation then damages other macromolecules (MM) (b). The coupling between two fatty acyl chain radicals terminates the domino effect but introduces an acyl chain cross-link (c). Lipid hydroperoxides can lead to fragmentation through the conversion to alkoxyl radicals via Fenton chemistry (d).

Yeast membranes do not contain polyunsaturated fatty acids, instead 30-50% of the phospholipid bilayer consists of monounsaturated palmitoleic acid. Polyunsaturated fatty acids are a better ROS target and therefore it is uncertain to which extent lipid peroxidation occurs in yeast membranes. The presence of malondialdehyde in yeast cells treated with H_2O_2 is sometimes seen as an indication for fatty acid degradation (Steels *et al.*, 1994), but this breakdown product can also be generated by ROS attack on amino acids and deoxyribose (Burger *et al.*, 1980).

2.2 Damage to proteins

Proteins have a general sensitivity to oxygen radicals (Davies, 1987) and the oxidative damage to proteins can involve amino acid oxidation, protein fragmentation, loss of catalytic activity, disassembly of iron-sulphur clusters, protein denaturation or protein cross-linking, leading to increased proteolytic susceptibility and decreased biological activity (Flint *et al.*, 1993; Cheng *et al.*, 1994). Mitochondrial and some glycolytic enzymes are among the most preferred targets for protein oxidation (Cabiscol *et al.*, 2000; Costa *et al.*, 2002; Shanmuganathan *et al.*, 2004) due to topographical and innate reasons, respectively. Thus, mitochondrial metabolism and glycolysis may slow down during oxidative stress. Oxidative attack on the polypeptide backbone (Fig. 2.8) is initiated by the $OH^{\bullet}$-dependent abstraction of the α-hydrogen atom of an amino acid residue to form a carbon-centred radical (a). This radical then rapidly reacts with O_2 to form an alkylperoxyl radical. Both carbon-centred radicals and peroxyl radicals contribute to the autocatalytic propagation (b) of damage to lipids, DNA, and other proteins (MM). A coupling between two carbon-centred radicals (c) terminates the transmission of radical injury but results in protein cross-linking. The alkylperoxyl radicals that are formed in the presence of oxygen also give rise to alkoxyl radicals via Fenton chemistry, resulting in peptide bond cleavage by either the α-amidation (d) or diamide (e) pathways (Berlett and Stadtman, 1997).

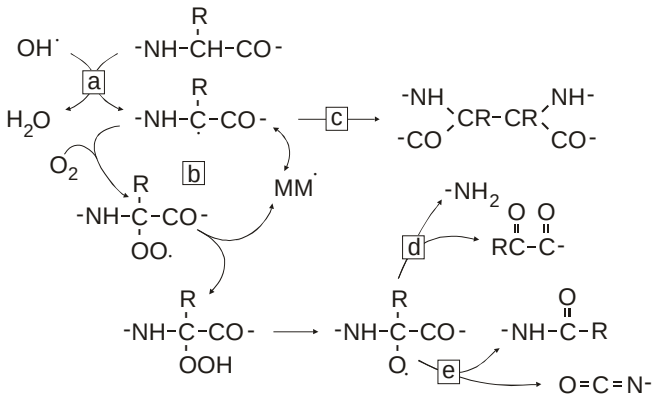

Fig. 2.8: Free radical damage to the backbone of proteins is initiated by hydrogen abstraction (a). Propagation then damages other macromolecules (MM) (b). The coupling between two carbon-centred radicals terminates the domino effect but introduces a protein cross-link (c). Protein hydro-peroxides can lead to fragmentation through the conversion to alkoxyl radicals via Fenton chemistry (d, e).

All amino acid residues of proteins are susceptible to $OH^{\bullet}$ oxidation, although aromatic amino acids are among the preferred targets (Stadtman, 1993; Berlett and Stadtman, 1997). In addition, cysteine and methionine residues are particularly sensitive to oxidation by almost all forms of ROS. When unwanted disulphide bonds are generated in cytosolic proteins, the situation is referred to as disulphide stress (Aslund and Beckwith, 1999). Intramolecular disulphide bridges alter protein conformation whereas intermolecular disulphide bridges between cysteine-rich molecules create dimers and multimers, potentially influencing association with other cellular proteins. Methionine residues are oxidised to methionine sulphoxide. Yeast contains disulphide reductases and methionine sulphoxide reductase that can convert the oxidised forms back to their unmodified ones. These are the only oxidative modifications of proteins that can be repaired. Based on the observation that preferential oxidation of several exposed methionine residues in some proteins has little effect on

their biological function, it was proposed that the cyclic oxidation-reduction of methionine residues serves as a "built-in" ROS scavenger system, thereby protecting such proteins from more extensive and irreversible oxidative modifications (Levine *et al.*, 1996; Moskovitz *et al.*, 1997). The sulphydryl groups of a cysteine residue can also be oxidised to a sulphenic acid (-SOH), sulphinic acid (-SSH) or a sulphonic acid (-SO_3H) and these modifications are irreversible (Toone *et al.*, 2001).

2.3 Damage to nucleic acids

In spite of the double-stranded nature, the compact structure and the histone-mediated packaging of genomic DNA (Enright *et al.*, 1992), it is still an important target for oxidative damage because iron can localise along the phosphodiester backbone (Imlay and Linn, 1988). A substantial portion of the H_2O_2 lethality involves DNA damage by oxidants generated from iron-mediated Fenton reactions (Luo *et al.*, 1994; Keyer and Imlay, 1996). Because the hydroxyl radical has high thermochemical reactivity and a diffusion-limited mode of action, its generation in DNA proximity is very likely to lead to DNA damage. Indeed, Fenton oxidants cause damage to the phosphodiester sugar backbone and the DNA bases, leading to a wide variety of lesions (Henle *et al.*, 1996; Luo *et al.*, 1996).

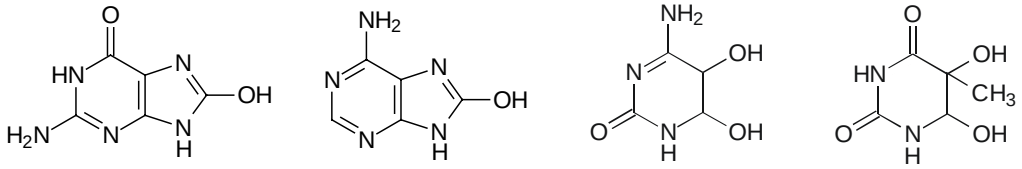

Fig. 2.9: Some of the end products of the $OH^{\cdot}$ attack on DNA bases. From left to right: 8-hydroxyguanine, 8-hydroxyadenine, cytosine glycol and thymine glycol. Adapted from Halliwel and Aruoma (1991).

Sugar damage is initiated by hydrogen abstraction from one of the deoxyribose carbons and results in deoxyribose fragmentation, eventually leading to strand breakage and release of a base-propenal, which subsequently decomposes to the free base and malondialdehyde (Janicek *et al.*, 1985; Bertoncini and Meneghini, 1995). Besides fragmentation, the deoxyribose moiety can be altered by a β-to-α conversion at the 1'-carbon, which disrupts the B-structure of the DNA helix (Ide *et al.*, 1994). Base damage is mainly initiated at the electron-rich double bonds, particularly the purine N7-C8 and the pyrimidine C5-C6 (Henle *et al.*, 1996; Luo *et al.*, 1996), leading to a spectrum of potentially mutagenic base lesions (Halliwell and Aruoma, 1991; Michaels *et al.*, 1992); (Demple and Harrison, 1994) such as 8-hydroxyguanine, 8-hydroxyadenine, cytosine glycol and thymine glycol (Fig. 2.9).

Nucleic acid damage can also result in DNA recombinations and DNA-protein cross-links. DNA-DNA intrastrand cross-links were not shown to be formed by oxygen radicals (Henle and Linn, 1997).

3. Antioxidant defence systems in yeast

In order to maintain ROS at physiologically harmless levels, yeast cells have developed at least four lines of defence against oxidative stress. The first mechanism aims at preventing the formation of ROS, mainly by maintaining metal ion homeostasis. The second line of defence is involved in the scavenging or interception of ROS using both enzymatic and non-enzymatic systems. The third defence system includes the repair of free radical-mediated cellular damage and the replacement of heavily damaged molecules via *de novo* synthesis. The fourth mechanism results in the adaptation to the adverse condition through the biosynthetic induction of antioxidant defence components. These four lines of defence are known as *prevention*, *interception*, *repair* and *adaptation*.

Some authors refer to interception and repair as being the primary and secondary defence mechanisms, respectively. But this numeric nomenclature ignores the preventive systems present in cells and is therefore somewhat confusing. Hence, the four-lines classification is strongly favoured (Noguchi *et al.*, 2000).

3.1 Prevention

3.1.1 Metallothionein

Metallothioneins are small, cysteine-rich metal-binding proteins (6 kDa) that play a central role in metal detoxification by scavenging metal ions, especially copper and iron (Hamer, 1986; Kagi and Schaffer, 1988). Yeast metallothioneins are encoded by the *CUP1* and *CRS5* genes (Butt *et al.*, 1984; Culotta *et al.*, 1994) and protect cells from elevated levels of metal ions via high-affinity binding and sequestering through cysteine thiolates (Kagi and Schaffer, 1988). The sequestration of these metal ions renders them unavailable from engaging in ROS-generating redox chemistry. Metallothioneins are thus involved in the first line of antioxidant defence. It was demonstrated that the Cup1 metallothionein shows an additional antioxidant function by scavenging the reactive oxygen species $O_2^{\bullet -}$ and $OH^{\bullet}$ in its Cu^{1+}-bound form. Therefore, Cup1 is involved in both the first and the second defence strategy (Felix *et al.*, 1993; Tamai *et al.*, 1993).

3.2 Non-enzymatic interception

3.2.1 Glutathione

Glutathione (GSH), a tripeptide δ-L-glutamyl-L-cystinylglycine, is one of the major antioxidant molecules and it is thought to play a vital role in buffering the cell against ROS (Meister and Anderson, 1983) and toxic electrophiles (Li *et al.*, 1997). The biological importance of GSH is dependent upon the redox-active sulphydryl moiety of a cysteine residue.

Glutathione is synthesised in two ATP-dependent steps (Fig. 2.10), catalysed by δ-glutamyl-cysteine synthetase (Gsh1) and glutathione synthetase (Gsh2) (Ohtake and Yabuuchi, 1991; Grant *et al.*, 1997). This mechanism of synthesis has two consequences: first, it is mRNA-independent and

second, the glutamic acid residue is linked to the cysteine residue via its γ-carbon atom which is thought to protect glutathione from proteolytic cleavage (Meister, 1988). Glutathione GSH rapidly undergoes interactions with reactive oxygen intermediates and H_2O_2, leading to a thiyl radical and oxidised glutathione (Fig. 2.11). This thiyl radical is less reactive than the oxygen free radical and therefore, acts as a chain breaker. Further diradical annihilation leads to oxidised glutathione GSSG.

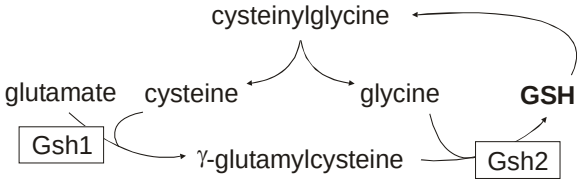

Fig. 2.10: The glutathione cycle in yeast.

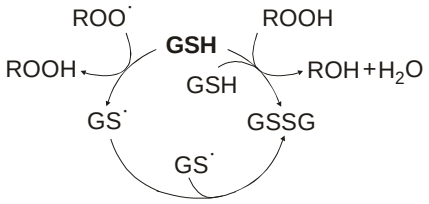

Fig. 2.11: ROS-glutathione interactions.

Glutathione GSH can also form mixed disulphides with protein –SH groups (Fig. 2.13) to prevent irreversible oxidative damage (Shenton and Grant, 2003). The protein S-thiolation is reversible and dethiolation can occur via direct reduction by GSH (Coan *et al.*, 1992; Thomas *et al.*, 1995). In the absence of oxidative stress glutathione GSH also reduces protein disulphides (Fig. 2.12).

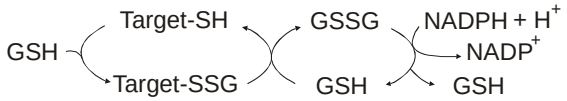

Fig. 2.13: GSH modification of proteins as protection against oxidative damage. GSH itself can reduce mixed disulphide if the GSH/GSSG ratio is high.

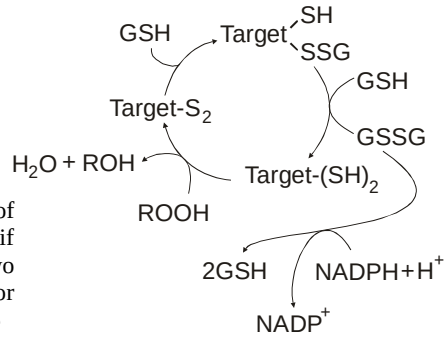

Fig. 2.12: Direct cleavage of protein disulphides by GSH if GSH/GSSG ratio is high. Two GSH molecules are needed for reducing one disulphide bridge

3.2.2 Thioredoxin

Thioredoxins are small, cysteine-rich proteins (11 kDa) with a redox-active dithiol/disulphide active site within the conserved sequence Trp-Cys-Gly-Pro-Cys (Holmgren, 1985, 1989). The amino-terminal Cys-residue of the active site has a low pK value and is the attacking nucleophile in disulphide reduction of proteins. The mechanism involves a transient mixed disulphide intermediate and a fast thiol-disulphide exchange in a hydrophobic environment (Holmgren, 1995). The reaction is reversible and thioredoxin may either form or break disulphides depending on the redox potential of its substrate. However, its low redox potential (E_0: -0.27V) ensures thioredoxin to be the major reductant that keeps thiol groups of intracellular proteins in the reduced form and protects proteins from aggregation and subsequent inactivation, due to the prevention of disulphide stress (Holmgren, 1984, 1985). Additionally, thioredoxins also serve as electron donors for enzymes, such as

thioredoxin peroxidase (Chae *et al.*, 1994a), ribonucleotide reductase (Holmgren, 1989) and 3'-PhosphoAdenosine-5'-Phosphosulphate (PAPS) reductase (Muller, 1991; Lillig *et al.*, 1999).
The reduced form of thioredoxin can be regenerated by reaction with NADPH, catalysed by the flavoprotein thioredoxin reductase (Holmgren, 1985), and these three components are part of the thioredoxin system (Fig. 2.14).

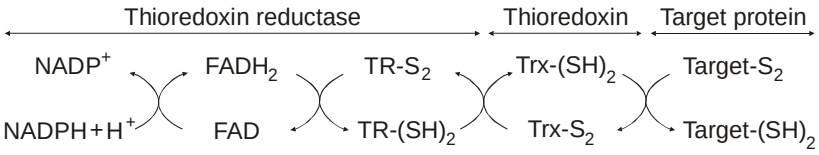

Fig. 2.14: Disulphide reduction by the thioredoxin system: a combined action of thioredoxin reductase (TR) and thioredoxin (Trx). Adapted from Holmgren (1985).

In yeast there are two such systems: one in the cytoplasm with two ThioRedoXins (Trx1 and Trx2) and one ThioRedoxin Reductase (Trr1) (Gan, 1991; Muller, 1991) and a second mitochondrial system (Pedrajas *et al.*, 1999) with one thioredoxin (Trx3) and one thioredoxin reductase (Trr2). It has been shown the mitochondrial and cytoplasmic thioredoxin systems cannot substitute for each other (Draculic *et al.*, 2000). In addition, the cytoplasmic thioredoxin system is required for protection against reductive stress in yeast (Trotter and Grant, 2002) and mitochondrial thioredoxin plays an important role in cell viability, respiration and the regulation of apoptosis in mammalia (Saitoh *et al.*, 1998; Nonn *et al.*, 2003). The thioredoxin and glutathione systems are not merely parallel redox systems, as glutathionylation of thioredoxin is known to regulate its enzyme activity and function (Casagrande *et al.*, 2002). Besides, the redox regulation of both systems appears to be independent and non-reciprocal (Trotter and Grant, 2003).

3.2.3 Glutaredoxin

Glutaredoxins are a class of small proteins (11-25 kDa) with a redox-sensitive active site, acting as glutathione-dependent disulphide reductants (Holmgren, 1989), but catalysing disulphide cleavage only in the presence of low levels of reduced glutathione. They may therefore act to reduce any disulphide formed following oxidative stress (Chrestensen *et al.*, 1995). Yeast contains glutaredoxins within two subfamilies differing in the number of cysteine residues at the active site.
The first GlutaRedoXin family (Grx1 and Grx2) contains a cysteine pair at the active site, reducing protein disulphides via a dithiol mechanism using glutathione as a cofactor (Luikenhuis *et al.*, 1998). GSH first reduces the protein disulphide, followed by reaction with glutaredoxin to release the reduced protein and forming a mixed disulphide between glutathione and glutaredoxin. Disulphide formation at the glutaredoxin active site releases the GSH cofactor and results in the fully oxidised glutaredoxin (Fig. 2.15). Moreover, these glutathione-dependent oxidoreductases can also directly reduce hydroperoxides in a catalytic manner (Collinson *et al.*, 2002), using the reducing power provided by NADPH, GSH and glutathione reductase (Fig. 2.16).

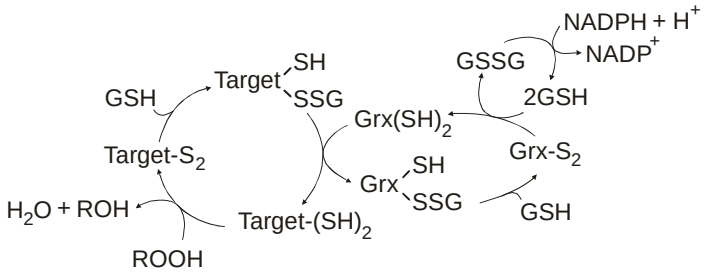

Fig. 2.15: Cleavage of protein disulphides by glutaredoxin (Grx) via a dithiol mechanism with GSH as cofactor. This system is operational during oxidative stress when GSH/GSSG is not that high.

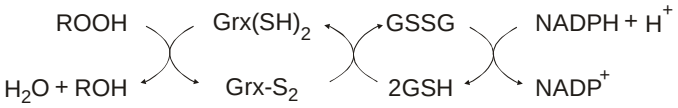

Fig. 2.16: Glutathione-peroxidase activity of glutaredoxin Grx1-2.

The second family (Grx3, Grx4, Grx5) contains a single cysteine residue at the putative active site and these glutaredoxins catalyse the reduction of mixed disulphides of GSH-modified proteins (Fig. 2.17) via a monothiol reaction mechanism (Bushweller *et al.*, 1992; Rodriguez-Manzaneque *et al.*, 1999).

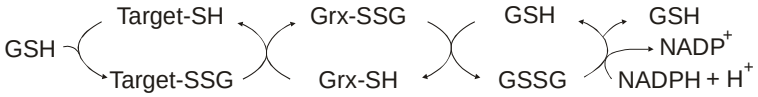

Fig. 2.17: Mixed disulphide reduction by the glutaredoxin (Grx3-5) system. This monothiol reaction mechanism is operational during oxidative stress when GSH/GSSG ratio is not that high.

Glutaredoxins differ from thioredoxins in that the reduced form is regenerated by reaction with GSH, which is then reduced in a NADPH-dependent reaction catalysed by glutathione reductase (Fig. 2.15, Fig. 2.17). The glutaredoxin system thus contains NADPH, the flavoprotein glutathione reductase and glutaredoxin itself (Holmgren, 1989).

Thioredoxins and glutaredoxins are structurally very similar and there appears to be considerable functional redundancy between the two systems; for example they both can act as reductant for ribonucleotide reductase and PAPS reductase (Holmgren, 1989; Lillig *et al.*, 1999).

3.3 Enzymatic interception

3.3.1 Catalase

Catalases (EC 1.11.1.6) are ubiquitous haemoproteins that catalyse the dismutation of hydrogen peroxide into water and molecular oxygen (Fig. 2.18). Yeast have two such enzymes, CaTalase-A and CaTalase-T, encoded by the *CTA1* and *CTT1* genes and located in the peroxisomes and the cytoplasm respectively (Seah and Kaplan, 1973; Hartig and Ruis, 1986; Cohen *et al.*, 1988).

The main physiological role of peroxisomal catalase A appears to remove H_2O_2 produced from fatty acid β-oxidation. The role of catalase T is less clear, although it protects yeast from oxidative stress

(Wieser *et al.*, 1991). Catalases are not important for oxidative stress defence in exponentially growing yeast cells. They have, however, an important role in the removal of H_2O_2 during stationary phase, demonstrated by the fact that an acatalasaemic strain only shows an impaired resistance towards hydrogen peroxide upon entering stationary phase (Izawa *et al.*, 1996).

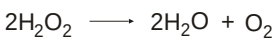

Fig. 2.18: Catalase dismutates hydrogen peroxide.

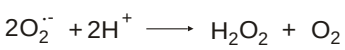

Fig. 2.19: Superoxide dismutase disproportionates superoxide.

3.3.2 Superoxide dismutase

Superoxide dismutases (EC 1.15.1.1) are a group of metallo-enzymes that catalyse the disproportionation of superoxide anions to hydrogen peroxide and molecular oxygen (Fig. 2.19) (Fridovich, 1995), playing an important role in protecting aerobic organisms against oxidative damage (van Loon *et al.*, 1986). Yeast possesses two intracellular SuperOxide Dismutases: the cytoplasmatically located copper/zinc-containing superoxide dismutase (Cu/Zn-SOD), encoded by the *SOD1* gene (Gralla and Kosman, 1992), and the mitochondrially located manganese-bound superoxide dismutase (Mn-SOD), encoded by *SOD2* (Bermingham-McDonogh *et al.*, 1988).

The yeast Cu/Zn-SOD is a homodimer of two 16-kDa subunits and each monomer binds to a single copper and zinc ion, which are absolutely required for catalysis. The yeast Mn-SOD is tetrameric, having four 23-kDa subunits, each of which binds to a single Mn-ion (Gralla and Kosman, 1992).

The Cu/Zn-SOD appears to be the major enzyme involved in removing superoxide anions from the cytoplasm and possibly the peroxisome (Gralla and Valentine, 1991), while Mn-SOD is the primary defence against the toxicity of superoxides generated in the mitochondria during respiration (Guidot *et al.*, 1993).

3.3.3 Cytochrome-c peroxidase

Cytochrome-c peroxidase (EC 1.11.1.5), a mitochondrial haem protein present in the intracristal space, degrades ROS within the mitochondria using cytochrome-c as an electron donor, as depicted in Fig. 2.20 (Mathews and Wittenberg, 1979; Poulos and Kraut, 1980).

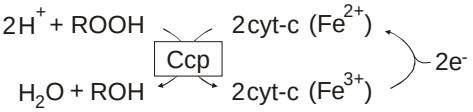

Fig. 2.20: Enzyme cycle of cyt-c peroxidase (Ccp). Two moles oxidised cyt-c are needed for each mole of peroxide to be degraded.

The double oxidised form of the Cytochrome-C Peroxidase Ccp1 contains both a Fe^{4+}ion and a radical that is centred on Trp^{191} of the protein. The 'free' radical probably plays a crucial role in the electron transfer mechanism (Poulos and Kraut, 1980) and it is thought to be important for the activation of the Skn7 transcription factor. Ccp1 is oxidised by hydroperoxides and is subsequently reduced by direct interaction with cyt-c.

3.3.4 Glutathione reductase and peroxidase

Crucial to the role of glutathione as an antioxidant is the maintenance of a high GSH/GSSG ratio inside the cell. This is ensured by the flavoenzyme glutathione reductase (EC 1.6.4.2) which catalyses the reduction of GSSG to GSH by using the reducing power of NADPH, generated from the pentose phosphate pathway via glucose-6-phosphate dehydrogenase and 6-phosphogluconate (Fig. 2.21) (Juhnke *et al.*, 1996; Slekar *et al.*, 1996). The gene encoding GLutathione Reductase has been identified (*GLR1*) (Collinson and Dawes, 1995); null mutants accumulate GSSG (Muller, 1996) and are hypersensitive to oxidants (Grant *et al.*, 1996), indicating a requirement of Glr1 for the protection against oxidative stress.

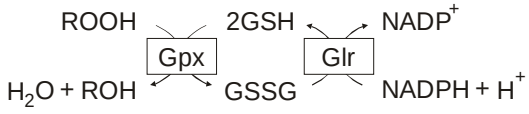

Fig. 2.21: Enzymatic defence against ROS by the combined activity of glutathione peroxidase (Gpx) and glutathione reductase (Glr). Adapted from (Carmel-Harel and Storz, 2000).

Glutathione PeroXidase (EC 1.11.1.19) catalyses the reduction of hydroperoxides using GSH as a reductant (Galiazzo *et al.*, 1987), thereby protecting cells against oxidative damage (Fig. 2.21). In yeast, three gene products (Gpx1, Gpx2 and Gpx3) with significant homology to mammalian glutathione peroxidases have been identified (Inoue *et al.*, 1999). Disruption of *GPX3* results in sensitivity to hydrogen peroxide and Gpx3 appears to account for the majority of the glutathione peroxidase activity in the cell (Inoue *et al.*, 1999). In addition, Gpx3 is known to be an H_2O_2 receptor involved in H_2O_2 scavenging (Delaunay *et al.*, 2002).

3.3.5 Glutathione S-transferase

Two genes encoding functional GlutaThione S-Transferases, designated *GTT1* and *GTT2*, have been identified in yeast. However, Gtt1 and Gtt2 share little sequence homology with each other and with glutathione S-transferases from other species, with the most conserved residues corresponding to the GSH binding site. Strains deleted for *GTT1* and *GTT2* are viable and show no increased sensitivity to ROS, indicating that they are not essential for protection against oxidative stress (Choi *et al.*, 1998). Lipid hydroperoxides need to be conjugated to GSH by the action of glutathione S-transferase before glutathione peroxidase can catalyse their reduction. In addition, glutathione S-transferase mediated conjugation of GSH to lipophilic Xenobiotics (X) also dictates their detoxification via cytosolic removal by the ATP-dependent GS-X pump Ycf1 (Szczypka *et al.*, 1994; Li *et al.*, 1996).

3.3.6 Thioredoxin peroxidase and reductase

Thioredoxin peroxidases reduce hydrogen peroxide and organic alkyl hydroperoxides by the use of hydrogens provided by thioredoxin, thioredoxin reductase and NADPH (Chae *et al.*, 1994a; Chae *et al.*, 1994b; Netto *et al.*, 1996) thereby protecting cellular components against oxidative damage

(Fig. 2.22). Thioredoxin reductase (EC 1.6.4.5) is a member of the family of dimeric flavoproteins that promote catalysis via FAD and a redox active disulphide (Fig. 2.14) (Williams, 1995).

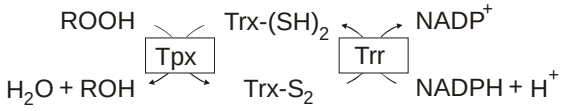

Fig. 2.22: Enzymatic defence against ROS by the combined activity of thioredoxin peroxidase (Tpx) and thioredoxin reductase (Trr). Adapted from (Carmel-Harel and Storz, 2000).

Tsa1 (Thiol-Specific Antioxidant) is an abundant cytoplasmatic thioredoxin peroxidase (Chae *et al.*, 1994a) and it was shown to constitute about 0.5% of the total soluble protein of aerobically grown yeast. A *tsa1* null mutant is hypersensitive to pro-oxidants (Chae *et al.*, 1993), indicating Tsa1 to have a physiologically important antioxidant activity. Due to its abundance, Tsa1 is likely to play a major role in protecting cytosolic proteins from oxygen radical damage (Park *et al.*, 2000), although it preferentially reduces H_2O_2 rather than alkyl hydroperoxides (Jeong *et al.*, 1999). The protection against alkyl hydroperoxide is exerted by Ahp1 (AlkylHydroperoxide Peroxidase), a cytoplasmic thioredoxin peroxidase (Lee *et al.*, 1999b; Wong *et al.*, 2002).

The proposed catalytic mechanism for Tsa1 and Ahp1 involves the reduction of the peroxide substrate by the amino-terminal active site Cys-residue that, upon oxidation, reacts with the carboxy-terminal Cys-residue of another subunit to form an intermolecular disulphide. This disulphide is subsequently reduced with electrons donated by thioredoxin, thioredoxin reductase and NADPH (Chae *et al.*, 1994c; Netto *et al.*, 1996). For Tsa1 as well as Ahp1, the existence of both Cys-residues is essential to maintain the thioredoxin-dependent peroxidase activity (Chae *et al.*, 1994c; Lee *et al.*, 1999b). Therefore, they belong to the 2-Cys Prx subfamily of peroxiredoxins, a large family of peroxidases not containing redox cofactors such as metals or prosthetic groups (Kang *et al.*, 1998; Kim *et al.*, 2000).

The products of three other open reading frames (*YDR453C*, *YBL064C* and *YIL010W*) show homology with Tsa1 and Ahp1 and the corresponding purified proteins were demonstrated to have thioredoxin-dependent peroxidase activity (Park *et al.*, 2000). The mitochondrial thioredoxin-peroxidase Prx1, encoded by *YBL064C*, was shown to belong to the 1-Cys Prx subfamily of peroxiredoxins (Pedrajas *et al.*, 2000) and it uses the mitochondrial thioredoxin system to protect against oxidative stress generated by mitochondrial metabolism.

The five thioredoxin-dependent peroxidases appear to have distinct physiological functions (Park *et al.*, 2000). The current knowledge about them is summarised in Table 2.1. Additionally, Tsa1 is known to have a more pronounced impact on the antioxidant defence when the functional state of the mitochondria is compromised (Demasi *et al.*, 2001). Tsa1 was found to be essential for the Yap1- and Skn7-dependent transcriptional response to oxidative stress (Ross *et al.*, 2000) and thus for the transcriptional induction of other components of the thioredoxin system. Indeed, Yap1 and Skn7 are involved in the regulation of *TSA1*, *TRX2* and *TRR1* expression in response to oxidative stress (Kuge and Jones, 1994; Morgan *et al.*, 1997; Lee *et al.*, 1999a). Together, this suggests Tsa1 functions in a

feedback loop in which at low/high levels of oxidative stress the Tsa1 redox state regulates the low/high expression of the thioredoxin system.

ORF Gene	*PRX Subfam*	*Cellular location*	*Knock-out effect*	*mRNA level during life span*	*Deduced role in cellular physiology*
TSA1	2-Cys	Cytoplasm	ROS^{ss} (t,h,d)	Constant	Critical house-keeping antioxidant Regulator of cellular stress response
AHP1	2-Cys	Cytoplasm	ROS^{ss} (t)	Up at stat phase	Lipid hydroperoxide peroxidase for stat phase survival
YDR453C	2-Cys	Cytoplasm	G_1 arrest	Gradual increase	Cell proliferation and growth arrest during oxidative stress
YBL064C	1-Cys	Mitochondria	ROS^{s} (d)	Up at stat phase	Mitochondrial protection against oxidative damage
YIL010W	?	Nucleus	ROS^{s} (d)	Constant	Protection of DNA against oxidative damage

Table 2.1: Physiological functionality of the five thioredoxin-peroxidases. Not all of them have a known ROS-defensive role.
d: diamide, t: tBuOOH. h: H_2O_2, s: sensitivity, ss: super-sensitivity and stat: stationary.

3.4 Overview

To protect their cellular constituents and to maintain cellular redox state, *S. cerevisiae* possesses a broad range of antioxidant molecules and enzymes, as was outlined above (See 3.1, 3.2 and 3.3). Table 2.2 summarises the data, presenting a quick reference concerning these antioxidant defences.

Gene	Protein	Function
CUP1	Copper-binding protein	Copper homeostasis; prevention of Fenton chemistry
CRS5	Copper-binding protein	Copper homeostasis; prevention of Fenton chemistry
CTA1	Catalase A	Dismutation of hydrogen peroxide (peroxisome)
CTT1	Catalase T	Dismutation of hydrogen peroxide (cytoplasm)
CCP1	Cyt-c peroxidase	Reduction of hydroperoxides (mitochondrial)
SOD1	Superoxide dismutase	Disproportionation of superoxide radicals (cytoplasm)
SOD2	Superoxide dismutase	Disproportionation of superoxide radicals (mitochondrial)
GSH1-2	Glutathione	Reduction of protein disulphides; ROS scavenging
GLR1	Glutathione reductase	Reduction of oxidised glutathione
GTT1-2	Glutathione transferase	Glutathione conjugation to lipid hydroperoxides
GPX1-3	Glutathione peroxidase	Reduction of hydroperoxides
GRX1-2	Glutaredoxin	Reduction of protein disulphides and hydroperoxides
GRX3-5	Glutaredoxin	Reduction of glutathione-modified mixed disulphides
TRX1-2	Thioredoxin	Reduction of protein disulphides (cytoplasm)
TRX3	Thioredoxin	Reduction of protein disulphides (mitochondial)
TRR1	Thioredoxin reductase	Reduction of oxidised thioredoxin (cytoplasm)
TRR2	Thioredoxin reductase	Reduction of oxidised thioredoxin (mitochondrial)
TSA1	Thioredoxin peroxidase	Reduction of hydrogen peroxide (cytoplasm)
AHP1	Thioredoxin peroxidase	Reduction of hydrogen peroxide (cytoplasm)

Table 2.2: Quick overview of the major antioxidant defence systems present in the yeast *S. cerevisiae*. The most typical defensive role(s) of each protein are indicated. Cellular localisation is mentioned if detailed information is known. Please, refer to the paragraphs 3.1, 3.2 and 3.3 for profound concepts.

4. Responses to oxidative stress in yeast

S. cerevisiae cells possess a degree of constitutive resistance towards oxidants. However, treatment of exponentially growing yeast cells with sublethal levels of oxidants results in a transient induction of protection against a subsequent exposure to an otherwise lethal dose (Collinson and Dawes, 1992; Davies *et al.*, 1995). This inducible, protective response to oxidative stress forms the basis of adaptation and is referred to as the adaptive response. It requires the synthesis of increased levels of existing antioxidants and generates new lines of oxidative stress protection via biosynthetic induction mechanisms (Stephen *et al.*, 1995). Therefore, cells need to be equipped with regulatory molecules to rapidly sense and respond to oxidative stress in order to adapt to these redox perturbations. Yeast was shown to have distinct adaptive responses to hydrogen peroxide, menadione and paraquat (Collinson and Dawes, 1992; Jamieson, 1992). Additionally, secondary and tertiary products of oxidative stress, namely lipid hydroperoxides and its breakdown product malondialdehyde, also induce adaptation (Turton *et al.*, 1997; Evans *et al.*, 1998).

4.1 Metabolic changes

The adaptation of *S. cerevisiae* to H_2O_2 was reported to be accompanied with the stimulated synthesis of 115 proteins and the repression of 52 others, as determined by comparative two-dimensional gel electrophoresis (Godon *et al.*, 1998). In addition to the ROS scavenging and antioxidant defence activities (Jamieson *et al.*, 1994), metabolic enzymes were found to be H_2O_2-responsive targets. Remarkable metabolic alterations tend to slow down glycolysis and to redirect the hexose phosphate pool to the pentose phosphate pathway and trehalose synthesis (Godon *et al.*, 1998), leading to the regeneration of NADPH , the most important cellular reducing power. Such a widespread genomic response suggests the existence of specific control pathways and different regulatory mechanisms.

4.2 Transcriptional regulation

A number of studies have presented evidence for much of the regulation of the oxidative stress response in yeast being at the level of transcription (Jamieson *et al.*, 1994; Stephen *et al.*, 1995; Lee *et al.*, 1996), although post-transcriptional control mechanisms may not be ruled out. In spite of the amount and variety of proteins that are induced by oxidative stress, the number of transcriptional control mechanisms turns out to be low and only the Yap1 and Skn7 transcriptional regulators and the general stress response pathway are known to be involved to date.

4.2.1 The bZIP family member Yap1

The Yap1 (Yeast AP1-like Protein) transcription factor was identified by its ability to bind to an AP1 Recognition Element (ARE) without any consideration of function (Harshman *et al.*, 1988; Moye-

Rowley *et al.*, 1989). The AP1 group of mammalian transcription factors consists of homo- and heterodimers of the Jun or Fos protein families and Yap1 was shown to have homology with the c-Jun oncogene. Similar to the AP1 factor, Yap1 contains a bZIP-domain (Fig. 2.23) consisting of a Basic DNA-binding region and a leucine ZIPper that mediates protein homo- or heterodimerisation.

Fig. 2.23: Domain structure of Yap1 protein. bZIP: basic region leucine zipper, nCRD/cCRD: amino-terminal/carboxy-terminal cysteine-rich domain.

In addition, the *YAP1* gene was isolated in an overexpression screen for functions able to overcome the xenobiotics-induced growth arrest (Hussain and Lenard, 1991; Bossier *et al.*, 1993; Wu *et al.*, 1993; Kuge and Jones, 1994). Mutants deficient in Yap1 were shown to be hypersensitive to oxidants (Schnell *et al.*, 1992) and in these null mutants the adaptive response to H_2O_2 was severely impaired (Stephen *et al.*, 1995), indicating the critical role of Yap1 in regulating this response. Expression analyses demonstrated Yap1 to directly regulate the expression of genes whose products play a major role in the oxidative stress response, including *TRR1* (Morgan *et al.*, 1997), *TRX2* (Kuge and Jones, 1994) and *TSA1* (Lee *et al.*, 1999a).

The search for transcription factor-dependent target genes has shifted to a new level by advances in proteomics and with the development of DNA microarrays, allowing the exploration of gene expression patterns on a genomic scale. In a comparative transcriptome analysis, 17 genes whose mRNA levels were increased more than 3-fold were identified in a Yap1-overexpressing strain (DeRisi *et al.*, 1997). A complementary approach, a comparative proteome analysis, identified 32 proteins not/less triggered upon H_2O_2 treatment in a Yap1 null mutant, indicating these to be Yap1-regulated (Lee *et al.*, 1999a).

Upon treatment with H_2O_2, there is no increase in Yap1 protein levels neither in its DNA-binding activity (Kuge and Jones, 1994). Instead, the localisation of the protein changes dramatically. Yap1 is present throughout the cell during normal growth, but it is concentrated in the nucleus when cells are treated with oxidants (Kuge *et al.*, 1997). The *S. cerevisiae* genome contains eight potential AP1-like proteins based on homology within the bZIP-domain (Fernandes *et al.*, 1997). However, oxidative stress-dependent changes in subcellular protein localisation have only been described for Yap1. Given the critical role of Yap1 in the adaptive response to H_2O_2, research has focussed on the regulation of the Yap1 transcription factor by oxidative stress.

In addition to the bZIP-domain, the Yap1 protein was found to contain two Cysteine-Rich Domains (Fig. 2.23), one located at the carboxy-terminus (cCRD) and the other somewhat in the middle of the protein (nCRD), more towards the amino-terminus (Wemmie *et al.*, 1997). The cCRD terminal domain carries three repeated Cys-Ser-Glu motifs embedded in a non-canonical Nuclear Export Signal (NES) (Kuge *et al.*, 1997), which interacts with the nuclear exportin Crm1 to maintain Yap1

in the cytoplasm (Kuge *et al.*, 1998; Yan *et al.*, 1998). Mutational analysis has designated the set of three Cys-residues contained in the cCRD as being required for the normal increase of the Yap1 transactivation upon oxidative stress (Kuge *et al.*, 1998), suggesting that the cellular redox state is regulating Yap1 localisation via the cCRD of the protein. Biochemical studies further revealed the role of the thioredoxin system in the Yap1-dependent response to oxidative stress (Izawa *et al.*, 1999; Delaunay *et al.*, 2000; Carmel-Harel *et al.*, 2001) and mass spectrometry could eventually demonstrate an intramolecular disulphide bond at the cCRD domain (Kuge *et al.*, 2001). Together, these data clearly indicate that the cCRD acts as a redox-sensitive nuclear export signal. Another study also demonstrated the Yap1 localisation to be solely regulated at the step of nuclear export, as the nuclear import mediated by the importin Pse1 was unaffected by oxidative stress (Isoyama *et al.*, 2001).

The nCRD internal domain also contains three cysteine residues and it was shown to be required for H_2O_2 resistance and prolonged nuclear localisation of Yap1 in response to H_2O_2 (Coleman *et al.*, 1999). It is suspected that a transient cCRD redox signal is converted to a more stable nCRD-cCRD form with a lower redox potential in order to prolong the nuclear localisation of Yap1 until the cellular oxidation status is recovered.

However, Yap1 is not directly oxidised by H_2O_2 and the glutathione peroxidase Gpx3 (See 3.3.4) is known to serve as a sensor and transducer of the hydrogen peroxide signal to Yap1 (Delaunay *et al.*, 2000; Delaunay *et al.*, 2002).

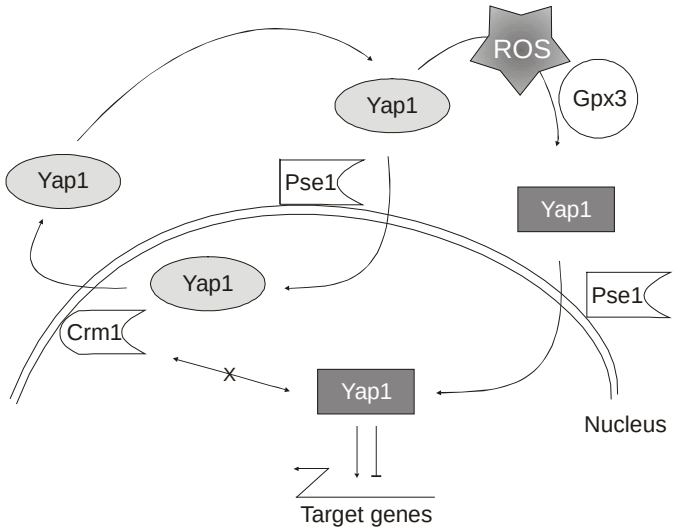

Fig. 2.24: Current model of Yap1 as a redox-sensor. Yap1 is controlled by oxidative stress-dependent changes in subcellular localisation. Under non-stress conditions Yap1 is continually imported into the nucleus and exported in a Crm1-dependent way immediately. Under conditions of oxidative stress the interaction between Crm1 and Yap1 is blocked and the Yap1 transcription factor accumulates in the nucleus. An intramolecular disulphide bond at the cCRD-domain or between cCRD and nCRD is responsible for the redox-dependent inhibition of export.

The current model (Fig. 2.24) suggests that (i) Yap1 is continually imported into the nucleus and exported in a Crm1-dependent way; (ii) disulphide bridging at the cCRD during oxidative stress leads to a conformational change masking the NES and promoting nuclear accumulation of Yap1; (iii) the conversion to a more stable nCRD-cCRD disulphide bridge prolongs nuclear localisation if required; and (iv) the deactivation via enzymatic reduction provides a mechanism for autoregulation.

In addition to its role as a cellular redox sensor, Yap1 was shown to control genes involved in regulating membrane integrity, intracellular trafficking and cell proliferation (Dumond *et al.*, 2000).

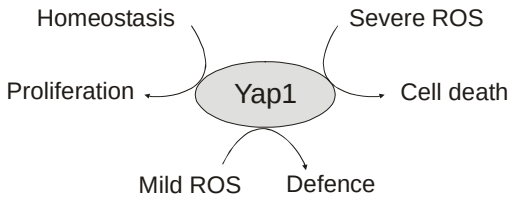

Fig. 2.25: Current model for Yap1 as a mediator of cell growth and cell death. Yap1 is thought to be involved in cell growth during homeostasis. In case of sublethal stress, Yap1 induces a ROS defence. If cellular damage is too high, Yap1 is thought to have an anti-proliferative function.

Accordingly, Yap1 is thought to stimulate cell division during normal aerobic growth and activate cellular defence and slow down proliferation after exposure to H_2O_2 (Fig. 2.25). The role of Yap1 in proliferation correlates well with the involvement of AP1 in either mitogenic or apoptotic processes (Karin *et al.*, 1997).

4.2.2 The response regulator-like Skn7

The isolation of a Skn7 mutant that exhibited an elevated sensitivity to hydrogen peroxide (Krems *et al.*, 1995), revealed the role of the Skn7 protein in the oxidative stress response. Gene expression analysis demonstrated that Skn7 is required for H_2O_2-induced expression of *TRX2* (Krems *et al.*, 1996) and it was found to bind to the *TRX2* promoter *in vitro* (Morgan *et al.*, 1997). A genetic cross of the Skn7 mutant with the Yap1 mutant resulted in a double null mutant with the same sensitivity to H_2O_2 as either single mutant, suggesting the function of Skn7 in oxidative stress protection is closely linked to that of Yap1 (Krems *et al.*, 1996). However, in contrast to Yap1, its function does not seem to be regulated by its cellular localisation, as it can be found in the nucleus independent of the presence or absence of oxidative stress (Raitt *et al.*, 2000). Furthermore, a comparative proteome analysis identified Skn7 to be required for 15 of the 32 Yap1-regulated proteins (Lee *et al.*, 1999a). In addition, G_0-arrested cells are dependent on Skn7 for survival (Aguilaniu *et al.*, 2001) as this growth arrest resulted in a respiratory transition from state 3 (phosphorylating) to state 4 (non-phosphorylating), associated with ROS generation via Q-cycle semiquinones.

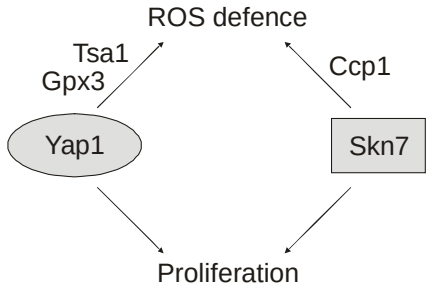

Fig. 2.26: Central role of Yap1 and Skn7. Both transcription factors are involved in the antioxidant defence that is controlled by the peroxidases Tsa1, Gpx3 and Ccp1. Both Yap1 and Skn7 are thought to be involved in cell proliferation when oxidative stress is absent.

A screen for genes involved in the ROS-dependent activation of Skn7, identified cytochrome-c peroxidase Ccp1 as a mitochondrial sensor protein that generates an oxidative stress signal leading to Skn7 activation (Fig. 2.26), and the subsequent transcriptional induction of target genes responding

to oxidative stress (Charizanis *et al.*, 1999b). In addition, an essential nuclear factor Fap7 was seen to be important for Skn7-dependent transcription upon oxidative stress (Juhnke *et al.*, 2000), possibly by stabilising the interaction between Skn7 and the transcriptosome complex.

The Skn7 protein contains a region with a high degree of homology to the receiver domain found in two-component signal transduction systems of prokaryotes (Krems *et al.*, 1996). In bacteria, these signal transduction systems are a common method for detecting and responding to environmental conditions and are composed of at least two crucial proteins: the 'sensor' protein and the 'response regulator', a cytoplasmic effector protein (Kofoid and Parkinson, 1988). The sensor is often associated with the plasma membrane and functions as an autophosphorylating histidine kinase whose activity is regulated by an environmental signal. Communication between the sensor kinase and its response regulator results in the phosphorylation of the latter at a highly conserved aspartic acid residue within the receiver domain (Parkinson, 1993; Lowry *et al.*, 1994), leading to large-scale conformational changes and activation of this protein.

The yeast Skn7 functions in a eukaryotic two-component regulatory pathway (Brown *et al.*, 1994) and its activity is modulated by the Sln1-Ypd1 osmosensor, which contributes to the regulation of the HOG (High Osmolarity Glycerol responsive) pathway (Ketela *et al.*, 1998; Li *et al.*, 1998). However, the signalling through the receiver domain of Skn7 does not appear to have any role in the oxidative stress response (Morgan *et al.*, 1997), although this was initially suggested by Krems and colleagues (1996).

Overexpression of Skn7 suppresses a cell wall defect associated with mutations of the *KRE9* gene (Brown *et al.*, 1993) and the growth defect seen in a *pkc1* null mutant (Brown *et al.*, 1994). As the PKC (Protein Kinase-C) MAP (Mitogen-Activated Pathway) kinase pathway is involved in cell wall biosynthesis (Nonaka *et al.*, 1995; Kamada *et al.*, 1996), this suggested Skn7 could play a role in the expression of cell wall genes. Further, Skn7 was shown to interact with Rho1, a regulator of the PKC MAP kinase pathway (Alberts *et al.*, 1998). A screen designed to isolate new components of the G_1/S-cell cycle transcription machinery, demonstrated the ability of Skn7 to bypass the requirement for the transcription factors SBF and MBF upon overexpression (Morgan *et al.*, 1995). SBF (Swi4/6 cell cycle box Binding Factor), composed of Swi4 and Swi6, controls genes encoding G_1-cyclins (Ogas *et al.*, 1991; Taba *et al.*, 1991). MBF (MluI-Binding Factor), comprising Swi6 and Mbp1, primarily regulates S-phase genes but is also important for G_1-cyclin expression (Dirick *et al.*, 1992; Koch *et al.*, 1993). Furthermore, Skn7 is able to heterodimerise with Mbp1, the DNA-binding component of the G_1/S transcription factor MBF and this new transcription factor could be involved in bud emergence and/or actin organisation (Bouquin *et al.*, 1999).

The Heat-Shock transcription Factor Hsf1 can also function as a Skn7 heterodimerising partner and this interaction was shown to be required for the induction of heat shock genes in response to oxidative stress (Raitt *et al.*, 2000). However, Skn7 has no role in the cellular response upon heat shock (Morgan *et al.*, 1995).

4.3 Regulatory pathways

The cAMP-PKA (Protein Kinase-A) pathway negatively regulates the antioxidant response in *S. cerevisiae*. Under optimal growth conditions, high cAMP levels confer high PKA activity, resulting in cellular proliferation and the absence of stress responses. In adverse growth conditions, cAMP levels are decreased and the concomitant low PKA activity inhibits cell growth and allows the activation of the cellular stress protection program.

The cAMP/PKA pathway inhibits the general stress response mediated by Msn2 and Msn4 (Wieser *et al.*, 1991; Kobayashi and McEntee, 1993). These two Cys_2His_2 zinc-finger proteins bind to STress REsponsive (STRE) promoter elements in yeast genes (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996), conferring their transcriptional induction upon a wide variety of stresses, like heat shock, DNA damage, osmotic shock and oxidative stress (Marchler *et al.*, 1993).

The cAMP/PKA pathway also inhibits the Yap1- and Skn7-dependent oxidative stress response (Fernandes *et al.*, 1997; Charizanis *et al.*, 1999a). In addition, Yap1 is thought to inhibit the PKA activity upon oxidative stress (Dumond *et al.*, 2000), which is in accordance with its indirect role in the STRE response (Gounalaki and Thireos, 1994).

5. References

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Chapter 3
Introduction to transcriptional profiling

Our modern concept of gene expression dates to 1961, when messenger RNA was discovered, the genetic code deciphered and the theory of genetic regulation of protein synthesis described (Jacob and Monod, 1961; Nirenberg and Matthaei, 1961). The first attempts at global gene expression surveys were undertaken in the mid-1970s (Bishop and Smith, 1974; Galau *et al.*, 1974; Roth, 2002) and interest in gene expression increased steadily. In the 1990s, a new era of large-scale gene expression studies unfolded, mainly resulting from complete genome sequences and technological advances (Fodor *et al.*, 1993; Schena *et al.*, 1995; Velculescu *et al.*, 1995). As the completion of a genome's sequence is just the first step in the understanding *how* it actually works, these gene expression profilings mark the transition from *structural* to *functional* genomics (Hieter and Boguski, 1997).

The aim of this chapter is to describe some techniques that can be used for expression analysis, more particular these involved in transcriptional profiling.

1. Concept of transcriptional profiling

The description of the functional state of a biological system by the quantitative measurements of the spatial and/or temporal fluctuations of the system's constituents is an essential area of biology. It is by now axiomic that gene expression patterns dynamically link the genome and the characteristics or phenotypes associated herewith (Velculescu *et al.*, 1997). Of course, gene expression is initially controlled at transcriptional level, but in fact the proteome (compendium of all proteins expressed) and metabolome (collection of all protein activities) are responsible for and ultimately cause the cellular responses. Thus, transcriptional profiling does not provide a complete picture of cellular function and therefore, is believed not to be sufficient in itself for the quantitative description of biological systems (Young, 2000). In addition, the correlation between mRNA and protein levels is not always that proportional (Gygi *et al.*, 1999; Celis *et al.*, 2000; Ideker *et al.*, 2001), making the prediction of protein abundance based on quantitative transcript data a risky business. But if mRNA is only an intermediate on the way to produce functional protein products, why measure it at all? Simply put, protein-based approaches are generally more difficult, less sensitive and have a lower throughput than RNA-based ones (Smith, 2000). Experimental tools that permit efficient systematic assessment of protein levels and protein modifications are developed in earnest (Persidis, 1998) and will be complementary to genome-wide transcriptional profilings (Pandey and Mann, 2000).

To date, transcriptional profiling is the most productive approach to study biological systems, representing a reproducible and comprehensive cell signature. In recent years, transcriptional profiling (transcriptomics) has become synonymous with *gene expression analysis*, largely because of the technical difficulties associated with proteomics and metabolomics. However, we prefer using *transcriptional profiling* to designate *mRNA expression pattern determination*.

2. Methods for transcriptional profiling

2.1 PCR-based methods

2.1.1 Differential display

Differential display was the first one-tube method to systematically analyse and compare transcribed genes in a bi-directional fashion (Liang and Pardee, 1992). The general strategy of Differential Display (DD) is to amplify partial cDNA sequences from subsets of mRNA by Reverse Transcription (RT) and the Polymerase Chain Reaction (PCR). Poly $(A)^+$ mRNA is converted into first-strand complementary DNA (cDNA) via a reverse transcription, using Anchoring Primers (AP) containing eleven consecutive thymidines (T_{11}) followed by two additional specificity-guaranteeing 'anchor' nucleotides at the 3'-end ($T_{11}XY$; X: A, G, C and Y: A, G, C, T). Next, the cDNA pools serve as templates for *hot* PCR reactions using the same $T_{11}XY$ AP-primer in pairwise combination with a set of random decameric ARbitrary Primers (ARP). Each of these primers will amplify a subset of all

cDNA molecules, resulting in the generation of up to hundred cDNA fragments in one tube. The cDNA fragment pattern from control and experimental samples are then 'displayed' side by side via autoradiography or phosphorescence, after size fractionation on a high-resolution denaturing gel. Comparison of these 'bar codes' identify differentially amplified cDNA fragments, which can then be eluted from the gel, reamplified, cloned and sequenced.

Since its introduction in 1992, a number of technical improvements (Bauer *et al.*, 1993; Liang *et al.*, 1993; Callard *et al.*, 1994; Liang and Pardee, 1995; Zhao *et al.*, 1995b) increased the successful application of DD even further (Table 3.1) and the subsequent use of fluorescently labelled amplification primers provided safer working conditions (Ito and Sakaki, 1997).

2.1.2 RNA fingerprinting

RNA fingerprinting differs from differential display (See 2.1.1) in the first step, in that arbitrary primers are used to initiate reverse transcription (Welsh *et al.*, 1992; Sokolov and Prockop, 1994). This PCR-based approach thus only uses combinations of arbitrary primers and is therefore referred to as Random Arbitrarily Primed RAP-PCR. The method can also be used to screen transcripts that are not polyadenylated, such as prokaryotic mRNA. However, there are definite advantages of DD over RNA fingerprinting for eukaryotic model systems. If total RNA is the starting substrate in RAP-PCR, there is no way to confine the arbitrary priming to the mRNA fraction only. Since ribosomal and transfer RNA are much more abundant than mRNA in the total RNA sample, most of the resulting cDNA fragments will derive from non-mRNA templates, so that the RAP-PCR gel may contain a high percentage of non-relevant data (McClelland *et al.*, 1995).

2.1.3 Selective fragment amplification

To counteract the problems associated with the low stringency PCR-based differential display (See 2.1.1) and RNA fingerprinting (See 2.1.2), Keygene in the Netherlands developed the cDNA-AFLP (Amplified Fragment Length Polymorphism) technique as a method for selective fragment amplification (Vos *et al.*, 1995; Bachem *et al.*, 1996; Breyne and Zabeau, 2001). The system is based on the use of highly stringent PCR conditions, afforded by the ligation of adaptors to the ends of the cDNA restriction fragments, which serve as primer sites during amplification. Selective fragment amplification is achieved by adding one or more bases to the PCR primers, which will then only be successfully extended if these primer extensions match the nucleotides flanking the restriction sites, thereby reducing the number of visualised bands. The method shows both good reproducibility and sensitivity, and correlates nicely with Northern analysis.

Other methods for selective fragment amplification have also been developed, like GeneCalling (Shimkets *et al.*, 1999), READS (Restriction Endonucleolytic Analysis of Differentially expressed Sequences) (Prashar and Weissman, 1999), RAGE (Rapid Analysis of Gene Expression) (Wang *et al.*, 1999) and TOGA (TOtal Gene expression Analysis) (Sutcliffe *et al.*, 2000).

2.1.4 Representational difference analysis

Originally developed to identify differences between two complex genomes (Lisitsyn and Wigler, 1993; Lisitsyn, 1995), Representational Difference Analysis (RDA) was adapted to analyse differential gene expression by taking advantage of both subtractive hybridisation and PCR (Hubank and Schatz, 1994). In a first step, mRNA from two different populations (tester and driver) is copied to cDNA. Tester cDNA represents the population in which the differentially expressed genes are to be expected, while driver cDNA functions as control only. Following digestion with a frequent-cutting restriction endonuclease, adapters are ligated to both ends of tester and driver cDNA molecules. During subsequent PCR amplification, 'representations' corresponding to simplified versions of the original transcriptomes are formed. Next, the adapters on the representations of both tester and driver are digested and new (different) linkers are ligated to both ends of tester cDNA only. Differentially expressed genes become enriched by hybridising tester cDNA with an excess of driver cDNA, mostly in a ratio ranging from 1:100 to 1:100.000, in order to promote hybridisation between single-stranded cDNA molecules common in both tester and driver cDNA pools. Tester-specific cDNA molecules are then exponentially PCR-amplified via adapter-specific primers. cDNA molecules present in both tester and driver are linearly amplified, while driver-specific cDNA molecules cannot be amplified at all. The amplicons resulting from the homoduplexes generated by tester cDNA are in fact the differential 'representations', which can be cloned and sequenced to identify these differential candidates (Table 3.1). The RDA subtractions are usually performed in forward and reverse directions in order to find both up- and down-regulated products, respectively.

2.1.5 Suppression subtractive hybridisation

Suppression Subtractive Hybridisation (SSH) (Table 3.1) is used to selectively amplify differentially expressed cDNA fragments and simultaneously suppress other DNA species by means of a suppression PCR strategy (Diatchenko *et al.*, 1996). First, cDNA is synthesised from the two cells types that are to be compared. The cDNA that contains specific (differentially expressed) genes is referred to as the 'tester', and the control cDNA as the 'driver'. The tester and driver cDNAs are digested with a restriction enzyme (e.g. *Rsa*I) to obtain short blunt-ended molecules. The tester DNA is then divided into two portions and each is ligated to *different* adaptors. The adaptor sequences are specifically engineered to prevent undesirable PCR amplification via the formation of the 'panhandle' structure, due to intramolecular annealing of the long inverted repeats, when the *same* adaptors are present on both DNA ends. Secondly, two rounds of hybridisation are performed. In the first hybridisation, an excess of driver is added to each tester sample, which results in the significant enrichment of differentially expressed sequences. During the second hybridisation, the two primary hybridisation samples are mixed together without denaturing, allowing the remaining to be equalised and the subtracted tester DNAs to form templates (with *different* adapters at both ends) for the subsequent PCR amplification. Next, by using suppression PCR, only the differentially expressed sequences are exponentially amplified, whereas the amplification of those sequences identical in

abundance in both populations is suppressed. The background is reduced and differentially expressed sequences are further enriched during a second PCR amplification with a pair of nested primers.

2.2 Nucleotide sequencing-based methods

2.2.1 Serial analysis of gene expression

Serial Analysis of Gene Expression (SAGE) is a highly sensitive and quantitative sequence-based method that allows the simultaneous analysis of a large number of transcripts (Velculescu *et al.*, 1995) (Table 3.1). SAGE is based on three principles: (i) a short nucleotide sequence tag of 9-10 base pairs contains enough information to unequivocally and uniquely identify a transcript, provided it is isolated from a defined position within the transcript; (ii) concatenation of these short sequence tags allows the efficient analysis of transcripts in a serial manner by the sequencing of multiple tags within a single clone; and (iii) comparison of the sequence data determines differences in expression of genes that have been identified by the tags. Briefly, mRNA is reverse transcribed in the presence of a biotinylated oligo-dT primer. The resulting cDNA is then digested with a frequent-cutting restriction enzyme (anchoring enzyme) that is expected to cleave most transcripts at least once (four-base recognition sequence: anchoring enzyme restriction site). The cDNA fragments corresponding to the 3'-ends of transcripts are isolated by binding to streptavidin-coated beads and then ligated via the anchoring enzyme restriction site to one of two linkers containing a type IIS restriction site (tagging enzyme). Type IIS restriction endonucleases cleave at a defined distance up to 20 bp away from their asymmetric recognition sites, resulting in the release of the linker with a short piece of the cDNA (referred to as SAGE tag). The two pools of SAGE tags, with different adaptor sequences attached, are then blunted, tail-tail ligated to one another to form 'ditags', and PCR amplified. The adaptors are then removed by digestion with the anchoring enzyme and ditags isolated. The sticky ends thus generated, enable the DNA fragments to concatenate by ligation and the resulting concatemers are ready to clone. Thus, a single anchoring enzyme recognition site separates ditags in the final construct and serves as a punctuation mark. It should be emphasised however, that identification of SAGE tags is only possible if matching cDNA sequences exist in public databases (Velculescu *et al.*, 1997). Further, SAGE was reported to cause a significant underestimation of the number of active genes and the fraction of genes expressed at low copy number (Stollberg *et al.*, 2000). Consequently, SAGE is most useful in providing *global* transcription profiles of well-characterised organisms (Gilles, 2001; Lievens *et al.*, 2001).

2.2.2 Massively parallel signature sequencing

The method of MPSS (Massively Parallel Signature Sequencing) (Brenner *et al.*, 2000a) for large-scale gene expression analysis is based on the construction of a microbead cDNA library by *in vitro*

cloning (Brenner *et al.*, 2000b). To achieve this, a collection of oligonucleotide tags is synthesised from a defined set of tetramers. This collection is so large that when attached to the 3' end of cDNA restriction fragments, tag-cDNA conjugates are obtained with virtually every polynucleotide having a unique tag. These conjugates are then amplified and hybridised to anti-tag sequences specific to separate paramagnetic microbeads. As a result, each microbead hybridises about 10^4-10^5 identical copies of a single cDNA species. Then, sequences on the free ends of the cloned templates on each microbead are simultaneously analysed using a fluorescence-based signature sequencing method involving type IIS restriction endonucleases and the tandem ligation of differential adaptors, one set of which is fluorescently labelled. Thus, a type IIS recognition site positioned in an adaptor allows cleavage to occur inside the template, hereby exposing further bases to identify in the following cycle. 'Encoded probes' are used permitting the identification of four bases in each cycle of ligation and cleavage, one base at a time. In each cycle, a full set of 1024 encoded adaptors is ligated to the cDNA molecules, so that each microbead has an equal number of four different adaptors attached, encoding the specific nucleotides at each position of the four-base overhang. The identity and ordering of nucleotides are then determined by specifically hybridising, one at a time, each of 16 'decoder probes' to the decoder binding sites of the successfully ligated adaptors. To collect the signature data, microbeads are tracked through successive cycles of ligation, dynamic hybridisation (Fan *et al.*, 1999) and cleavage. Sequencing progress is monitored optically by collecting and imaging the fluorescent signals generated by the entire planar microbead array onto a CCD (Charge-Coupled Device) detector, followed by image processing. Sequences of 16-20 bases are routinely obtained, which are longer than those from SAGE analysis (See 2.2.1), thus helping to resolve redundancy of matches to cDNA databases. With this method, a highly abundant mRNA will have its sequence found on a large number of microbeads.

2.3 Nucleic acid hybridisation-based methods

2.3.1 Subtractive hybridisation

Subtractive Hybridisation (SH) allows the identification of mRNA molecules uniquely present in a certain experimental condition, through the removal, via liquid hybridisation, of cDNA molecules common to the experimental and control condition. While there are numerous protocols for SH (Sargent and Dawid, 1983; Hedrick *et al.*, 1984; Welcher *et al.*, 1986; Sive and St John, 1988; Barr and Emanuel, 1990; Aasheim *et al.*, 1997), the principle remains the same. The SH process begins with the synthesis of biotinylated first-strand cDNAs from each mRNA species present in the control (driver) cell population. To obtain these mRNA moleculeS (mRNAs) specifically present in the experimental (target) cell population, the biotinylated driver cDNAs are prepared at ≥ twenty-fold molar excess and hybridised to the mRNA pool from the target cells. The duplexes of driver cDNAs and target mRNAs (common transcripts) and unhybridised driver cDNAs are then removed via binding of the biotin moieties to streptavidin, resulting in a pool of target mRNAs preferentially

enriched in transcripts expressed only by the target cell population. In fact, several rounds of mRNA:cDNA hybridisation are required to remove abundant mRNAs common to both cell populations. To determine the down-regulated mRNAs in the experimental cell population, the SH must be carried out in reverse, with biotinylated cDNAs synthesised from the experimental cell type now serving as the driver, and the cDNAs are hybridised to the mRNAs of the control cell population. Although this technique was initially successful for isolating a number of important genes (Davis *et al.*, 1984; Davis *et al.*, 1986; Duguid *et al.*, 1988; Basset *et al.*, 1990; Krady *et al.*, 1990), it is usually inefficient in detecting small differences between tester and driver and obtaining low abundance transcripts.

2.3.2 Macroarray or genefilter

The macroarray technology is a hybridisation-based system for the semiquantitative and large-scale transcriptional analysis of all or most of an organism's genes in parallel (Zhao *et al.*, 1995a; Pietu *et al.*, 1996; Cox *et al.*, 1999) (Table 3.1) A macroarray consists of an ordered collection of amplified cDNA molecules, covalently attached to a nylon membrane, with spot sizes of about 300-500 μm or larger. mRNA populations to be analysed are radioactively labelled during a first-strand cDNA synthesis and hybridised to sequences present on the macroarray surface (Lennon and Lehrach, 1991). Only those spots representing mRNAs present in the sample will give emission signals, the intensity of which is a measure of the expression level of that particular mRNA in the examined cell population (Schena *et al.*, 1995; Zhao *et al.*, 1995a). Besides a radioactivity-based phorphorimaging, chemiluminescent signal detection of genefilter hybridisation seems to be a good alternative (Guiliano *et al.*, 1999).

The signal intensity for a given hybridisation spot on the macroarray is affected by a number of factors in addition to the amount of transcript actually represented in the labelled probe. Factors such as GC-content, presence of repetitive elements, and the amount of DNA deposited on the membrane will all contribute to the final signal detected for each clone. These variable factors (Cheung *et al.*, 1999) make comparisons of expression between clones on the same membrane difficult.

Transcriptome comparison via macroarray technology requires the hybridisation of each sample to separate duplicate filters (parallel experiment), or to a single filter that must be stripped and hybridised sequentially (serial experiment) (Bowtell, 1999). Identification of differentially expressed genes can then be easily performed due to the ordered concept of the array, and these expression patterns have great chance for an exact verification via Northern analysis (Cox *et al.*, 1999; Rep *et al.*, 2000). Of course, the cross-hybridisation of multiple cDNA species against a single target cannot be identified, but only seems to contribute to spot signal intensity for genes with a sequence similarity ≥ 70-80% (Spellman *et al.*, 1998).

Hybridisation experiments with cDNA arrays preferably detect mRNA species expressed at middle to high abundance (Pietu *et al.*, 1996). Background hybridisation limits the detection of rare

transcripts and the detection sensitivity of macroarrays was reported to be 5-10 copies/cell at maximum (Nguyen *et al.*, 1995; Zhao *et al.*, 1995a). The detection of low abundant transcripts requires the use of enriched probes with reduced complexity, lacking the abundantly expressed genes. To produce such probes DD (Gonzalez *et al.*, 1999) (See 2.1.1), RDA (Geng *et al.*, 1998) (See 2.1.4), RAP (Trenkle *et al.*, 1998) (See 2.1.2) or SSH (von Stein *et al.*, 1997) (See 2.1.5) were demonstrated to be ideal techniques. In addition, the presence of dideoxynucleotides in the reverse transcription mixture was reported to exclude systematic repriming of the most abundant transcripts and to increase the genefilter sensitivity, thereby improving the detection of the least abundant transcripts in these expression profiling experiments (Decraene *et al.*, 1999).

2.3.3 Microarray technology

A microarray is a solid support to which single-stranded DNA molecules are attached at fixed locations (spots), the diameter of which is typically about 100-200 μm. Like macroarrays, also microarrays use the preferential binding of complementary, single stranded nucleic-acid sequences to predefined targets. Microarray hybridisation is a sensitive (detection limit: 1 copy/cell) (Ramsay, 1998), accurate and quantitative (Schena *et al.*, 1995; Wodicka *et al.*, 1997; Ishii *et al.*, 2000) transcriptional profiling technique (Table 3.1) allowing far-reaching parallelism and automation. More than 10^5 genes can be analysed on one single microarray (Chee *et al.*, 1996) with a surface area as little as two square cm. In addition, due to miniaturisation, small reaction volumes (2 μl) fasten up hybridisation kinetics by increasing probe concentrations (Schena *et al.*, 1995). The porosity, autofluorescence and signal scattering of membranes do not permit such very dense array spacing, prompting the use of non-porous alternatives such as glass or silicon (Ross *et al.*, 1992). In addition, these matrices also allow hybridisation detection by fluorescence, which is known to have very little signal dispersal.

Usually, one has to compare mRNA abundance in two different samples (sample A and sample B). In this case, cDNAs from sample A and sample B are labelled with two different fluorescent dyes (e.g. a red label for the cDNAs from sample A and a green label for sample B). This labelling can be performed either during the reverse transcription process, through direct incorporation by reverse transcriptase, or afterwards by chemical conjugation. Next, the two cDNA populations are allowed to hybridise to the same microarray slide, the signals of which are detected via laser scanning (Schena *et al.*, 1995; Wodicka *et al.*, 1997). Separate scans performed at wavelengths that are required for each fluorophore will generate two images that are to be combined during data analysis. Specialised software is then used to determine the individual signal intensities. If a particular mRNA from the sample is in excess, the spot with the complementary probe will be red; if the concentration of the particular mRNA is higher in the control, the spot will be green. If both samples contain the same amount of a given mRNA, the spot will be yellow; if neither contains a certain transcript, its spot will appear black. Thus, the relative expression levels of the genes can be estimated from the

fluorescence intensities and colours for each spot (Chen, 1997). The hybridisation signal and the RNA expression level show a linear relationship, providing the target DNA is present on the array in at least a ten-fold excess (Lemieux *et al.*, 1998).

By using a single reference sample as the control for a series of experimental samples collected over time, one can compare relative levels of transcript abundance among samples. This in turn allows the identification of gene expression profile trends (Kozian and Kirschbaum, 1999).

Microarray analysis does not give information about absolute gene expression levels in the samples. This is because the intensity of the fluorescent signals is not solely dependent on the number of fragments, but also on the length of these fragments and the number of fluorescent labels each fragment carries, i.e. labelling density.

Fluorescence technology, which is the most commonly used detection method for microarray analysis, is known to be limited in sensitivity. Therefore, chemoluminescence, diode array detectors, direct electrical charge detection, and piezoelectric readouts are all being developed as alternative detection methods (Gabig and Wegrzyn, 2001).

2.3.3.1 The oligonucleotide array format

Oligonucleotide microarrays can be generated by two different methods. In the SYNTHESIS concept, the microarray is build by a sophisticated method that combines *photolithography* and solid-phase chemical synthesis (Fodor *et al.*, 1991) for the consecutive *in situ* synthesis of the 20-25 mers directly onto a solid matrix (silicon wafer). Synthesis is performed in a step-wise fashion using a different light-impermeable mask for each round (Pease *et al.*, 1994). These arrays are routinely created with densities of 10^4-10^5 probes/cm^2. For each of the genes, both 'perfect match' and 'mismatch' oligonucleotides are synthesised (Lockhart *et al.*, 1996). The mismatch oligonucleotides assess local background variation and can identify cross-hybridisations up to a similarity ≤ 93% (Lipshutz *et al.*, 1999). Affymetrix (http://www.affymetrix.com) is currently producing these high-density oligonucleotide microarrays. An alternative *in situ* synthesis concept was developed by Rosetta Inpharmactics (Kirkland, WA) (http://www.rii.com), based upon the delivery of chemical reagents via *ink-jet technology* (Hughes *et al.*, 2001). More particularly, the nucleotides are sprayed separately by the ink-jet heads and linked on the glass slide into oligonucleotides. Agilent (Palo Alto, CA) has licensed this technolgy. In the DEPOSIT (micro dispensing) concept, oligonucleotides are orderly deposited in small amounts on the glass surface of the microarray. Both passive dispensing (contact printing) (Schena *et al.*, 1996; Schena *et al.*, 1998) or active printing via piezoelectric delivery or syringe-solenoid deposition (Marshall and Hodgson, 1998) are used. In contact deposition, solid, hollow, or split-open pen designs are used for transfer. Contact deposition required less target nucleic acid solution than the non-contact methods and also results in smaller spots that can be packed more densely on the microarray surface.

Methods based on synthetic oligonucleotides offer the advantage that sequence information is sufficient to generate the DNA to be arrayed; no time-consuming handling of cDNA resources is

required. Furthermore, probes can be designed to represent the most unique part of a given transcript (Lockhart *et al.*, 1996), making the detection of closely related genes possible. However, these short oligonucleotides may result in less specific hybridisation and reduced sensitivity. The arraying of larger oligonucleotides (80-100 mers) was developed to counteract these disadvantages (Kane *et al.*, 2000). In addition, the strength and specificity of hybridisation can also be improved considerably by using Peptide Nucleic Acid (PNA) oligonucleotides due to the high stability of PNA-DNA duplexes (Weiler *et al.*, 1997; Kozian and Kirschbaum, 1999).

2.3.3.2 The cDNA microarray format

The first cDNA microarray was developed by Schena and Shalon (Schena *et al.*, 1995) in Patric Brown's and Davis' laboratories at Stanford University. This array was made by PCR amplification of approximately 50 cDNAs of *Arabidopsis thaliana* and printing them onto a microscopic glass slide with a high-speed arrayer. Microarrays prepared by high-robotic speed printing of cDNAs on glass (Schena *et al.*, 1995) can be used for quantitative transcriptional analysis of all (DeRisi *et al.*, 1997; Lashkari *et al.*, 1997) or a relevant subset (Doughery and Geschwind, 2002) of an organism's genes in parallel. Cross-hybridisation was shown to contribute to the signal intensity only for genes with a sequence similarity ≥ 70-80% (Girke *et al.*, 2000). However, a poor correlation has been observed between the Stanford type cDNA microarrays and Affymetrix oligonuleotide microarrays (Kothapalli *et al.*, 2002; Kuo *et al.*, 2002).

2.3.3.3 The fibre-optic microarray format

A microarray format that starts looking promising, utilizes fibre-optic technology (Ferguson *et al.*, 1996; Healey *et al.*, 1997; Michael *et al.*, 1998). A bundle of optical fibres is assembled in a stainless steel tube, with each fibre carrying a different oligonucleotide probe immobilized on its distal end. The hybridisation of fluorescently labelled probes to the microarray can be monitored via the imaging system connected at the tube's proximal end.

A new modification has been published for the screening of unlabeled DNA probes via Molecular Beacons (MB) immobilized to arrayed microspheres that are etched in an optical imaging fibre (Steemers *et al.*, 2000). This BeadArray is commercialised by Illumina (San Diego, CA).

2.3.3.4 The microelectronic microarray format

A novel microarray technology was developed as part of the Genosensor Consortium (Beattie *et al.*, 1993) and uses the property of electronic-based addressing to manoeuvre DNA molecules to specific test sites on an electronic microchip. These active microelectronic array devices have the ability to produce reconfigurable electric fields (more specifically, electrophoretic fields) on the microarray surface that allow rapid and controlled transport of charged DNA molecules to any test site. The DNA targets are immobilized onto a microscopic electronic biosite by direct attachment to the permeation layer (Tu *et al.*, 2000). Hybridisation of sample DNA to these targets is detected by a

low-voltage alternating electric field that discriminates between the DNA dielectric relaxation frequency of hybridised probes versus nonhybridised probes. Alternatively, electronic hybridisation can be carried out by selective application of a direct current positive bias to the individual microelectrodes beneath the selected test sites. This causes rapid transport and concentration of negatively charged nucleic acid molecules over selected locations on the microelectronic array. This rapid concentration leads to a dramatic reduction in time for hybridisation when compared to passive hybridisation techniques. Reversal of the electric potential then allows rapid removal of unhybridised molecules, as well as continuous adjustment of stringency (Heller and Tu, 1993; Edman *et al.*, 1997; Sosnowski *et al.*, 1997). This electrokinetic technology is licenced by Nanogen (San Diego, CA).

2.3.3.5 The gel pad microarray format

Although originally described as a method for DNA Sequencing by Hybridisation with an Oligo Matrix (SHOM) (Khrapko *et al.*, 1989; Khrapko *et al.*, 1991), the microarray consisting of 100 μm gel pads with immobilised prefabricated oligonucleotides has potential for gene expression analysis. The oligonucleotides attached to the gel pads appear to have similar hybridisation specificity and sensitivity compared to the one deposited on a glass slide. This technique is licensed by Motorola Life Sciences (Northbrook, IL), but has till now limited commercial acceptability.

2.3.3.6 Microarray applications

The following is a compilation of array publications in some of the broad areas of application: gene expression analysis for cancer (Cossman, 2001; Graveel *et al.*, 2001; Okabe *et al.*, 2001), drug discovery, metabolism and toxicity (De Backer *et al.*, 2001; Gerhold *et al.*, 2001; De Backer *et al.*, 2002), for neuroscience applications (Geschwind, 2000; Cavallaro *et al.*, 2001), microbiological and infectious diseases (Heller *et al.*, 1997; Coombes and Mahony, 2001; Kagnoff and Eckmann, 2001), genotyping (Hacia and Collins, 1999; Mahalingam and Fedoroff, 2001), disease diagnostics (Helmberg, 2001; Petrik, 2001), cell division (Cho *et al.*, 1998; Spellman *et al.*, 1998), chemical or genetic perturbations (DeRisi *et al.*, 1997; Holstege *et al.*, 1998; Marton *et al.*, 1998; Galitski *et al.*, 1999; Hughes *et al.*, 2000), RNA decay (Bernstein *et al.*, 2002; Bernstein *et al.*, 2004) and molecular phenotyping (Holstege *et al.*, 1998; Marton *et al.*, 1998; Golub *et al.*, 1999; Perou *et al.*, 1999; Alizadeh *et al.*, 2000).

In addition, microarrays can be used to study translational control via the examination of polysome profiles (Kuhn *et al.*, 2001) and protein-DNA interactions by chromatin immunoprecipitation (ChIP-chip) (Horak and Snyder, 2002; Lee *et al.*, 2002).

Further, considerable effort is now being directed to the development and use of microarrays for the immobilization of cell and tissue samples and for proteomic applications (Mousses *et al.*, 2001).

	Transcriptional profiling method		
Characteristics	DD	RDA	SSH
Sensitivity	high	moderate	moderate
False positive rate	high	low	low
Global analysis	no	no	no
Sample comparison	any number	two	two
Expression detection	up and down	one direction	one direction
Measurement	relative	relative	relative
DNA identity	no	no	no
Identification	sequencing	sequencing	sequencing
Redundancy	moderate	moderate	low
Labour intensity	high	moderate	moderate
Financial cost	low	low	low
Publications	9746	1463	703

	Transcriptional profiling method		
Characteristics	SAGE	macroarray	microarray
Sensitivity	high	moderate	high
False positive rate	NA	moderate	low/moderate
Global analysis	yes	yes	yes
Sample comparison	any number	any number	any number
Expression detection	up and down	up and down	up and down
Measurement	absolute	relative	relative
DNA identity	yes	no	yes/no
Identification	sequencing	radioactivity	fluorescence
Redundancy	high	NA	NA
Labour intensity	high	moderate	moderate
Financial cost	high	moderate	high
Publications	1561	147	6447

Table 3.1: Comparison of transcriptional profiling technologies. DD: Differential Display (See 2.1.1); RDA: Representational Difference Analysis (See 2.1.4); SSH: Suppression Subtractive Hybridisation (See 2.1.5); SAGE: Serial Analysis of Gene Expression (See 2.1.1). For the microarray technique (See 2.3.3), both the oligo (left part) and cDNA format (right part) are included.

DNA identity indicates whether the genome sequence should be availabe. NA: not applicable.

Adapted from (Roth, 2002; Stein and Liang, 2002; Boothroyd *et al.*, 2003).

The impact of these technologies in gene expression analysis (publications) was determined via Medline searches conducted on June 9, 2004 by PubMed at the NCBI (National Centre for Biotechnology Information) website (http://www.ncbi.nlm.gov/entrez) using keywords depicting each methodology.

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Chapter 4
Experimental methodology

The aim of this chapter is to describe the experimental details, concepts and strategies used throughout this work, hereby focusing on the project-specific methods. Standard recombinant DNA methodologies and basic molecular biology techniques are not provided in detail.

1. Materials

1.1 Strains and growth conditions

The *E. coli* MC1061 strain (F^- *ara*D139 Δ(*ara leu*)7697 Δ*lac*X74 *gal*U *gal*K *hsd*R2(rk^-, mk^+) *mcr*B1 *rps*L str^R) (Wertman *et al.*, 1986) was used for the construction and the amplification of plasmids. The *Saccharomyces cerevisiae* strains used were INVSc1 (*MAT*α *his*3Δ1 *leu*2-3, 112 *trp*1-289 *ura*3-52) (Invitrogen, San Diego, CA), BY4742 (*MAT*α *his*3Δ1 *leu*2Δ0 *lys*2Δ0 *ura*3Δ0), BY4743 (*MAT*a/*MAT*α *his*3Δ1/*his*3Δ1 *leu*2Δ0/*leu*2Δ0 *lys*2Δ0/*lys*2Δ0 *ura*3Δ0/*ura*3Δ0) and their knockout derivatives (Euroscarf, Frankfurt, Germany). Yeast strains were grown at 30°C and 250 rpm in rich YPD-medium (2% glucose, 2% bactopeptone, 1% yeast extract), rich YPGly-medium (3% glycerol, 2% bactopeptone, 1% yeast extract), or SD-medium (2% glucose, 0.67% yeast nitrogen base, 0.5% ammonium sulphate), either minimally supplemented (minimal SD-medium) with amino acids (0.2 mM leucine, 0.2 mM lysine, 0.1 mM histidine, 0.1 mM tryptophan) and 0.2 mM uracil, or fully supplemented (synthetic SD-medium) with a complete amino acid mix containing uracil (CSM: Complete Synthetic Medium) (BIO101 Inc., Carlsbad, CA). For the selection of transformants leucine was dropped out (CSM – leucine) (BIO101 Inc.) (selective SD-medium). SG- and SGR-medium were used for the induction of galactose regulated promoters. SG-medium differs from SD-medium only by the replacement of glucose with 2% galactose as carbon source. SGR-medium is based on SG-medium, containing additional 2% raffinose. The liquid cultures never exceeded 20% of the vessel volume. To prepare plates, medium was solidified with 2% agar.

2. Methods

2.1 Cloning of the mouse Bax-α coding sequence

cDNA (complementary DNA) encoding the mouse Bax-α protein (EMBL L22472) (European Molecular Biology Laboratory) was amplified from an EL4/13.18 thymoma cDNA library (BCCM/LMBP-LIB15) by PCR (Polymerase Chain Reaction) using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and the primers 5'-ATGGACGGGTCCGGGAGCAG-3' and 5'-TCAGC-CCATCTTCTTCCAGATGGTGAG-3'. The resultant PCR product was sequence verified and cloned using standard procedures (Sambrook *et al.*, 1989) in a *Hinc*II-opened pUC19 plasmid (LMBP 1128) (Yanisch-Perron *et al.*, 1985), to produce pUC19B.

2.2 Regulation of *GAL* transcription

In *S. cerevisiae*, the utilisation of exogenous galactose requires the induction of an enzyme system consisting of a specific galactose transport activity (Douglas and Condie, 1954; Cirillo, 1968) and the Leloir pathway enzymes (Kosterlitz, 1943; Leloir, 1951; Kalckar *et al.*, 1953; Douglas and

Hawthorne, 1964). The enzymes of the Leloir pathway convert galactose to galactose 1-phosphate (Gal1; galactokinase), galactose 1-phosphate to glucose 1-phosphate (Gal7; α-D-galactose-1-phosphate uridyltransferase), and uridine diphosphogalactose (UDP-Gal) to uridine diphosphoglucose (UDP-Glu) (Gal10; uridine diphosphoglucose 4-epimerase). Glucose 1-phosphate is converted to glucose 6-phosphate by phosphoglucomutase (Gal5), which is constitutively expressed. The regulatory genes *GAL3, GAL4* and *GAL80* exert a tight transcriptional control of the inducible galactose pathway enzymes. In non-inducing conditions, the *GAL4* product is bound to the Upstream Activating Sequences for galactose (UAS_G), but is prevented from activating transcription by the Gal80 inhibitor (Lohr *et al.*, 1995; Reece and Platt, 1997). Rapid induction by galactose (> 1000 fold) requires the product of *GAL3* (Nogi, 1986; Torchia and Hopper, 1986; Lohr *et al.*, 1995). When Gal3 is bound to galactose, it interacts directly with Gal80 in the presence of ATP (Suzuki-Fujimoto *et al.*, 1996; Zenke *et al.*, 1996; Yano and Fukasawa, 1997; Platt and Reece, 1998). This interaction occurs in the cytoplasm and is thought to influence the shuttling of Gal80 between the nucleus and the cytoplasm (Peng and Hopper, 2000), thereby reducing the effective concentration of the Gal80 inhibitor in the nucleus. When glucose is present in the medium, the transcription of *GAL* genes is rapidly shut off. This phenomenon is known as glucose repression and is mediated by the binding of the Mig1 complex to the *GAL4* promoter (Griggs and Johnston, 1991; Nehlin *et al.*, 1991; Lamphier and Ptashne, 1992) and the promoters of the other inducible *GAL* genes (Johnston *et al.*, 1994), leading to transcriptional repression and preventing Gal4 from binding to the UAS_G.
The *GAL1* promoter is one of the strongest regulated promoters in yeast (Louis and Borts, 1995). The *GALL* promoter, which is a deletion variant of the *GAL1* promoter lacking one of the three UAS_G, exhibits the same galactose-dependent regulation but transcriptional induction is reduced to half of the *GAL1* level (Mumberg *et al.*, 1994).

2.3 Plasmid constructions

The 2-mu ori and the *URA3* marker gene were consecutively excised from pUT332 (Gatignol *et al.*, 1990) by digestion followed by self ligation using *Cla*I and *Bgl*II, to produce YIpUT332. A *Bam*HI-*Hin*dIII fragment of the vector pSP64GAL1m (LMBP 2118), containing the *GAL1* promoter, was ligated into the *Bgl*II-*Hin*dIII-opened YIpUT332plasmid, thereby obtaining YIpUTGAL1p. This plasmid was linearised with *Hin*dIII, end-blunted, digested with *Xba*I, and ligated to a *Fsp*I-*Xba*I fragment isolated from pEMBLyex4 (LMBP 2423) (Murray *et al.*, 1987) that contained the *FLP1* terminator, creating plasmid YIpUT. Insertion of a Ty-δ element as a blunt-ended *Eco*RI-*Bsa*AI fragment derived from pUD208 (LMBP 2504), in the *Kpn*I-*Aat*II-opened and blunted YIpUT resulted in YIpUTy. Subsequent insertion of the *LEU2* marker gene, as a blunted *Bsa*AI-*Bsr*GI fragment from pFV17 (Volkert and Broach, 1986), in the *Bam*HI-opened and blunted YIpUTy created pSCTyGAL1-L. Mouse *bax* cDNA was excised from pUC19B by digestion with *Xba*I and *Hin*dIII and subcloned into the *Xba*I-*Hin*dIII-opened plasmid pSCTyGAL1-L (Fig. 4.1), obtaining

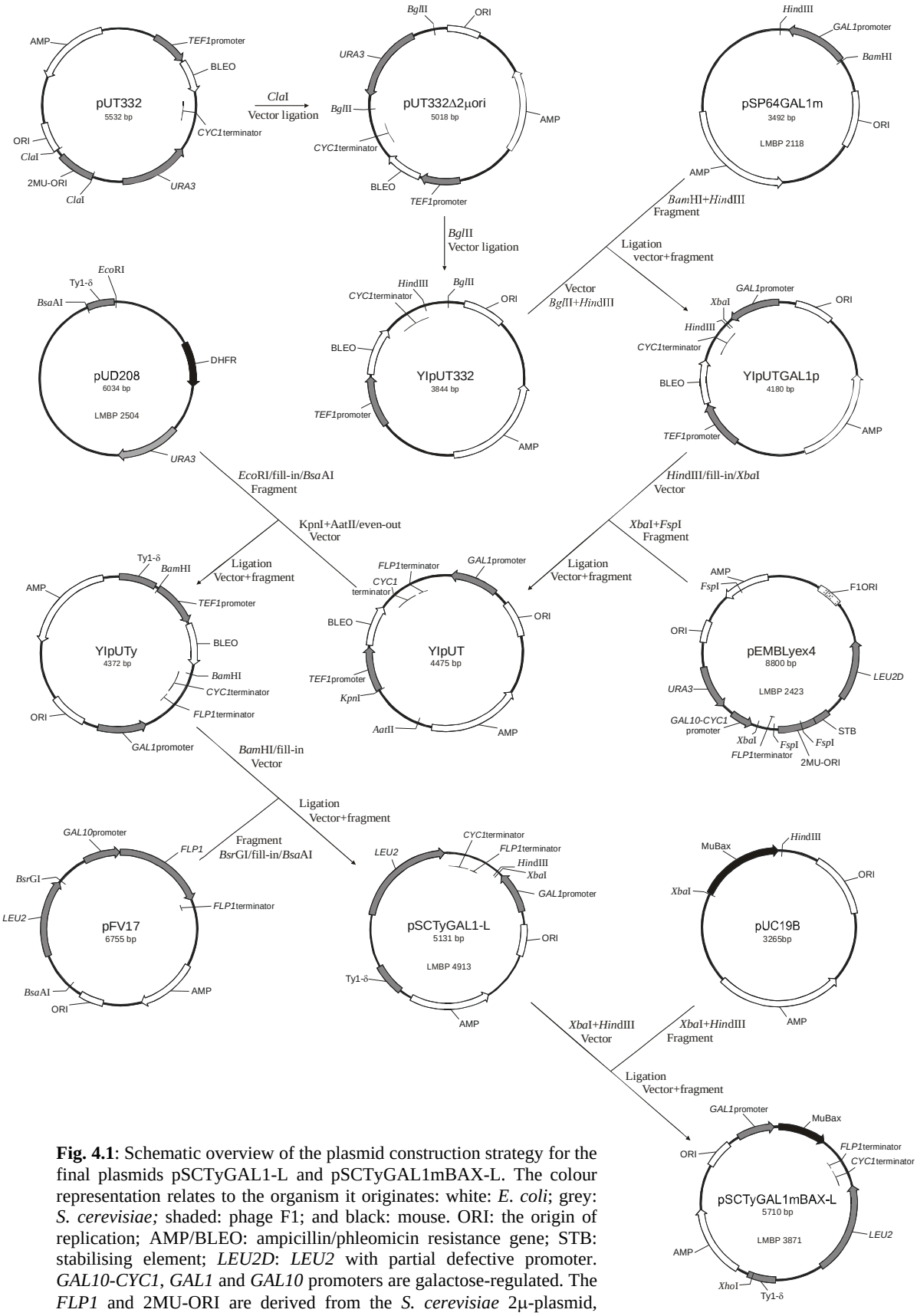

Fig. 4.1: Schematic overview of the plasmid construction strategy for the final plasmids pSCTyGAL1-L and pSCTyGAL1mBAX-L. The colour representation relates to the organism it originates: white: *E. coli*; grey: *S. cerevisiae;* shaded: phage F1; and black: mouse. ORI: the origin of replication; AMP/BLEO: ampicillin/phleomicin resistance gene; STB: stabilising element; *LEU2D*: *LEU2* with partial defective promoter. *GAL10-CYC1*, *GAL1* and *GAL10* promoters are galactose-regulated. The *FLP1* and 2MU-ORI are derived from the *S. cerevisiae* 2μ-plasmid, indicating the flippase-encoding gene and the replication origin respectively. Even-out: 3'-overhang flushing via the removal of nucleotides; fill-in: 5'-overhang flushing via nucleotide addition. Please refer to the text for details.

pSCTyGAL1mBAX-L (LMBP 3871) as a final integrative expression plasmid. The pSCTyGAL1-L and pSCTyGAL1mBAX-L plasmids were linearised at the Ty δ element via *Xho*I prior to transformation to ensure targeted integration (Zhu, 1986). The *S. cerevisiae* INVSc1 strain was transformed by the lithium acetate method (Gietz *et al.*, 1992) and the resultant transformants designated as INVSc1::pSCTyGAL1-L and INVSc1::pSCTyGAL1mBAX-L. The plasmid pSCTyGAL1mBAX-L was digested with *Bsg*I and *Sap*I, blunted and the fragment containing the *GAL1* promoter, the mouse *bax* cDNA and *FLP1* terminator was isolated. This fragment was inserted into the *Ecl*136II-opened pRS415 (Simons *et al.*, 1987) (LMBP 4059), to produce the episomal expression vector pRS415GAL1mBAX (LMBP 4327) (Fig. 4.2). Mouse *bax* cDNA was excised from pUC19B by digestion with *Xba*I and *Hin*dIII and subcloned into the *Xba*I-*Hin*dIII-opened plasmid p415GALL (Mumberg *et al.*, 1994) (LMBP 4032), to obtain the episomal expression vector p415GALLmBAX (LMBP 4575) (Fig. 4.2) for *GALL*-regulated Bax protein expression. The *S. cerevisiae* strains BY4742 and BY4743 and knockout derivatives were used for all episomal transformation strategies. Plasmids indicated with a LMBP number are deposited at the public LMBP collection (Laboratory of Molecular Biology Plasmid) of BCCM (Belgian Coordinated Collection of Microorganisms) (http://www.dmbr.Ugent.be/lmbp).

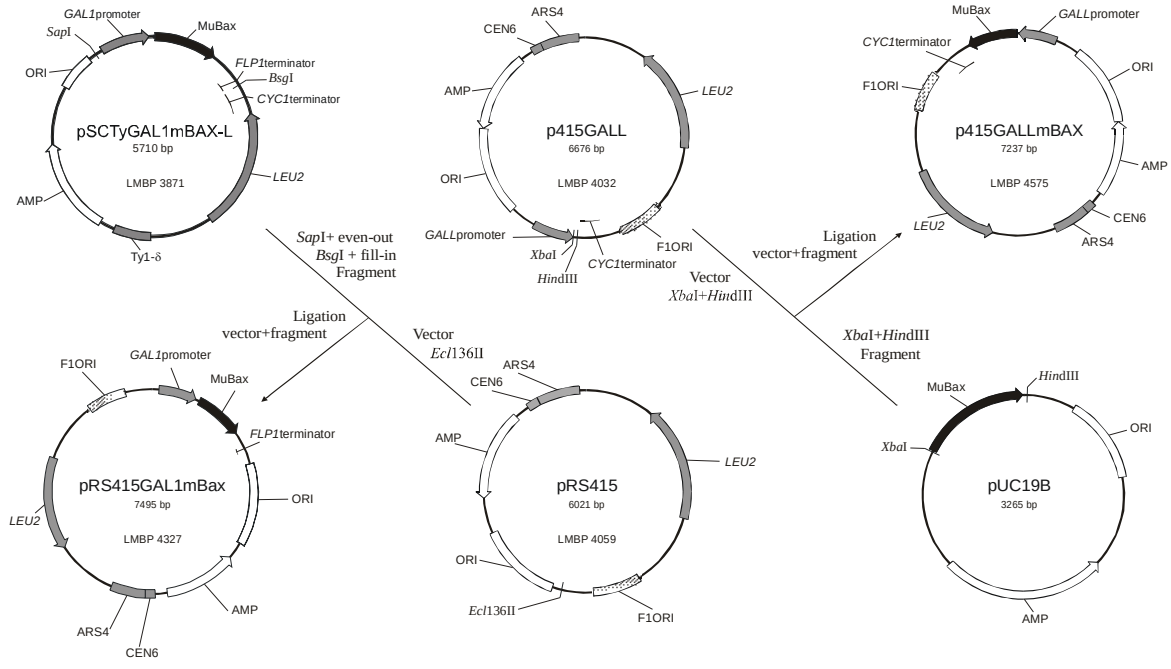

Fig. 4.2: Schematic overview of the plasmid construction strategy for the final plasmids pRS415GAL1mBAX and p415GALLmBAX. The colour representation relates to the organism it originates: white: *E. coli*; grey: *S. cerevisiae;* shaded: phage F1; and black: mouse. ORI: the origin of replication; *GAL1* and *GALL* promoters are galactose-regulated. ARS4: autonomously replicating sequence; CEN6: centromeric sequence. Even-out: 3'-overhang flushing via the removal of nucleotides; fill-in: 5'-overhang flushing via nucleotide addition. Please refer to the text for details.

2.4 Alkaline Southern blot

The yeast strains INVSc1::pSCTyGAL1-L and INVSc1::pSCTyGAL1mBAX-L were grown overnight in 10 ml YPD medium. About $5x10^8$ cells were pelleted by centrifugation at 2,200 g, washed twice with sterile dH_2O, and used for genomic DNA purification using the DNA-pure™ Yeast Genomic Kit (CPG® Inc., Lincoln Park, NJ) according to the guidelines provided by the manufacturer. Briefly, cells were resuspended in 540 µl Resuspension Buffer I and 60 µl Lysis Buffer, incubated at 70°C for 10 min and cooled for 10 min at room temperature. Proteins were precipitated with 300 µl Protein Precip Buffer, cooled on ice for 2 min, and the debris pelleted by centrifugation at 20,000 g for 5 min. The supernatant was transferred to a new tube, and after adding 600 µl isopropanol the DNA was pelleted by centrifugation at 20,000 g for 10 min. Next, the pellet was washed with 200 µl ice-cold 70% ethanol, any residual ethanol was allowed to evaporate. DNA was then carefully resuspended in 50 µl of Resuspension Buffer II by tube tapping. Further, 20 µl of each genomic DNA resuspension (>15 µg) was digested overnight with 15 units *Xho*I/µg DNA. Samples were phenolised, salt-precipitated, and run on a 0.8% agarose gel for 7 hours at 100 V. Subsequently, the gel was depurinated by soaking in 0.25M HCl for 20 min, followed by thorough rinsing with dH_2O. Fragments were transferred to a Hybond-N^+ nylon filter (Amersham Biosciences, Piscataway, NJ) via Southern blot (Southern, 1975) with 0.4M NaOH as tank buffer. The filter was air-dried and placed in a small hybridisation flask (35x250 mm) (Amerham Pharmacia Biotec, Piscataway, NJ) filled with 10 ml prehybridisation buffer [1 mM EDTA, 0.5M sodium phosphate pH 7.2, and 7% SDS] and incubated at 68°C for 5 hours in a tube roller (Amersham Biosciences, Piscataway, NJ) at 10 rpm. The 618 bp *Bst*XI-*Bst*EII pSCTyGAL1-L fragment, randomly labelled with α-[^{32}P] dCTP via High Prime (Roche, Basel, Switserland), was added to the prehybridisation buffer for overnight *LEU2/leu2* hybridisation. Further, the filter was washed three times for 30 min with the first wash buffer [1mM EDTA, 40 mM sodium phosphate pH 7.2, 5% SDS] at 68°C and twice with the second wash buffer [1mM EDTA, 40 mM sodiumphosphate pH 7.2, 1% SDS] at 68°C for 30 min. The filter was then placed in a PhosphorImager™ cassette (Molecular Dynamics, Sunnyvale, CA) with a storage phosphor screen. After 4 hours of exposure, a digital image of this screen was obtained with the Personal Molecular Imager® FX (BioRad, Richmond, CA) by scanning at a resolution of 100 µm.

2.5 RNA extraction following Bax induction or H_2O_2 treatment

2.5.1 Induction of Bax expression

Precultures of yeast strains INVSc1::pSCTyGAL1mBAX-L and INVSc1::pSCTyGAL1-L were grown overnight in selective minimal SD-medium. They were diluted at least 500-fold in the same growth medium and grown to an OD_{600} of 1.0. Subsequently, the cells were washed three times with sterile dH_2O, resuspended in 100-ml selective minimal SG-medium to induce Bax, and incubated for an additional period of 30 min, 1 hour, 2 hours, 3 hours, 6 hours or 15 hours.

2.5.2 Challenge with hydrogen peroxide

The yeast strain INVSc1::pSCTyGAL1-L was grown as described above, but the selective minimal SG-medium was supplemented with different concentrations of H_2O_2 ranging from 0.1 to 0.5 mM.

2.5.3 Total RNA extraction

After Bax protein induction, H_2O_2- or mock-treatment, about 10^9 cells were harvested by centrifugation at 2,200g for 5 min at 4°C and washed with ice-cold sterile dH_2O. Cells were then combined with 1 ml RNApure™ reagent (Genhunter Corporation, Nashville, NY) and 1 g of glass beads (0.5 mm), and broken by thorough mixing during two 2-minutes periods. Samples were placed on ice in-between to avoid RNA degradation. The lysate was combined with 150 µl chloroform and centrifuged at 20,000 g for 10 min at 4°C. The RNA in the supernatant was precipitated with an equal volume of isopropanol for 10 min on ice, pelleted at 20,000 g for 10 min at 4°C, and washed with 70% ice-cold ethanol. The RNA was resuspended in 50 µl RNAse-free dH_2O. Typically, about 1 mg total RNA could be extracted from 10^9 cells. The purity and quality of the RNA preparations were checked by spectrophotometry ($A_{260}/A_{280} \geq 2.0$ and $A_{260}/A_{230} \geq 1.8$) and by an RNA assay using the Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany).

2.6 Gene expression profiling using macroarray technology

2.6.1 Synthesis of cDNA probes

Probes with high specific activity were prepared by first-strand cDNA synthesis via α-[^{33}P] dCTP incorporation as follows: 2 µg oligo-dT was added to 20 µg of total RNA, the volume was raised to 10 µl with RNAse-free dH_2O and the mixture incubated at 70°C for 10 min. After cooling on ice, the following components were added: first strand buffer (Life Technologies, Paisley, UK), 3.3 mM dithiothreitol, 40 units RNase Block (Stratagene, La Jolla, CA), 1mM each of dATP, dGTP and dTTP (Amersham Pharmacia Biotec, Piscataway, NJ), 300 units Superscript II Reverse Transcriptase (Life Technologies) and 100 µCi 3000 Ci/mmol α-[^{33}P] dCTP (Amersham Pharmacia Biotec). The 30 µl mixture was incubated for 2 hours at 37°C and unincorporated label was removed by Sephadex G-50 column passage (Amersham Pharmacia Biotec). The incorporated radioactivity was measured by liquid scintillation.

2.6.2 GeneFilter® hybridisation

The yeast GeneFilter® (Invitrogen, Gaithersburg, MD) (990809C-20, 990809D-20, 990825C-98 and 990958D-98) was sequentially hybridised with the α-[^{33}P] dCTP labelled cDNA probes using formamide-containing MicroHyb™ (Invitrogen). The GeneFilter® was placed in a hybridisation flask (35x250 mm) (Amerham Pharmacia Biotec, Piscataway, NJ), filled with 5 ml MicroHyb™ supplemented with 1 ng/ml poly dA (Invitrogen) and incubated for 24 hours at 42°C in a tube roller

(Amersham Biosciences, Piscataway, NJ) at 10 rpm. The cDNA probe, denatured for 3 min at 100°C, was added to the prehybridisation mixture and allowed to hybridise overnight at 42°C and 10 rpm. Hybridisation solution was then gently decanted and the filters were washed twice with 2x SSC, 1% SDS at 50°C for 20 min. Filters were transferred to a plastic box and washed once with 0.5x SSC, 1% SDS at room temperature for 15 min on an orbital shaker. The GeneFilter® was then placed in a PhosphorImager™ cassette (Molecular Dynamics, Sunnyvale, CA) with a storage phosphor screen. After 4 days of exposure, a digital image was obtained with the Personal Molecular Imager® FX (BioRad, Richmond, CA) by scanning at 50 μm resolution (finest pixel size available). Filters were kept moist during the whole procedure to facilitate stripping of bound probe in-between. Filters were stripped by submersion in boiling 0.5% SDS and orbital shaking for at least 1 hour. Stripping efficiency was checked via phosphorimaging and was typically more than 95%. The GeneFilter® was re-used at maximum 6 times.

2.6.3 Data analysis

Conversion of the digital images to a 16 bit TIFF (Tag Image File Format) using the Quantity One® program (BioRad, Richmond, CA) preserved image data and was necessary for file import into Pathways™ 2.01 (Invitrogen, Gaithersburg, MD). This software was used for quantification of hybridisation signals, which were normalised against all data points. These normalised data were then compared pairwise to determine the induction/repression ratio. The ratios were calculated by dividing the spot intensity after Bax induction or after H_2O_2 challenge by the spot intensity after mock-treatment. They were estimated significant when (i) expression level changes were at least two-fold and (ii) the normalised signal intensity of both spots was more than tenfold above the average background value.

2.6.4 Cluster analysis

Clusters were established using a hierarchical cluster algorithm (Hartigan, 1975) to display genome-wide expression patterns (Eisen *et al.*, 1998). This agglomerative algorithm sorts through all the data to find the pairs of genes that behave most similarly in each experiment and then progressively adds other genes to the initial pairs to form clusters of apparently coregulated genes. The process continues until all individual profiles and nodes have been joined to form a single hierarchical tree, the branch lengths of which reflect the degree of similarity between gene expression responses. A hierarchical clustering (centred, average-linkage) of the expression data was performed, using the program Cluster 2.11 and the resultant expression maps were visualised with Treeview 1.50. Both programs are publicly available at http://rana.standford.edu/software. Prior to the application of the cluster algorithm to the expression matrix, linear ratios were $\log_2$-transformed and files had to be saved in a tab-delimited format.

2.7 Gene expression profiling using microarray technology

2.7.1 Construction of the microarrays

The Yeast microarrays consisted of 70-mer oligos (Qiagen, Hilden, Germany) spotted on Type VII silane-coated slides and represented 6307 well-characterised yeast genes. The microarrays were kindly provided by the Microarray Department of the Swammerdam Institute for Life Sciences at the University of Amsterdam.

2.7.2 RNA amplification and labelling

RNA was amplified using a modified protocol of *in vitro* transcription (Puskas *et al.*, 2002). Briefly, 5 µg total RNA was converted to double stranded cDNA using an anchored oligo-dT + T7 promoter 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAG-GCGG-T_{24}(ACG)-3' (Eurogentec, Belgium). From this cDNA, RNA was transcribed using T7-*in vitro* transcriptase to produce 10-30 µg amplified RNA (aRNA). The aRNA (1 µg) was labelled with the CyScribe Direct™ mRNA labelling kit (Amersham Biosciences, Piscataway, NJ) in either Cy3 or Cy5 (Amersham Biosciences) and purified using the CyScribe™ GFX™ purification kit (Amersham Biosciences). The resultant probes were analysed for amplification yield and incorporation efficiency by measuring the RNA concentration at 280 nm, the Cy3 incorporation at 550 nm and the Cy5 incorporation at 650 nm using a Nanodrop spectrophotometer (NanoDrop Technologies, Rockland, DE). A good probe had an average labelling efficiency of 1 fluorochrome every 30-80 bases. For each probe, 40 pmol of incorporated Cy5 or Cy3 were mixed in 210 µl hybridisation solution containing 50 % formamide and 0.1 % SDS in 1x Hybridization Buffer (cat# RPK0325, Amersham BioSciences).

2.7.3 Array hybridisation and post-hybridisation processes

Hybridisation and post-hybridisation washing were performed at 37ºC in an Automated Slide Processor (Amersham BioSciences, Piscataway, NJ). Post-hybridisation washing was performed in 1x SSC, 0.1% SDS, followed by 0.1x SSC, 0.1% SDS, and finally 0.1x SSC. Arrays were scanned at 532 nm and 635 nm using the Agilent DNA MicroArray scanner (Agilent Technologies, Waldbronn, Germany). Images were analysed with ArrayVision™ (Imaging Research Inc, Ontario, Canada) and spot intensities were measured as median intensities corrected for local background (sMedianDensity). Data were normalised for dye intensity differences using a Lowess-procedure (Yang *et al.*, 2002) between ratios [$\log_2$ (Cy5/Cy3)] and average signal intensities [$\log_2$ (Cy5 x Cy3)]. Subsequently, between-slide normalisation was performed with the MARAN web application (http://www.esat.kuleuven.ac.be/maran), using a generic model for sequential ANalysis Of VAriance (ANOVA) (Engelen *et al.*, 2003). Regulated candidate genes were selected when expression level changes were at least two-fold and the variation coefficient among the ratio's < 0.5.

2.8 Subtractive gene expression profiling strategy

The normalised data of the Bax-responsive genes, selected from the 1-hour time point, were compared with the normalised data obtained after a 1-hour H_2O_2 treatment in order to eliminate the common gene expression changes. The Bax-specific gene expression changes within the pool of Bax-responsive genes were subtractively selected, choosing only the Bax-responsive genes for which the normalised signal intensity after Bax induction was at least two-fold more manifest or with a different tendency compared to the corresponding signal intensity after oxidative stress.

2.9 Real-time quantitative polymerase chain reaction

Total RNA (5 μg of the same pool as for the primary gene expression profiling) was incubated with 2 units of RQ1 RNAse-free DNAse (Promega, Madison, WI) at 37°C for 30 min. The volume was adjusted to 200 μl with RNAse-free dH_2O and the mixture desalted using Microcon-YM100 (Millipore, Billerica, MA). Briefly, the sample was added to the column, centrifuged at 500 g for 8 min and flow-through discarded. Application of 500 μl RNAse-free dH_2O was followed by a 20-min centrifugation at 500 g. The column was reversed, placed in a new vial, and centrifuged again for 3 min at 1000 g. The eluate, typically about 5 μl, was added to 2 μg oligo-dT, the volume adjusted to 10 μl with RNAse-free dH_2O, and the mixture incubated at 70°C for 10 min. After cooling on ice, the following components were added: first-strand buffer (Life Technologies, Paisley, UK), 3.3 mM dithiothreitol, 40 units RNase Block (Stratagene, La Jolla, CA), 1mM of each dXTP (Amersham Pharmacia Biotec, Uppsala, Sweden), and 200 units Superscript II reverse transcriptase (Life Technologies). The 25 μl mixture was incubated for 1 h at 42°C. The resulting first-strand cDNA was used as a template for the subsequent Real-Time Quantitative PCR (RT Q-PCR), which was performed in 3.5 mM $MgCl_2$, 0.2 mM of each dXTP, 300 nM primers, 0.025 units of Ampli*Taq* Gold® DNA polymerase, and 1x SYBR Green PCR buffer containing ROX as passive reference (Eurogentec, Seraing, Belgium). SYBR Green was used at a 1:66,000 dilution. RT Q-PCR amplification was performed in triplicate using the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The gradual increase in fluorescence due to formation of a complex between SYBR Green and double-stranded DNA was monitored in real-time using an ABI prism 7700 (Perkin-Elmer Applied Biosystems, Foster City, CA). Primers were designed with the assistance of the PrimerExpress™ software (Perkin-Elmer Applied Biosystems). Specificity of the amplicons generated during the PCR reactions was confirmed by melting curve generation (Ririe *et al.*, 1997), using Dissociation Curves 1.0 supplied by the manufacturer (Perkin-Elmer). Gene expression was quantified on the basis of the threshold cycle values (C_t) at which a statistically significant increase in ROX-normalised fluorescence intensity was first detected (Livak and Schmittgen, 2001; Muller *et al.*, 2002). The C_t-value (as the mean of three independent RT Q-PCR reactions) for the gene of interest (target) was normalised to an endogenous reference gene within each sample, and this normalised target value was compared

between the samples of interest (Bax expression or H_2O_2 treatment) and the control sample (untreated), generating a $\Delta\Delta C_t$ value. The normalised level of target mRNA in the sample relative to the control was expressed as $2^{-\Delta\Delta Ct}$.

2.10 Growth assay

Precultures were grown overnight in selective synthetic SD-medium, diluted at least 500-fold in the same growth medium and grown to early-logarithmic phase. The cultures were washed three times with sterile dH_2O and resuspended at $5x10^7$ cells/ml in sterile dH_2O. A sample of 5 µl from each suspension (250,000 cells) was then inoculated in 195 µl selective synthetic SD- or SG-medium (Honeywell plate format). Alternatively, a sample of 1 µl from each suspension (50,000 cells) was inoculated in 195 µl selective synthetic SD- or SGR-medium. Cells were grown at 30°C for 72 hours with intermittent shaking (medium intensity) for 10 sec at 10 min intervals. Growth was assayed using a Bioscreen C (Labsystems, Helsinki, Finland), measuring optical densities every hour, and growth curves were generated automatically. The data were fitted to a logistic growth equation (Richards, 1959) using iterative applications aiming to mathematically define the actual growth curve by equation parameters. The growth rate of each culture was calculated based on the parameters of the fitted growth equation ($R^2 > 0.999$).

2.11 Clonogenic survival assay

2.11.1 Following Bax expression or H_2O_2 treatment

Precultures were grown overnight in synthetic SD-medium (leucine drop-out in the case of transformants), diluted at least 500-fold in the same growth medium and grown to early-logarithmic phase. The cells were washed three times with sterile dH_2O if carbon source had to be switched from glucose to galactose. For induction of Bax protein expression, cells were resuspended in selective synthetic SG-medium. At specific time intervals, 1000 cells, based on measurement of OD_{600}, were plated on synthetic semi-solid SD-medium (leucine-deficient in the case of transformants) using 4-mm diameter sterile glass beads. The plates were incubated for 3 days. Colonies were counted automatically with the Scanalyzer (LemnaTec, Würselen, Germany). For each time point, percentage of survival was calculated with reference to the mock-treatment.

2.11.2 Following total nutrient depletion except for glucose

Yeast cells were grown in 10 ml synthetic SD-medium to a density of $5x10^7$ cells/ml and further incubated in the same growth medium for 2 days to reach stationary phase. The cells were pelleted at 2,200 g, washed three times with 40 ml sterile dH_2O and resuspended in 10 ml of 2% glucose at 10^7 cells/ml to start Sugar-Induced Cell Death (SICD). Samples were taken at 24 h intervals and clonogenic survival was determined as mentioned above.

2.12 Life span analysis

Replicative life span was determined as described (Kennedy *et al.*, 1994). The strains BY4742 and BY4742 Δ*OYE3* were grown in synthetic SD-medium to early-logarithmic phase and cells were spread on synthetic SG-plates at low density. Virgin cells were isolated by micromanipulation (Singer Instruments, Roadwater, England) and used as starting mothers, the life span of which was determined by the successive removal of all subsequent daughters. Cells were grown at 30°C during the day and kept at 4°C during the night. Each experiment involved the analysis of at least 60 mothers.

2.13 Flow cytometric cell death detection

Cells were washed twice, resuspended at 10^6/ml in sterile dH_2O, and analysed for dye exclusion using Propidium Iodide (PI). To 1 ml aliquots, 1 µl PI (10 mg/l) was added and suspensions were incubated for 10 min with periodic agitation. Samples were analysed via flow cytometry, using the FACSort™ (Becton Dickinson, NJ, USA) equipped with an argon-ion laser tuned to 488 nm. The emitted PI fluorescence (dye penetration) was displayed as FL3-H fluorescence (660 nm) using the following log-mode settings: FSC: E-1 with 150V threshold, SSC: 300V and FL3: 675V.

2.14 Immunological detection of Bax protein

About 10^8 cells were pelleted by centrifugation at 2,200 g and washed twice with sterile dH_2O. They were broken with 0.5 g glass beads (0.5 mm) by a vigorous vortexing for 5 min in 200 µl ice-cold lysis buffer (50 mM TrisHCl pH 8.0, 0.1% TritonX100, 0.5% SDS) containing 1x Complete™ protease inhibitor (Roche, Basel, Switserland). The lysate was then centrifuged at 6,000 g for 5 min at 4°C to remove cell debris and glass beads. Protein concentrations were determined with the Bicinchonic Acid (BCA) Protein assay (Pierce, Rockford, IL). Samples (30 µg) were fractionated by SDS-PAGE (15% acrylamide) and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, FRG). Membranes were blocked overnight with 2% skimmed milk in PBS supplemented with 0.05% Tween-20, and probed with a mouse anti-Bax monoclonal antibody (B9, sc-7480) diluted 1:100 in PBS, 0.05% Tween-20 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by a 1000-fold diluted horseradish-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO). The protein bands were visualised chemoluminescently using Western Lightning™ (Perkin Elmer Life Sciences, Boston, MA).

2.15 NADPH assay

NADPH was assayed spectrophotometrically (Shimadzu, Kyoto, Japan) as described (Zhang *et al.*, 2000), with minor modifications. To extract the pyridine nucleotides from the yeast, about 10^8 cells

were harvested, washed three times with sterile ice-cold dH_2O, and resuspended in 250 µl ice-cold extraction buffer (100 mM TrisHCl pH 8.0, 10 mM EDTA and 0.05% Triton X-100). Cells were broken with 0.5 g glass beads (0.5 mm) by a vigorous vortexing for 5 min at 4°C. The lysates were centrifuged at 6,000 g for 5 min at 4°C, and the supernatants were analysed immediately. The total amount of NADPH + NADH in the sample was determined by measuring the absorbance at 340 nm (A1) of 50 µl of extract resuspended in 950 µl extraction buffer. The NADPH present in a 50 µl aliquot of extract was then converted to $NADP^+$ by 5 units of glutathione reductase (Sigma, St. Louis, MO) in a prewarmed reaction mixture (100 mM phosphate buffer pH 7.6, 50 µM EDTA, 0.05% Triton X-100, 5 mM glutathione GSSG) in a total volume of 1 ml. After incubation at 25°C for 5 min, the absorbance measured at 340 nm reflected the amount of NADH present (A2). The total amount of NADPH in the sample aliquot was represented by A1 – A2. To determine the NADPH concentration in the samples, an NADPH standard curve was generated using NADPH concentrations ranging from 10 µM to 100 µM. NADPH concentrations were normalised to the protein content of the samples, as determined via the Bicinchonic acid (BCA) protein assay (Pierce, Rockford, IL).

2.16 Assay for oxidative protein damage

Approximately $3x10^8$ cells were harvested by centrifugation at 2,200 g, washed twice with sterile dH_2O and broken with 0.5 g glass beads (0.5 mm) in 200 µl ice-cold breaking buffer (15% glycerol, 2 mM EDTA) containing 1 x Complete™ protease inhibitor (Roche, Basel, Switserland). Beads and cell debris were removed by centrifugation at 6,000 g for 5 min at 4°C and protein concentrations were determined via the Bicinchonic Acid (BCA) Protein assay (Pierce, Rockford, IL). Oxidative carbonyl-modifications were immunoassayed according to (Levine *et al.*, 1994), using the OxyBlot™ Oxidised Protein Detection Kit (Intergen, Purchase, NY). Briefly, 15 µg of protein (3 µg/µl) were denatured by adding 5 µl of 12% SDS, combined with 10 µl of 1x DNPH, and incubated for 15 min at ambient temperature. Derivatisation was stopped by adding 10 µl of Neutralization Solution. One-third of each derivatised sample was dot-blotted onto nitrocellulose (Schleicher & Schuell, Dassel, FRG), and the membranes were blocked overnight with 2% Bovine Serum Albumine (BSA) in PBS supplemented with 0.05% Tween-20. DNP-derivatised proteins were detected using a rabbit anti-DNP antibody, diluted 1:150 in PBS, 0.05% Tween20, and a 300-fold diluted horseradish-conjugated goat anti-rabbit IgG. The protein dots were visualized chemoluminescently using Western Lightning™ (Perkin Elmer Life Sciences, Boston, MA) and the signals were analysed with LumiAnalysis™ (Roche, Basel, Switserland). The dot blots were then washed extensively, and the proteins were stained with Amido Black (0.1% in 45% methanol/10% acetic acid) for 15 min. After destaining with 45% methanol/10% acetic acid, the signal was analysed using the Quantity One® program (BioRad, Richmond, CA), and the immunostaining signals were normalised to the total protein content of the dot.

2.17 Assay for oxidative mitochondrial DNA damage

Approximately $3x10^8$ cells were pelleted by centrifugation at 2,200 g, washed twice with sterile dH_2O, and used for DNA purification using MasterPure™ (Epicentre, Madison, WI), according to the guidelines provided by the manufacturer. Briefly, cells were resuspended in 300 µl Yeast Cell Lysis Solution, incubated at 65°C for 15 min and chilled on ice for 5 min. Proteins were precipitated with 150 µl MPC reagent and the debris pelleted by centrifugation at 20,000 g for 10 min. The DNA in the supernatant was precipitated with 500 µl isopropanol, pelleted at 20,000 g for 10 min, and washed twice with 500 µl 70% ethanol. The DNA was carefully resuspended in 50 µl of TE buffer (10 mM TrisHCl, 1 mM EDTA, pH 7.5) by tube tapping. DNA concentration was determined using the PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Eugene, OR) and the CytoFluor fluorescence reader (PerSeptive Biosystems, Framingham, MA) (excitation: 485 nm, emission: 530 nm), employing λ DNA as standard. Oxidative mitochondrial DNA damage was measured via a Quantitative PCR (Q-PCR) as the degree by which the amplification of a large (error-prone) amplicon (6.9 kb) was hampered relative to an short (error-resistant) amplicon (0.3 kb) of the mitochondrial *COX1* gene (Santos *et al.*, 2002). The PCR reaction was performed with the following components: 15 ng total DNA, 100 ng/µl Bovine Serum Albumin (BSA), 200 µM of each dXTP (Amersham Biosciences, Piscataway, NJ), 1.3 mM $Mg(OAc)_2$, 400 nM of 5'-GTGCGTATATTTC-GTTGATGCGT-3' (sense primer), 400 nM of 5'-GTCACCACCTCCTGCTACTTCAA-3' as antisense primer for long amplicon or 5'-TTCACACTGCCTGTGCTATCTAA-3' as antisense primer for short amplicon, and 2 units of r*Tth* DNA polymerase XL (Roche, Basel, Switserland) in a total volume of 50 µl. Amplification of the short *COX1* amplicon was performed in triplicate under the following conditions: 94°C for 2 min and 80°C for 2 min (hot start), followed by 15 cycles at 95°C for 15 s, 60°C for 1 min, and 72°C for 10 min. Amplification of the long *COX1* amplicon was performed in triplicate using the following conditions: 94°C for 2 min and 80°C for 2 min (hot start), followed by 17 cycles at 95°C for 15 s, 60°C for 7 min, and 72°C for 10 min. Subsequently, DNA content was determined using the PicoGreen® dsDNA Quantitation kit (Molecular Probes). The normalised level of oxidative mtDNA damage was determined as reversely related to the abundance of the long amplicon upon normalisation for mtDNA copy number (short amplicon abundance).

2.18 Assay for lipid peroxidation

The extent of lipid peroxidation was measured by ThioBarbituric Acid (TBA) reactivity towards MalonDiAldehyde (MDA) as described previously (Quinlan *et al.*, 1988), but with some minor modifications. About $5x10^8$ cells were washed with sterile dH_2O, and resuspended in 125 µl 25% HCl. Next, 125 µl of 1% (w/v) TBA in 50 mM NaOH were added. Tubes were incubated at 85°C for 30 min and cooled. The chromogen was extracted by the addition of 250 µl 1-butanol, vigorous vortexing, and centrifugation for 1 min at 6,000 g. The upper organic phase was used for fluorescent analysis (excitation: 530 nm, emission: 590 nm) using a CytoFluor fluorescence reader (PerSeptive

Biosystems, Framingham, MA). To determine the MDA concentration in the samples, 1,1,3,3-tetramethoxypropane, an acid-hydrolysable MDA precursor (Yagi, 1976), was used as a standard at concentrations ranging from 2 μM to 200 μM.

2.19 Sterol quantification assay

Total intracellular sterol was extracted as reported (Arthington-Skaggs *et al.*, 1999), but with slight modifications. About 10^8 cells were pelleted, washed twice with sterile dH_2O and the wet weight of the pellet determined. Cells were resuspended in 0.5 ml 25% alcoholic KOH solution (25 g KOH and 35 ml of sterile dH_2O, adjusted to 100 ml with 100% ethanol), transferred to borosilicate glass screw-cap tubes, incubated at 85°C for 1 hour and cooled. Sterols were extracted by the addition of 150 μl sterile dH_2O and 500 μl n-heptane, vigorous vortexing, and centrifugation at 6,000 g for 1 min. The upper organic layer, containing non-saponifiable material, was scanned between 200 nm and 300 nm via spectrophotometry (Shimadzu, Kyoto, Japan). Ergosterol content was calculated by the following equation: % ergosterol = $[(A_{281.5}/290)-(A_{230}/518)]$/pellet weight, with 290 and 518 as the E-values (in %/cm) determined for crystalline ergosterol and 24(28)-dehydroergosterol, respectively (Breivik and Owades, 1957).

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Chapter 5
Kinetics of the Bax-responsive genes

Only two decades ago, the concept of simultaneously measuring the concentrations of every transcript in the cell in one single experiment would have seemed like science fiction to most researchers. The advent of array technology, however, has empowered genome researchers to do just that, and added a new dimension to our understanding of cell physiology (Bassett *et al.*, 1999).

The work presented in this chapter investigates the transcriptional responses of *S. cerevisiae* to the mammalian pro-apoptotic Bax protein via macroarray technology. A kinetic concept is used in an attempt to unravel Bax toxicity in yeast at the molecular level.

1. Functionality of the Bax expression system

To create a Bax expression system controlled by the *GAL1* promoter, a cDNA encoding the full-length mouse Bax-α protein was subcloned into the integrative plasmid pSCTyGAL1-L, resulting in pSCTyGAL1mBAX-L. *S. cerevisiae* cells (strain INVSc1) were transformed with the expression plasmid pSCTyGAL1mBAX-L or the parental control plasmid pSCTyGAL1-L and the resulting transformants were designated as INVSc1::pSCTyGAL1mBAX-L and INVSc1::pSCTyGAL1-L, hereby indicating genomic integration of these plasmids. The Ty-δ element of the parental plasmid allowed a single-copy integration of pSCTyGAL1mBAX-L or pSCTyGAL1-L in the INVSc1 genome, as confirmed by Southern analysis (Fig. 5.1A). The galactose-inducible nature of the Bax protein production was demonstrated by Western analysis (Fig 5.1B).

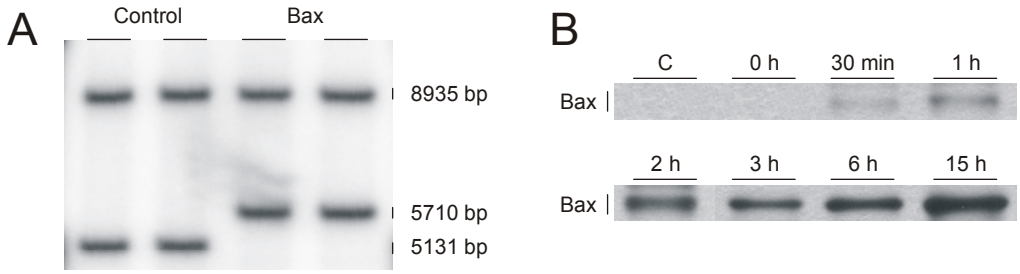

Fig. 5.1: Verification of the Bax expression system at the molecular level. (A) Southern analysis of INVSc1::pSCTyGAL1-L (Control) and INVSc1::pSCTyGAL1mBAX-L (Bax) transformants. The upper bands (8935 bp) represent the endogenic *leu2* gene. The lower bands represent the linearised plasmids pSCTyGAL1-L (5131 bp) and pSCTyGAL1mBAX (5710 bp). The intensity of the lower bands visualises the copy number of the integrated plasmids relative to the single copy *leu2* reference. (B) Western analysis of the induction of Bax protein expression following resuspension in galactose-containing medium for the timepoints indicated. C: negative control. Total protein extracts (30 μg) were subjected to SDS-PAGE, followed by immunoblot analysis with an anti-mouse Bax monoclonal antibody.

Consistent herewith, striping cells of INVSc1::pSCTyGAL1mBAX-L transformants on semisolid medium that contained galactose resulted in essentially complete inhibition of colony formation (Fig. 5.2A), whereas the colony formation on glucose-based medium occurred with about the same efficiency as observed for INVSc1::pSCTyGAL1-L transformants. However, plating of INVSc1::pSCTyGAL1mBAX-L transformants in increasing concentrations on semisolid galactose-containing medium favoured the spontaneous outgrowth of colonies able to survive the otherwise lethal Bax induction (Fig 5.2B). This is in agreement with the observation of Xu and colleagues (Xu *et al.*, 2000), based upon which they suggested spontaneous mutations, rendering yeast resistant to Bax, are selected for. A clonogenic survival assay was performed for discerning whether induction of Bax expression induced a genuine cell death or growth arrest. The lack of growth resumption, in the absence of further Bax protein production, would indicate the cells have passed the "point of no return" in the cell death process. On the other hand, if Bax expression would merely causes a growth

inhibition, cells should recover from cell cycle arrest and resume growth when Bax expression is terminated. Particularly, the clonogenic survival assay demonstrated that the conditional, galactose-inducible nature of the Bax protein production resulted in a time-dependent increase in the percentage of dead cells and a 30-min, 1-hour, 2-hours, 3-hours, 6-hours and 15-hours induction of Bax expression resulted in about 5%, 10%, 24%, 33%, 65% and 82% of dead cells (Fig. 5.2C). As Bax expression is known to induce an oxidative stress, the elevated ROS levels could have been triggering a G_1 cell cycle arrest only (Lee *et al.*, 1996; Wanke *et al.*, 1999). Therefore, the observed Bax lethality was interpreted as indicative for a serious distortion of cellular homeostasis.

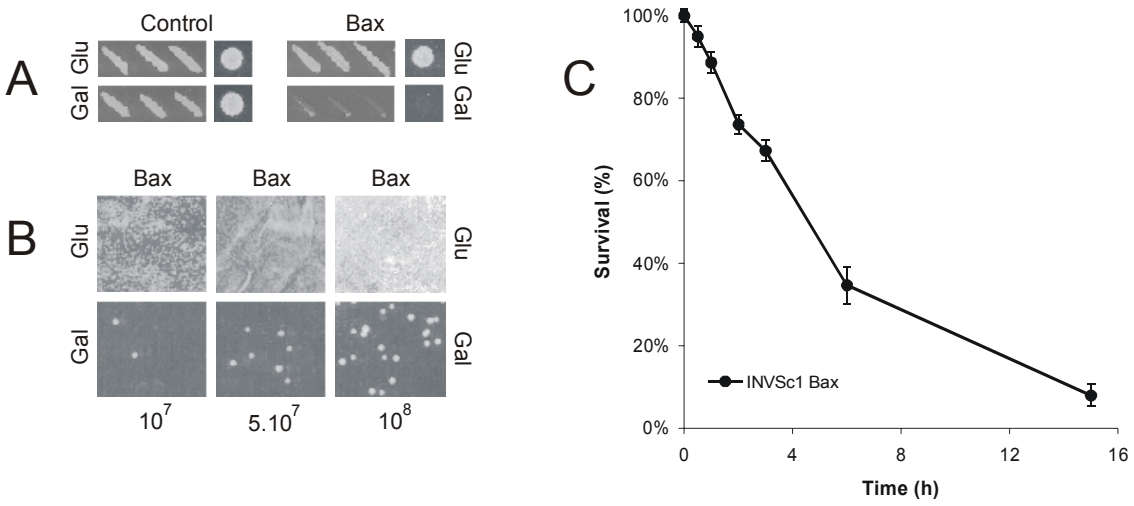

Fig. 5.2: Conditional expression of Bax confers a lethal phenotype in yeast cells. (A) Striping or spotting of control transformants on glucose- or galactose-based medium does not reveal growth differences. For yeast cells transformed with pSCTyGAL1mBAX-L, growth on galactose-based medium was inhibited due to the galactose-inducible nature of the Bax protein production. (B) Plating of increasing amounts of pSCTyGAL1mBAX-L-transformed cells on galactose-containing semisolid medium favoured the outcome of spontaneous survivors. (C) Clonogenic survival was determined by recovering cells at various times from galactose-based medium and plating 1000 cells on glucose-based semisolid medium. Induction of Bax protein expression resulted in a time-dependent exponential increase in the percentage of dead cells. Error bars represent the SD (Standard Deviation) of the mean of three independent experiments.

Intriguinly, a homogeneous dying behaviour of the cell population may have been expected. The absence of an all-or-nothing effect, however, is not that rare, and survival assays of heat-shock induced cell death reveal a similar course (Van Uden, 1984). Lewis also argued that yeast populations might consist of persistors with a disabled death program in order to guarantee future generations in case of a uniform challenge by a lethal factor (Lewis, 2000), which could explain the spontaneous outgrowth of surviving colonies of INVSc1::pSCTyGAL1mBAX-L cells on galactose-containing plates.

2. Bax-induced transcriptional changes

Global gene expression during early (30 min, 1 h), middle (2 h, 3 h) and later stages (6 h, 15 h) of Bax-induced toxicity was determined via a GeneFilter® (Research Genetics, Invitrogen) assembled from PCR-amplified open reading frames. Yeast cultures containing INVSc1::pSCTyGAL1-L or INVSc1::pSCTyGAL1mBAX-L cells were grown to mid-log phase in minimal selective SD-medium and then switched to galactose-containing minimal selective SG-medium for 30 min, 1 h, 2 h, 3 h, 6 h or 15 h. RNA was extracted from the cells at these time points and used for serial Genefilter® experiments. The hybridisation images are presented in Fig. 5.3 (30 min, 1 h, 2 h) and in Fig. 5.4 (3 h, 6 h, 15 h). The raw data were normalised to allow comparison between all the experimental datasets, as specific activity of the probe and exposure time cannot be exactly reproduced. The procedure divides the raw value for each spot by the sum of values for all spots and then multiplies by the number of elements in the macroarray. This normalisation against all data points finally reports normalised values, the intensity of which thus represent the proportion of total expression. For each of the investigated time points, the expression value of each gene in the control and Bax-induced samples was compared in a scatter plot (Fig. 5.5). The normalised signal intensity of a gene in the control is assigned to the x-axis and the expression value upon Bax expression to the y-axis. Genes with equal expression values in both experiments thus fall along the diagonal. Genes that are differentially expressed fall off the diagonal, the deviation of which reflecting the difference in the expression of that given gene. Points lying above the upper or below the lower diagonal parallel represent genes for which expression due to Bax was 2-fold higher or 2-fold lower compared to the control. The 2-hours and 3-hours time points demonstrated to be associated with the lowest fraction of transcriptional changes due to Bax protein expression. In contrast, the early 30-min and 1-hour conditions provide most changes, although downregulations due to Bax expression are more represented. In the later time points, 6-hours and 15-hours, we noticed similar changes. We analysed the frequency distribution of the expression levels (signal intensities) of all genes for control cells and Bax-treated cells for each of the time points tested and the results are given in Fig. 5.6. The most remarkable conclusion that can be made hereof comprised the large amount (≅ 50%) of hybridisation signals to be within background range (intensity ≤ 500), clearly demonstrating a major drawback of macroarray profiling: weakly expressed genes are under-represented in these analyses. Although the macroarray technology is – in theory – capable of determining gene expression patterns at the complete genome level, its expression pictures are thus far from complete. In Fig. 5.7, the hybridisation signal changes of all genes are depicted for all time points tested, thus representing an overall picture of the changes induced by Bax expression. We clearly see that most genes do not change due to the toxic Bax protein production, since the curves nicely centre on factor 1. The zoom-in boxes display the curve tails; more particularly at the regions were the expression factors change from -2 till -12 (left) and from +2 till +12 (right). In fact, these graphs represent a lateral view of the data cloud along the diagonal of the corresponding scatter profiles in Fig. 5.5.

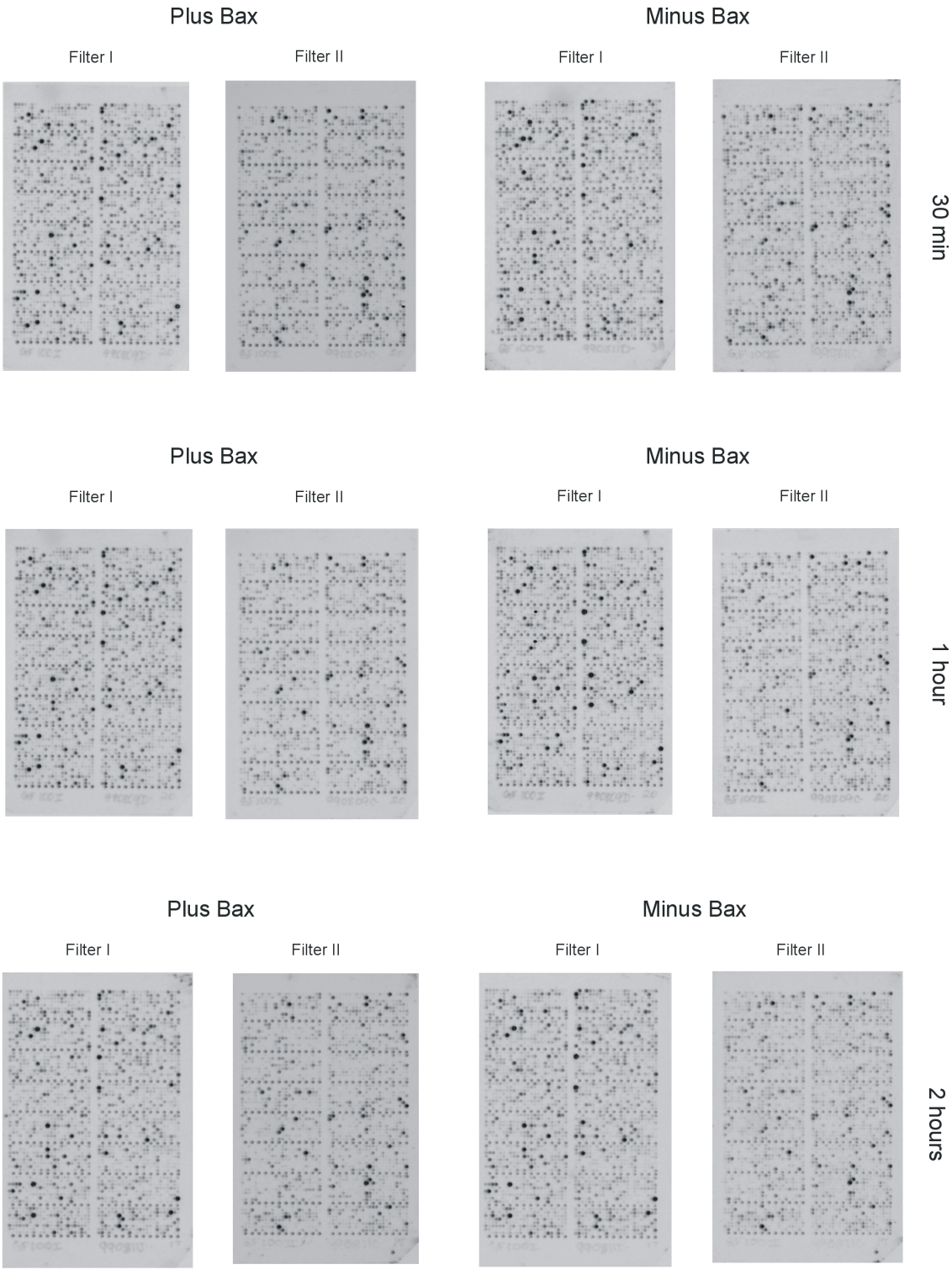

Fig. 5.3: Images of the serial GeneFilter® hybridisations for three time points tested: 30-min, 1-hour and 2-hours. Plus Bax indicates that cells transformed with pSCTyGAL1mBAX-L were used, Minus Bax refers to control cells; The GeneFilter® macroarray contains 6144 open reading frames spotted on two nylon membranes (Filter I and Filter II). Each Filter contains 3072 open reading frames organised in two fields; each field is divided into 8 grids. The grids are organised in 24 rows and 9 columns. The right hand column of each grid contains spots of total yeast genomic DNA, placed on every other row, serving as a hybridisation control.

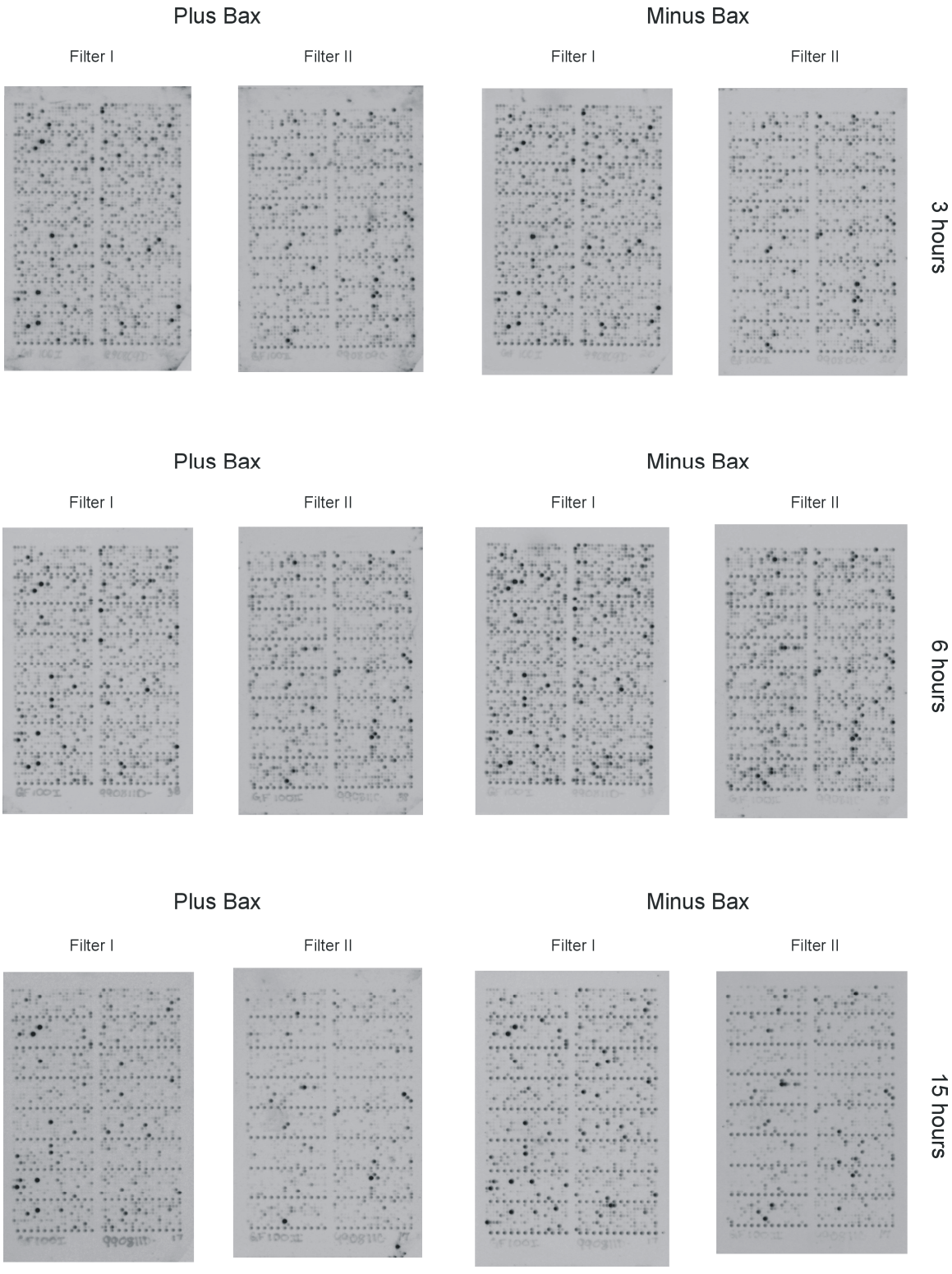

Fig. 5.4: Images of the serial GeneFilter® hybridisations for three time points tested: 3-hours, 6-hours and 15-hours. Plus Bax indicates that cells transformed with pSCTyGAL1mBAX-L were used, Minus Bax refers to control cells; The GeneFilter® macroarray contains 6144 open reading frames spotted on two nylon membranes (Filter I and Filter II). Each Filter contains 3072 open reading frames organised in two fields; each field is divided into 8 grids. The grids are organised in 24 rows and 9 columns. The right hand column of each grid contains spots of total yeast genomic DNA, placed on every other row, serving as a hybridisation control.

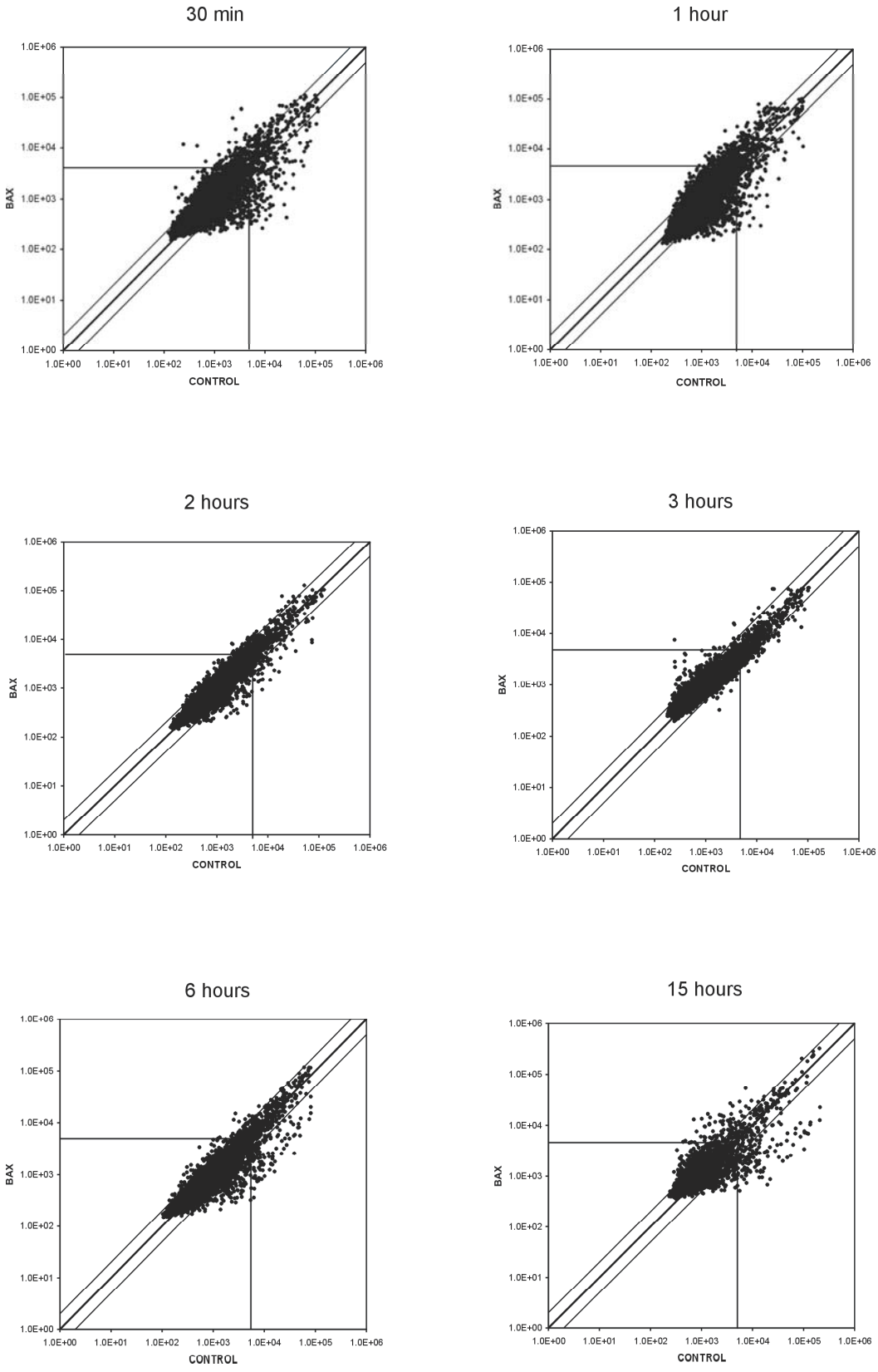

Fig. 5.5: Correlation graphs for all time points tested. Normalised hybridisation signals for the 6144 open reading frames present on the GeneFilter® from control sample and Bax sample after galactose-resuspension of INVSc1::pSCTyGAL1-L (control) or pSCTyGAL1mBax-L (Bax) cells for the indicated time period. Data points with a differential expression less than 2-fold fall within the parallels of the diagonal. Spots with an intensity of at least 10-fold above background (≥ 5000) could be distinguished clearly.

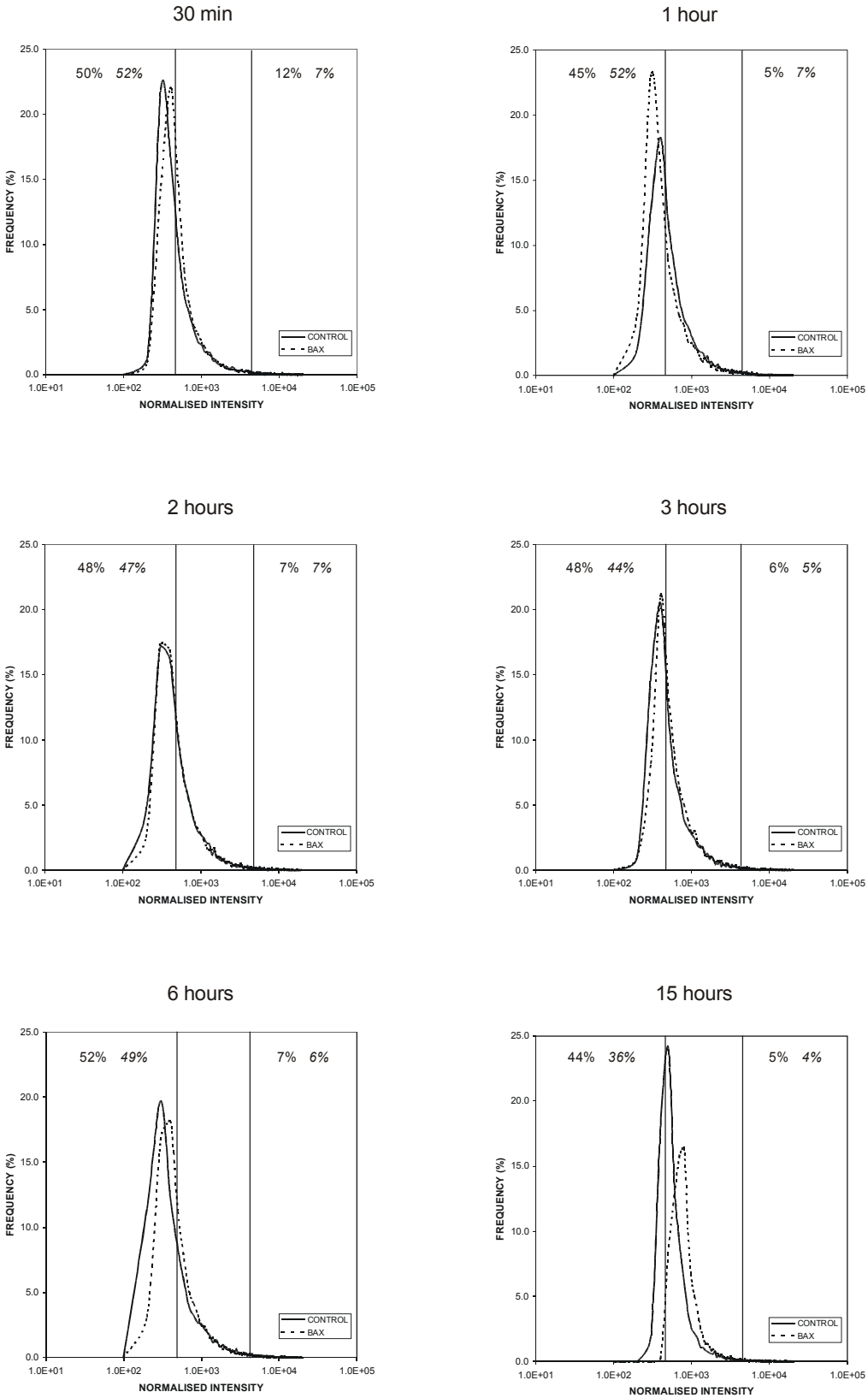

Fig. 5.6: Frequency distribution of the normalised signal intensities of the 6144 open reading frames for both control cells (full line) and Bax-treated cells (dashed line), for each of the time points tested. The percentage of signals below background value (≤ 500) is indicated in the upper left corner of each panel for the control sample (regular) or Bax-induced sample (italics), and is typically about 40-50%. The fraction of genes with a normalised signal intensity of at least 10-fold above background is represented in the upper right corner of each panel for control sample (regular) or Bax-induced sample (italics).

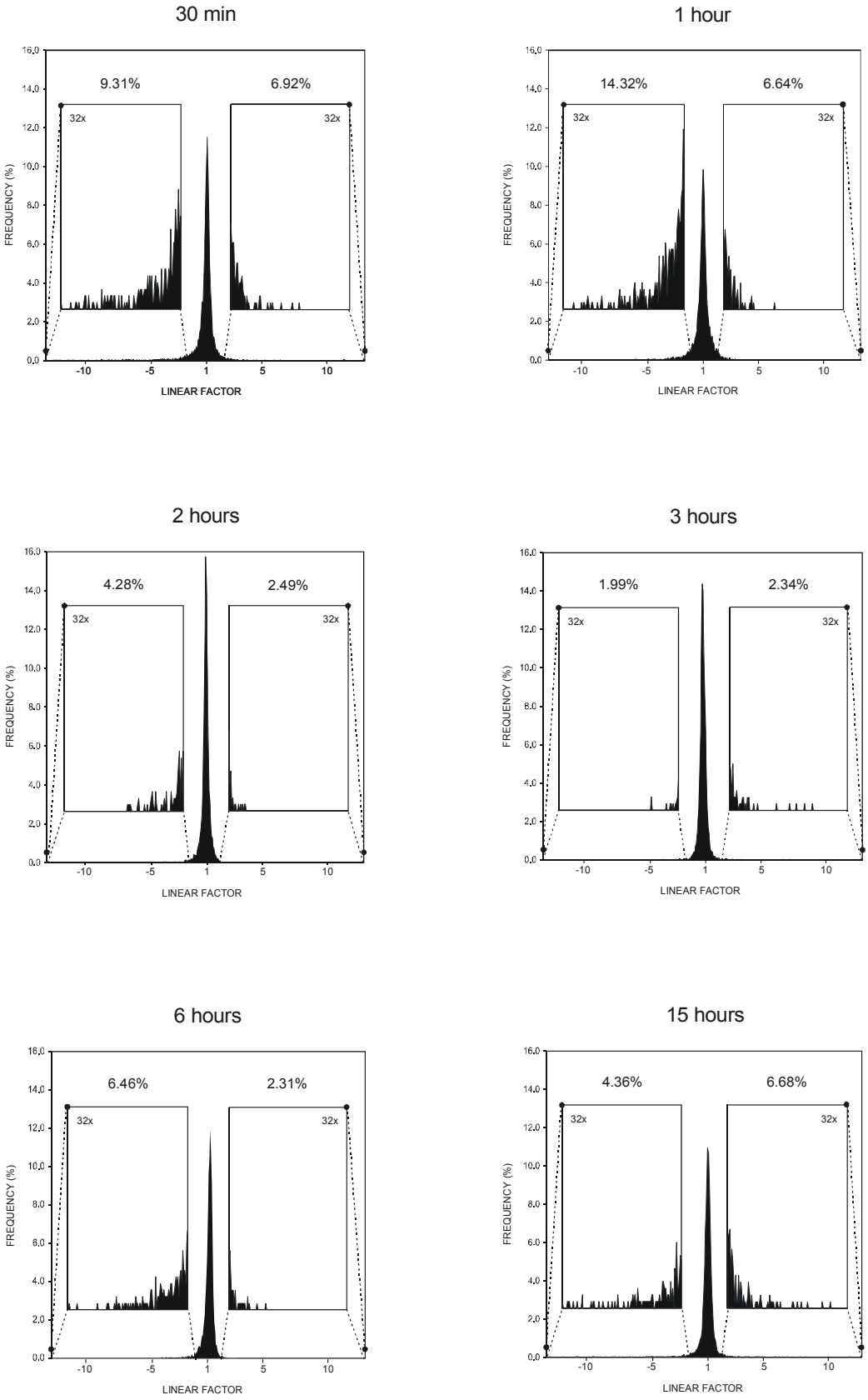

Fig. 5.7: Hybridisation signal changes due to Bax protein expression for all genes present on the GeneFilter®, for each of the timepoint tested. Factor 1 indicates no change in gene expression due to Bax induction. Factor 5: five-fold upregulation. Factor –5: five-fold downregulation. The zoom-in (32x) boxes display the curve tails at the regions where expression levels are 2- to 12-fold up- or downregulated.

3. Identification of 208 Bax-responsive genes

The criterion to define a gene as differentially expressed in a significant manner, included (i) a change in expression level of at least 2-fold, as this was observed to be significant for GeneFilter® transcriptional profiling (Yale and Bohnert, 2001); and (ii) a normalised signal intensity of the control and/or sample spot at least 10-fold above the background value (intensity ≥ 5000), as the variability of signal intensity is higher for spots of a lower intensity (Hess *et al.*, 2001). Using these cut-offs, the mRNA level of a total of 109 genes demonstrated to change significantly after 30 min of Bax induction [46 genes: up (42%); 63 genes: down (58%)]. For the 1-hour time point, we observed 104 differentially expressed genes [74 genes: up (71%); 30 genes: down (29%)]. For the 2-hours time point, we observed 52 differentially expressed genes [23 genes: up (46%); 29 genes: down (54%)]. For the 3-hours and 6-hours time points, we observed respectively 28 [4 genes: up (15%); 24 genes: down (85%)] and 61 [10 genes: up (17%); 51 genes: down (83%)] differentially expressed genes. For the 15-hours time point, 65 differentially expressed genes were observed [19 genes: up (25%); 46 genes: down (75%)]. Based on the observation that most of the significant downregulations are seen in the later timepoints, it can be argued that the transcriptional changes in these stages do not represent an active response to a toxic protein being expressed any longer, but merely enrich for the consequences of a dying cell. In total, by setting up an expression matrix including all the genes whose expression level changed at least once, we observed 208 Bax-responsive genes (3.3% of the genes encoded in the yeast genome or 6.6% of the genes detected by the macroarray) along the Bax-induction kinetics (Table 5.1). It is remarkable that 50% of the Bax-responsive genes showed an early response. We grouped the affected genes into functional categories in an attempt to identify the biochemical processes involved in Bax-induced cell death. Induction of Bax expression elicited a cellular stress condition affecting both expected and many unexpected categories (Table 5.1), including: amino-acid metabolism, cell cycle control, signal transduction, transcription factors/regulators, respiration, vesicular transport, carbohydrate metabolism, protein synthesis/degradation, cellular integrity, DNA structure/function, protein folding/translocation, RNA processing/modification, and small molecule transport. A bold typeface (Table 5.1) was used to indicate the gene was selected at that particular time point. Indications in regular typeface are meant to be informative only and represent expression level changes less than 2-fold (part (i) of criterion not fulfilled). However, they were included to assure the continuity of the expression profile kinetics. Indications with ‘nd’ represent signals below our intensity cut-off (part (ii) of criterion not met). We do not argue these genes not to fluctuate due to Bax expression, but the criterion used here did not allow evaluation. The classification of the expression profiles according to the cellular functions of the affected genes did not group similar profiles together. The only exception seemed to be the transcriptional behaviour of the ribosomal proteins. Due to the diversity of the affected cellular functions, the linear concept of the Bax-induced effect on cellular physiology is likely to be simplistic and outdated. Indeed, a portion of the Bax-responsive genes is also

controlled by other stress conditions and there are at least two explanations for this stress response overlap: (i) different conditions can often occur in nature simultaneously, and therefore any type of stress stimulates a broad response. In this case, a certain function may not be necessary to cope with all the stresses that induce its production; (ii) the specific stress disturbs cellular functions leading to another type of stress; (iii) Bax-induced ROS can damage a wide variety of cellular targets and the individual cells may lose viability as a result of different types of damage.

3.1 Genes known to affect Bax toxicity

The 208 Bax-responsive genes included some of those previously reported or predicted to have an effect on sensitivity to Bax expression when knocked-out, including *ATP1*, *ATP3*, *ATP4*, *ATP15*, *ATP16* and *ATP17* (Matsuyama *et al.*, 1998). The functionality of the F_0F_1-ATPase was reported to be necessary for Bax cytotoxicity, as an *atp4* and *atp16* strain demonstrated Bax-resistance. However, our macroarray read-out did not depict a coordinated up- or down-regulation of these subunits during Bax-induced cell death and some transcripts merely yo-yo along this process. It is worth mentioning that enhanced *ATP1* expression was observed during yeast ACD induced by histone deacetylase deletion (Yamaki *et al.*, 2001).

Manon and colleagues (Manon *et al.*, 1997) reported the downregulation of the cytochrome-c oxidase activity to accompany Bax-induced cell death and indicated that the enzyme activity of Yme1 is required for cytochrome-c oxidase protein degradation (Manon *et al.*, 2001). *COX7*, *COX12* and *COX14* transcripts are upregulated at the 1-hour time point, possibly attempting to compensate for the decreasing protein levels. Strikingly, *COX14* upregulation was also observed in *petite* cells (Traven *et al.*, 2001). In addition, the protein levels of the ubiquinol cyt-c reductase complex were also demonstrated to decrease during Bax expression (Manon *et al.*, 2001) and perhaps the upregulation of *QCR6*, *QCR8*, *QCR9* and *QCR10* can be seen as compensatory too. On the other hand, significant changes of *ANT1-3* or *VDAC1-2* could not be detected due to near-background hybridisation signals.

3.2 Genes with homologues implicated in apoptosis

The *S. cerevisiae* protein Cse1, similar to the human CAS (Cellular Apoptosis Susceptibility) protein (Brinkmann *et al.*, 1995) did not transcriptionally respond to the Bax trigger. Neither did Bir1, a member of the IAP (Inhibitor of APoptosis) protein family (Uren *et al.*, 1998). However, Yip2, homologous to the human Adenomateous Polyposis Coli protein 1 (APC1) tumour-suppressor, was transcriptionally downregulated during Bax-induced cell death. Further, the transcriptional repression of the APC1 protein during apoptosis was observed (Choi *et al.*, 2001), and thus our data extends this observation.

3.3 Genes involved in the cellular stress response

Cell death pathway(s) should oppose cellular stress responses, aimed to favour cellular survival, and thus a functional integration between these two basic responses is likely (Beere and Green, 2001). Indeed, it was demonstrated that some highly conserved Heat Shock ProteinS (HSPs) have a function in the regulation of apoptosis (Garrido *et al.*, 2001). HSPs, accumulating in mammalian cells exposed to heat and a variety of other stressful stimuli, include both anti-apoptotic (HSP27 and HSP70) and pro-apoptotic (HSP10 and HSP60) proteins and their expression levels can determine cellular fate in response to a death stimulus. The gene expression profiles upon Bax expression revealed the transcriptional downregulation of Hsp12, Hsp30 and Ssa4 (Hsp70 family). In addition, *YOR285W* and *YOR286W*, encoding proteins similar to *D. melanogaster* heat shock protein 67B2 and 67B3 respectively, are also repressed in the earliest time point tested.

ORF	Gene	Description	30 min	1h	2h	3h	6h	15h
Amino-acid metabolism								
YGR155W	*CYS4*	Cystathionine beta-synthase	**4.61**	nd	nd	nd	nd	nd
YIL074C	*SER33*	3-phosphoglycerate dehydrogenase	**4.20**	nd	nd	nd	nd	nd
YOR302W		Arginine attenuator peptide	nd	**2.32**	nd	**0.48**	1.25	nd
YPR035W	*GLN1*	Glutamine synthetase	0.69	**0.36**	**0.36**	0.78	0.93	nd
Carbohydrate metabolism								
YAL060W	*FUN49*	Stereospecific (2R, 3R)-2,3-butanediol dehydrogenase	nd	nd	**0.49**	nd	nd	nd
YBR019C	*GAL10*	UDP-glucose 4-epimerase; part of galactose metabolism	nd	nd	nd	**4.41**	0.87	nd
YBR149W	*ARA1*	Subunit of $NADP^+$-dependent D-arabinose dehydrogenase	**0.27**	**0.44**	**0.31**	1.56	0.98	nd
YDR178W	*SDH4*	Membrane anchor subunit of succinate dehydrogenase complex	**0.09**	1.29	**0.42**	1.82	1.21	nd
YEL071W	*DLD3*	D-lactate dehydrogenase; induced by retrograde signalling	**3.09**	1.81	1.29	1.16	1.74	0.89
YGR192C	*TDH3*	Glyceraldehyde-3-phosphate dehydrogenase 3 (glycolysis)	0.92	**0.37**	0.86	1.05	nd	**0.30**
YJR096W		Protein with similarity to aldehyde reductases	**0.49**	1.46	1.04	nd	1.07	nd
YKL060C	*FBA1*	Fructose-bisphosphate aldolase II; fourth step in glycolysis	1.20	0.65	1.31	0.62	**0.31**	**0.29**
YKR097W	*PCK1*	Phosphoenolpyruvate carboxykinase (gluconeogenesis)	nd	1.67	0.80	**0.41**	**2.64**	nd
YOL126C	*MDH2*	Malate dehydrogenase; probably functions in the glyoxylate cycle	nd	1.91	0.87	**0.32**	**2.73**	nd
YOR374W	*ALD4*	Mitochondrial aldehyde dehydrogenase (fermentation)	nd	**0.08**	nd	nd	nd	nd
Cell cycle control/Cell polarity								
YBR133C	*HSL7*	Adapter of regulatory pathway, relieves Cdc28 phosphorylation	**2.00**	**2.84**	1.28	0.59	1.22	1.10
YBL085W	*BOI1*	Protein required for cell polarisation and bud formation	**2.42**	1.52	1.01	0.62	1.23	nd
Cell stress								
YCL035C	*GRX1*	Putative glutaredoxin; has similarity to thioltransferase Grx2p	**0.16**	0.62	**0.45**	1.01	**0.41**	nd
YCR021C	*HSP30*	Heat shock protein with a role in cellular pH homeostasis	nd	nd	**0.45**	nd	nd	nd
YDL059C	*RAD59*	Protein involved in DNA repair	**6.72**	nd	nd	nd	nd	nd
YDR256C	*CTA1*	Vacuolar catalase A; removes hydrogen peroxide	nd	0.79	**0.44**	1.04	1.83	nd
YHR053C	*CUP1A*	Metallothionein (copper chelatin); copper-dependent antioxidant	1.68	**2.05**	1.94	**3.67**	**5.33**	1.55
YHR055C	*CUP1B*	Metallothionein (copper chelatin); copper-dependent antioxidant	1.27	**2.33**	**4.12**	**3.42**	**4.06**	1.52
YHR179W	*OYE2*	NADPH dehydrogenase; possibly involved stress response	**17.58**	0.70	1.14	0.51	nd	nd
YIR037W	*GPX3*	Glutathione peroxidase involved in oxidative stress response	**2.77**	1.10	1.01	1.07	**2.01**	nd
YLR043C	*TRX1*	Thioredoxin I; reduces specific disulfide bonds in proteins	**0.08**	0.79	**0.36**	0.97	**0.47**	**0.24**
YLR109W	*AHP1*	Alkyl hydroperoxide reductase; H_2O_2 reduction with thioredoxin	0.92	**0.15**	0.64	1.01	nd	nd
YML028W	*TSA1*	Thioredoxin peroxidase, cytoplasmic antioxidant protein	**0.43**	**0.17**	nd	nd	nd	nd
YMR173W	*DDR48*	Protein induced by heat shock, DNA damage, osmotic stress	**0.30**	**0.23**	0.76	nd	0.58	nd
YMR251W-A	*HOR7*	Protein involved in responsiveness to hyperosmolarity	**0.02**	0.63	**0.24**	1.63	**0.31**	1.14
YOL151W	*GRE2*	Protein involved in diamide tolerance, induced by osmotic stress	**9.20**	nd	nd	nd	nd	nd
YOR031W	*CRS5*	Metallothionein-like protein	0.97	**8.16**	**2.41**	0.78	1.71	**5.83**
Cell wall maintenance								
YER150W	*SPI1*	Protein induced in stationary phase	**0.19**	0.63	**0.40**	1.23	nd	nd
YKL097W-A	*CWP2*	Mannoprotein of the cell wall	nd	**0.30**	**0.38**	0.75	**0.12**	nd
YKR076W	*ECM4*	Protein possibly involved in cell wall structure or biosynthesis	**4.88**	**0.40**	nd	nd	nd	nd
YLR110C	*CCW12*	Cell wall mannoprotein	**0.05**	**0.11**	**0.16**	nd	0.53	**0.13**
YLR390W	*ECM19*	Protein possibly involved in cell wall structure or biosynthesis	**2.77**	**2.41**	1.72	**0.48**	nd	nd
Chromatin/chromosome structure								
YBL002W	*HTB2*	Histone H2B; overproduction causes chromosome instability	**0.21**	nd	1.54	nd	0.92	1.20
YBR009C	*HHF1*	Histone H4	**0.25**	**4.03**	1.15	nd	**0.48**	**0.22**
YDR224C	*HTB1*	Histone H2B	**0.34**	**4.07**	1.22	0.71	1.09	1.17
YDR545W	*YRF1-1*	Y'-helicase protein	1.77	**2.43**	1.86	**0.50**	1.47	1.11
YNL030W	*HHF2*	Histone H4	**0.25**	**2.26**	0.94	nd	0.64	**0.28**
YNL031C	*HHT2*	Histone H3	**0.20**	**0.09**	**0.18**	nd	0.58	nd

Table 5.1: Total macroarray expression matrix including all the genes whose expression level changed at least once during the Bax-induced cell death process. A bold typeface indicates the gene was selected at that particular time point. Indications in regular typeface are informative only and represent expression level changes less than 2-fold. Indications with 'nd', meaning no data, represent signals below the intensity cut-off. Based on data obtained in Chapter 6, expression matrix validation is questionable. Please, use this table with great care.

ORF	Gene	Description	30 min	1h	2h	3h	6h	15h
Energy generation								
YBL099W	*ATP1*	Alpha subunit of F_1-ATP synthase	nd	**3.22**	1.59	0.74	nd	nd
YBR039W	*ATP3*	Gamma subunit of F_1-ATP synthase	**0.32**	**0.37**	**0.26**	1.96	**0.50**	nd
YDL004W	*ATP16*	Delta subunit of F_1-ATP synthase	**0.08**	1.68	0.90	1.45	**0.44**	1.35
YDR377W	*ATP17*	Subunit f of F_0-ATP synthase	**0.05**	nd	1.10	nd	**0.50**	**0.27**
YDR529C	*QCR7*	Subunit 7 of ubiquinol cytochrome c reductase complex	**0.15**	1.24	0.67	1.34	0.63	**0.35**
YEL039C	*CYC7*	Cytochrome-c isoform 2	**0.20**	**2.34**	nd	nd	0.94	nd
YFR033C	*QCR6*	Subunit 6 of ubiquinol cytochrome c reductase complex	1.31	**3.72**	1.46	0.82	1.02	nd
YGR008C	*STF2*	Stabilising factor of F_1F_0-ATPase	**0.17**	0.58	nd	nd	nd	nd
YGR183C	*QCR9*	Subunit 9 of ubiquinol cytochrome c reductase complex	0.95	**3.53**	0.97	**0.18**	1.40	0.78
YHR001W-A	*QCR10*	Subunit 10 of ubiquinol cytochrome c reductase complex	1.04	**2.92**	1.89	0.78	0.96	nd
YJL166W	*QCR8*	Subunit 8 of ubiquinol cytochrome c reductase complex	0.60	**3.42**	1.58	0.67	0.70	1.50
YKL150W	*MCR1*	Mitochondrial NADH-cyt b5 reductase;	**0.29**	1.39	0.86	0.73	0.73	1.10
YLR038C	*COX12*	Subunit VIb of cytochrome c oxidase	**0.10**	**2.72**	0.93	0.72	0.75	0.53
YLR294C		Protein of unknown function; possibly involved in respiration	1.17	**5.74**	**2.43**	0.58	1.11	1.48
YLR327C		Protein with strong similarity to Stf2p	**0.47**	1.04	**2.48**	0.75	1.95	1.85
YML129C	*COX14*	Protein required for assembly of cytochrome oxidase	0.90	**2.60**	1.91	0.59	1.10	**7.54**
YMR256C	*COX7*	Subunit VII of cytochrome c oxidase	1.27	**3.79**	**2.18**	0.66	1.15	1.12
YPL078C	*ATP4*	Subunit 4 of F_0-ATP synthase	**0.22**	**3.28**	1.22	0.72	1.01	0.91
YPL271W	*ATP15*	Epsilon subunit of F_1-ATP synthase	1.09	**4.78**	**2.40**	0.59	1.25	**3.17**
Protein degradation								
YBR139W		Protein with similarity to serine-type carboxypeptidases	nd	**0.24**	**0.38**	nd	nd	nd
YDL147W	*RPN5*	Non-ATPase subunit of 26S proteasome complex	1.66	1.69	**2.14**	0.90	1.34	1.79
YFR052W	*RPN12*	Non-ATPase subunit of 26S proteasome complex	**2.66**	1.58	1.62	0.92	1.69	nd
YGR132C	*PHB1*	Prohibitin; stabilises mitochondrial proteins	**0.36**	nd	1.61	1.22	0.93	0.78
YGR135W	*PRE9*	Proteasome subunit a3;	**0.14**	**2.00**	0.69	0.85	0.66	**0.33**
YOR261C	*RPN8*	Non-ATPase subunit of 26S proteasome complex	0.62	**2.59**	1.54	0.61	1.38	0.89
Protein folding								
YDR171W	*HSP42*	Heat shock protein, restoration of the cytoskeleton during stress	1.35	0.73	0.98	0.99	**2.02**	nd
YER103W	*SSA4*	Protein chaperone of the Hsp70 family	nd	**0.08**	1.80	nd	nd	nd
YFL014W	*HSP12*	Heat shock protein of 12 kDa; induced by stress	**0.45**	0.74	**0.38**	1.48	0.71	**10.47**
YKL117W	*SBA1*	Co-chaperone of Hsp90 family;	**0.15**	**0.08**	**0.13**	nd	nd	nd
YLR216C	*CPR6*	Cyclophilin (peptidylprolyl cis-trans isomerase)	**3.24**	1.95	1.61	0.83	**2.18**	**4.60**
YPL037C	*EGD1*	Betasubunit of nascent polypeptide-associated complex	**0.17**	1.84	1.08	0.62	**0.46**	nd
Protein synthesis								
YBL072C	*RPS8A*	Ribosomal protein RPS8A	0.95	1.06	nd	0.67	**0.19**	**0.16**
YBL092W	*RPL32*	Ribosomal protein RPL32	nd	nd	nd	1.05	**0.11**	nd
YBR189W	*RPS9B*	Ribosomal protein RPS9B	nd	nd	nd	0.60	**0.21**	nd
YBR191W	*RPL21A*	Ribosomal protein RPL21A	nd	nd	nd	0.61	**0.12**	**0.15**
YDL075W	*RPL31A*	Ribosomal protein RPL31A	nd	nd	nd	nd	**0.22**	nd
YDL083C	*RPS16B*	Ribosomal protein RPS16B	nd	nd	nd	nd	**0.14**	**0.08**
YDL136W	*RPL35B*	Ribosomal protein RPL35B	0.63	nd	nd	0.65	**0.20**	**0.13**
YDL191W	*RPL35A*	Ribosomal protein RPL35A	0.71	nd	nd	0.72	**0.21**	**0.13**
YDR064W	*RPS13*	Ribosomal protein RPS13	nd	nd	nd	nd	**0.16**	**0.24**
YDR418W	*RPL12B*	Ribosomal protein RPL12B	nd	nd	nd	0.63	**0.11**	nd
YDR450W	*RPS18A*	Ribosomal protein RPS18A	**0.13**	nd	nd	0.86	**0.15**	**0.06**
YDR471W	*RPL27B*	Ribosomal protein RPL27B	nd	nd	nd	**0.46**	**0.18**	**0.05**
YER102W	*RPS8B*	Ribosomal protein RPS8B	nd	nd	nd	nd	**0.11**	**0.12**
YGL123W	*RPS2*	Ribosomal protein RPS2	nd	nd	nd	0.74	**0.18**	**0.32**
YGL147C	*RPL9A*	Ribosomal protein RPL9A	1.62	1.62	1.85	0.66	**0.12**	**0.06**
YGR085C	*RPL11B*	Ribosomal protein RPL11B	0.64	nd	1.79	0.56	**0.14**	**0.11**
YGR118W	*RPS23A*	Ribosomal protein RPS23A	nd	nd	nd	nd	**0.09**	**0.04**
YHR010W	*RPL27A*	Ribosomal protein RPL27A	1.36	**6.00**	**2.68**	**0.27**	**0.18**	**0.08**
YHR021C	*RPS27B*	Ribosomal protein RPS27B	nd	nd	nd	nd	**0.10**	**0.11**
YHR141C	*RPL42B*	Ribosomal protein RPL42B	nd	nd	nd	nd	**0.09**	**0.07**
YIL148W	*RPL40A*	Ribosomal protein RPL40A	1.43	**4.20**	**2.30**	0.54	**0.20**	**0.15**
YJL138C	*TIF2*	Translation initiation factor 4A	**0.47**	**0.35**	0.63	0.78	**0.36**	0.55
YJL189W	*RPL39*	Ribosomal protein RPL39	**2.56**	**4.45**	**3.92**	**0.33**	**0.19**	**0.07**
YJL190C	*RPS22A*	Ribosomal protein RPS22A	nd	nd	nd	nd	nd	**0.08**
YKL156W	*RPS27A*	Ribosomal protein RPS27A	**0.41**	nd	nd	0.54	**0.47**	**0.40**
YKR094C	*RPL40B*	Ribosomal protein RPL40B	0.95	**3.14**	**2.54**	**0.44**	**0.15**	**0.14**
YLR029C	*RPL15A*	Ribosomal protein RPL15A	1.39	**2.12**	1.46	**0.40**	**0.25**	**0.17**
YLR167W	*RPS31*	Ribosomal protein RPS31	**0.25**	1.59	1.38	0.61	**0.07**	**0.03**
YLR325C	*RPL38*	Ribosomal protein RPL38	0.76	**4.33**	**2.40**	**0.34**	**0.17**	**0.06**
YML026C	*RPS18B*	Ribosomal protein RPS18B	nd	nd	nd	0.70	**0.14**	**0.05**
YML063W	*RPS1B*	Ribosomal protein RPS1B	nd	nd	nd	0.80	**0.07**	**0.07**
YMR143W	*RPS16A*	Ribosomal protein RPS16A	nd	nd	1.16	nd	nd	**0.09**
YMR230W	*RPS10B*	Ribosomal protein RPS10B	nd	nd	nd	**0.44**	**0.30**	**0.22**
YOR096W	*RPS7A*	Ribosomal protein RPS7A	1.61	**3.64**	**2.22**	**0.34**	**0.13**	**0.07**
YOR293W	*RPS10A*	Ribosomal protein RPS10A	1.13	**2.79**	**2.01**	**0.39**	**0.23**	**0.11**
YOR312C	*RPL20B*	Ribosomal protein RPL20B	0.99	1.41	1.64	**0.47**	**0.11**	**0.08**
YPL079W	*RPL21B*	Ribosomal protein RPL21B	1.14	nd	1.87	**0.43**	**0.16**	**0.29**
Protein translocation								
YNL131W	*TOM22*	Part of mitochondrial outer membrane receptor complex	**0.10**	1.08	0.67	0.71	0.79	nd
RNA processing/modification								
YER112W	*LSM4*	U6 snRNA-associated protein of the Sm-like group	**2.46**	**2.66**	**2.04**	0.83	0.99	0.93
YGR250C		Protein with similarity to human 64K polyadenylation factor	nd	nd	**2.02**	nd	nd	nd
YNL112W	*DBP2*	ATP-dependent RNA helicase	**2.82**	0.84	0.79	0.77	1.52	1.93
YPL190C	*NAB3*	Nuclear polyadenylated RNA-binding protein	**2.80**	1.88	1.06	0.82	1.77	nd

ORF	Gene	Description	30 min	1h	2h	3h	6h	15h
Signal transduction								
YGR023W	*MTL1*	Putative sensor for cell wall integrity signaling	**2.67**	0.83	1.46	nd	nd	nd
YGR070W	*ROM1*	GDP-GTP exchange factor for Rho1, activate the *PKC1* pathway	**2.34**	0.78	0.78	1.09	1.67	1.44
YHR135C	*YCK1*	Casein kinase I; phosphorylation of plasma membrane H^+-ATPase	nd	**2.12**	1.42	0.88	1.14	nd
YOL100W	*PKH2*	Serine/threonine protein kinase; phosphorylates Pkc1 *in vitro*	1.64	**2.74**	nd	0.67	1.67	nd
Small molecule transport								
YDR276C	*SNA1*	Small, abundant plasma membrane proteolipid protein	**0.07**	0.98	0.64	1.09	0.86	**2.74**
YDR342C	*HXT7*	High-affinity hexose transporter of the hexose transport family	**0.36**	**0.34**	**0.36**	1.25	1.36	0.99
YDR343C	*HXT6*	High-affinity hexose transporter of the hexose transport family	**0.42**	**0.14**	**0.12**	1.09	1.38	0.88
YDR345C	*HXT3*	Low-affinity hexose transporter of the hexose transport family	**0.04**	0.53	0.74	1.58	0.96	nd
YGR197C	*SNG1*	Probable transport protein	nd	nd	**2.20**	nd	nd	nd
YHR039C-B	*VMA10*	Subunit of vacuolar V_0-ATPase	**0.14**	1.56	**0.50**	1.33	0.54	0.68
YHR094C	*HXT1*	Low-affinity hexose transporter of the hexose transport family	**0.06**	**0.46**	0.60	nd	1.02	nd
YMR011W	*HXT2*	High-affinity hexose transporter of the hexose transport family	**0.30**	**0.42**	0.54	0.77	0.94	nd
YOR382W	*FIT2*	Protein possibly involved in iron uptake	**4.02**	nd	1.85	nd	nd	nd
Transcription activator								
YDR073W	*SNF11*	Component of SWI-SNF global transcription activator complex	**2.54**	**2.13**	1.89	nd	1.35	nd
YOR372C	*NDD1*	Positively affects transcription of a subset of genes	**2.19**	1.92	0.91	0.77	1.57	1.17
Transcription factor								
YBR090C-A	*NHP6B*	Regulator of Swi6p-dependent gene transcription	**0.28**	nd	1.70	nd	1.01	nd
YBR112C	*SSN6*	General repressor of RNA polymerase II transcription	**2.45**	1.29	0.87	0.89	1.86	1.02
YBR289W	*SNF5*	Component of SWI-SNF global transcription activator complex	**2.18**	1.59	1.11	0.90	1.72	1.19
YDR145W	*TAF61*	Component of the TAF(II) complex	1.89	**2.77**	0.92	1.14	1.68	1.21
YDR216W	*ADR1*	Zinc-finger transcription factor, regulation of peroxisomal genes	**0.29**	**3.11**	1.23	0.80	0.97	1.06
YDR253C	*MET32*	Tanscriptional regulation of methionine metabolism	**2.58**	1.20	0.85	nd	nd	1.33
YEL009C	*GCN4*	General transcription factor of basic leucine zipper (bZIP) family	**0.18**	0.84	0.78	1.08	0.60	nd
YKR092C	*SRP40*	Nucleolar protein, maybe involved in nucleocytoplasmic transport	**2.70**	nd	nd	nd	nd	nd
YMR043W	*MCM1*	Transcription factor of the MADS box family	**2.15**	**2.56**	1.16	0.78	1.86	1.40
YMR273C	*ZDS1*	Protein that regulates SWE1 and CLN2 transcription	**2.42**	1.15	1.02	0.73	1.39	1.11
YPL089C	*RLM1*	Transcription factor of the MADS box family	1.94	**2.74**	1.22	0.66	1.35	0.99
Unknown								
YBL048W		Protein of unknown function	nd	nd	nd	1.28	1.56	**7.31**
YBL109W		Protein of unknown function	1.89	**2.76**	1.51	0.77	1.37	0.96
YBR050C	*REG2*	Regulator of Glc7p	**3.07**	**2.11**	0.99	0.78	1.97	nd
YBR062C		Protein of unknown function	0.99	**4.34**	**2.02**	0.85	1.25	1.42
YCR004C	*YCP4*	Similar to a *Sch. pombe* brefeldin A resistance protein	**4.09**	**2.11**	1.41	0.58	1.99	nd
YCR013C		Protein of unknown function	**3.80**	nd	nd	1.31	nd	nd
YDL125C	*HNT1*	Member of the histidine triad (HIT) protein family	nd	0.56	1.25	nd	**3.29**	**13.33**
YDR133C		Protein of unknown function	1.32	0.76	0.83	0.77	1.60	**0.41**
YDR134C		Protein of unknown function with similarity to Ccw12p	**0.04**	**0.20**	**0.13**	1.68	0.62	**0.34**
YDR154C		Protein of unknown function	**3.55**	**2.65**	1.58	0.90	1.97	**2.20**
YDR366C		Protein of unknown function	1.44	**3.18**	**2.34**	0.78	1.16	0.69
YDR442W		Protein of unknown function	nd	nd	nd	0.87	**0.15**	**0.05**
YDR504C		Protein of unknown function	nd	**2.52**	nd	0.90	**2.09**	1.41
YDR544C		Protein of unknown function	1.99	**2.55**	1.53	**0.47**	1.22	0.99
YGL072C		Protein of unknown function	**0.16**	0.86	0.53	1.02	0.65	**0.48**
YGL080W		Protein of unknown function	**0.14**	0.81	0.51	1.01	0.72	0.62
YGR146C		Protein of unknown function	nd	**2.68**	nd	1.37	1.40	nd
YGR182C		Protein of unknown function	1.03	**4.24**	1.92	0.67	1.06	0.60
YGR236C	*SPG1*	Protein of unknown function	**0.32**	**3.64**	1.85	0.98	1.44	**7.25**
YHL021C		Protein of unknown function	1.05	**0.19**	1.13	nd	0.82	nd
YHR056C	*RSC30*	Protein of unknown function	0.80	**2.41**	**2.05**	1.80	**0.39**	1.50
YHR095W		Protein of unknown function	**2.42**	0.77	0.60	1.03	1.54	1.36
YHR162W		Protein of unknown function	0.81	**2.41**	1.27	1.18	0.75	nd
YIL057C		Protein of unknown function	**0.03**	**0.33**	**0.13**	**2.75**	**0.50**	nd
YJL060W		Protein of unknown function	**3.60**	**0.27**	nd	nd	nd	nd
YJL142C		Protein of unknown function	**2.40**	**2.29**	1.65	0.55	**2.02**	nd
YJL144W		Protein of unknown function	nd	**0.46**	**0.30**	nd	nd	nd
YJL161W		Protein of unknown function	**0.16**	1.07	nd	nd	1.01	nd
YJL188C	*BUD19*	Protein of unknown function	**2.95**	**4.09**	**4.20**	**0.35**	**0.21**	**0.07**
YKL054C	*VID31*	Protein of unknown function	**2.13**	**2.37**	0.82	0.74	1.73	1.46
YKL065C	*YET1*	Structural homolog of human BAP31	**0.18**	1.25	0.64	0.95	1.05	1.36
YKL066W		Protein of unknown function	1.11	**2.76**	1.87	0.69	1.49	1.52
YKL123W		Protein of unknown function	nd	**2.11**	nd	nd	nd	nd
YKR040C		Protein of unknown function	1.98	0.57	0.77	1.24	1.86	**2.23**
YLR053C		Protein with similarity to xylose isomerase	**2.50**	nd	1.91	1.96	1.24	1.47
YLR311C		Protein of unknown function	**3.02**	**3.99**	**2.22**	**0.45**	1.45	**2.14**
YLR414C		Protein of unknown function	nd	**2.63**	0.65	0.59	0.60	nd
YML053C		Protein of unknown function	1.04	**2.80**	0.88	0.67	1.24	0.70
YML128C	*MSC1*	Protein of unknown function	**0.17**	0.87	**0.44**	0.60	1.46	**4.01**
YML132W	*COS3*	Member of the COS family of subtelomerically-encoded proteins	0.97	1.49	1.42	**0.48**	1.10	1.37
YMR009W		Protein of unknown function	0.59	0.58	**0.46**	1.45	0.81	1.35
YMR099C		Protein of unknown function	1.61	**2.73**	1.63	0.68	1.12	nd
YMR107W		Protein of unknown function	0.15	**5.86**	0.95	0.94	0.98	1.85
YMR110C		Protein similar to aldehyde dehydrogenases	1.08	**2.05**	1.53	0.57	1.61	**2.09**
YMR173W-A		Probable membrane protein of unknown function	0.56	1.58	1.47	**0.48**	1.04	**5.18**
YNL134C		Protein of unknown function	**2.25**	**0.41**	1.52	0.65	nd	nd
YNL179C		Protein of unknown function	**2.88**	0.57	**0.50**	0.58	1.66	1.95
YNL190W		Protein of unknown function	nd	**0.05**	nd	nd	nd	nd
YNL338W		Protein of unknown function similar to Ybl109p and Ydr544p	1.78	**2.79**	1.62	**0.35**	1.14	1.18
YOL048C		Protein of unknown function	**0.25**	**0.22**	0.57	nd	0.78	**8.04**

ORF	Gene	Description	30 min	1h	2h	3h	6h	15h
Unknown (continued)								
YOL099C		Protein possibly interacting with Erg3p	**2.10**	**3.14**	1.63	0.60	1.82	**2.23**
YOL106W		Protein of unknown function	1.15	**3.51**	**2.44**	0.53	1.26	0.82
YOL109W	*ZEO1*	Protein that leads to zeocine resistance upon overproduction	**0.45**	**0.38**	**0.29**	1.30	0.54	0.68
YOL150C		Protein of unknown function	**17.69**	**2.08**	nd	**0.27**	nd	nd
YOR121C		Protein of unknown function	1.54	**3.40**	1.63	0.65	1.13	1.07
YOR131C		Protein with unknown function	**2.81**	**2.44**	1.30	0.56	0.81	nd
YOR285W		Protein similar to *D. melanogaster* heat shock protein 67B2	**0.46**	1.26	1.12	0.70	0.82	1.21
YOR286W		Protein similar to *D. melanogaster* heat shock protein 67B3	**0.05**	nd	nd	nd	**0.49**	nd
YPL201C		Protein of unknown function	**0.28**	**2.11**	0.97	0.58	1.49	nd
Vesicular transport								
YBL078C	*AUT7*	Protein required for delivery of autophagic vesicles to the vacuole	**0.44**	1.66	0.60	1.28	0.76	**5.49**
YHR138C		Protein possibly involved in vacuolar fusion	0.19	**2.38**	0.99	0.91	1.01	**2.20**
YHR161C		Protein proposed to coordinate early stages of endocytosis	**2.30**	**2.99**	1.29	0.91	1.56	1.21
YKL196C	*YKT6*	v-SNARE homologue; essential for ER-Golgi transport	**0.15**	nd	**0.34**	nd	**0.50**	0.74
YLR206W	*ENT2*	Clathrin binding protein; required for endocytosis	1.69	**2.40**	0.83	0.59	1.76	1.27
YPL085W	*SEC16*	Protein required for coatomer COPII vesicle formation	**2.28**	1.05	0.83	0.65	1.86	1.66
YPR028W	*YIP2*	Protein involved in vesicular transport	**0.08**	**0.27**	**0.36**	nd	0.55	nd

3.4 Genes involved in protein synthesis

Ribosomal proteins are strongly repressed in amino-acid starved (Natarajan *et al.*, 2001) or heat-shocked (Lashkari *et al.*, 1997) cells, and ribosome synthesis requires a functional secretory pathway (Mizuta and Warner, 1994; Nierras and Warner, 1999). Therefore, the transcriptional status of ribosomal proteins can be seen as a sensor for the well being of a yeast cell (Warner, 1999). It was shown that the ribosomal protein S3a and S29 play important roles in apoptosis (Naora, 1999; Khanna *et al.*, 2000). However, the yeast homologues of S3a and S29 are not observed to be Bax-responsive. Other findings also implicate the expression level changes of individual ribosomal proteins to modulate and to participate in a wide variety of activities associated with cell growth and death. The ribosomal proteins L21, L27 and L35 were demonstrated to be downregulated during apoptosis (Lin *et al.*, 1994) and the transcripts of the corresponding yeast homologues, respectively *RPL21A*, *RPL21B*, *RPL27A*, *RPL27B*, *RPL35A* and *RPL35B*, are dramatically repressed due to Bax-induced cytotoxicity. In *A. thaliana*, a mutation in the ribosomal protein S18 is associated with growth retardation (Van Lijsebettens *et al.*, 1994) and the corresponding yeast homologues *RPS18A* and *RPS18B* are downregulated during Bax-induced cell death. Naora and colleagues suggested that proteins playing a 'house-keeping' role as part of the basic cellular machinery – such as ribosomal proteins – might also participate in more highly specialised functions, such as cell death (Naora, 1999).

3.5 Genes regulated by retrograde signalling

Retrograde regulation implicates a signalling pathway from mitochondria to the nucleus and appears to function as a homeostatic or stress response mechanism by which cells adapt to the alterations in the functional state of the mitochondria (Parikh *et al.*, 1987; Butow *et al.*, 1988). For example, the expression of *YEL071W* (*DLD3*) is dependent on the mitochondrial state and all of the three Rtg proteins (Chelstowska *et al.*, 1999). The *DLD3* transcript is elevated about 10-fold in the ρ° strain, compared with the ρ^{+} strain. We observed the *DLD3* transcript to be about 4-fold upregulated in the

earliest time point measured, perhaps pointing to the Bax effect on mitochondrial physiology. On the other hand, *CIT2*, another gene regulated by retrograde signalling, was not observed to be significantly upregulated due to Bax expression.

3.6 Genes involved in small molecule transport

HXT1, *HXT2*, *HXT3*, *HXT6* and *HXT7* encode HeXose Transporters controlling the glycolytic flux. The transcripts of all these genes are downregulated from the early time point on, thereby suggesting the cells to become more dependent on respiration for cellular energy generation (Lagunas *et al.*, 1982). As expression of Bax disturbs the mitochondrial physiology in *S. cerevisiae*, based on the observation that Bax-survivors are *petites* unable to respire (Harris *et al.*, 2000), the reduced fermentation rate combined with a respiratory collapse may culminate in an energetic depletion in the affected yeast cells.

3.7 Genes of unknown function

YDR134C, encoding a protein similar to Ccw12, displays a Bax-responsive profile resembling the one observed for *CCW12* itself. The unknown proteins Ynl338p, Ybl109p and Ydr544p are similar to one another and their Bax-induced responses are also alike. One could interpret these observations as a good correlation between protein similarity (and perhaps functional similarity) and their transcriptional response to the toxic protein Bax. However, macroarray analysis cannot exclude cross-hybridisation from contributing to the final hybridisation signal. This would flatten the differences in Bax response among cross-hybridising transcripts, thus finally resulting in the 'same' profile.

YKL065C encodes the integral ER membrane protein Yet1, a structural homologue of the human BAP31; the latter being an ER-specific caspase-activating adapter protein important for manifesting the cytoplasmatic apoptotic events, perhaps via a blockade of the ER to Golgi transport (Ng *et al.*, 1997; Ng and Shore, 1998; Nguyen *et al.*, 2000). Yet1 was transcriptionally downregulated at the 30-min time point but it is unclear whether this has any impact on the ER physiology.

4. Clustering of the Bax-responsive genes

A natural first step in extracting information from a macroarray analysis is to examine the extremes with outlying changes in gene expression due to Bax expression. For instance, at the 30-min time point the expression of *YOL150C* and *HOR7* are more than 17-fold upregulated and 20-fold downregulated, respectively. However, such analysis does not address the full potential of genome-scale experiments. Indeed, a holistic approach is needed and therefore, we arranged the kinetic profiles of the Bax-responsive genes (Fig. 5.9) according to their similarity in gene expression

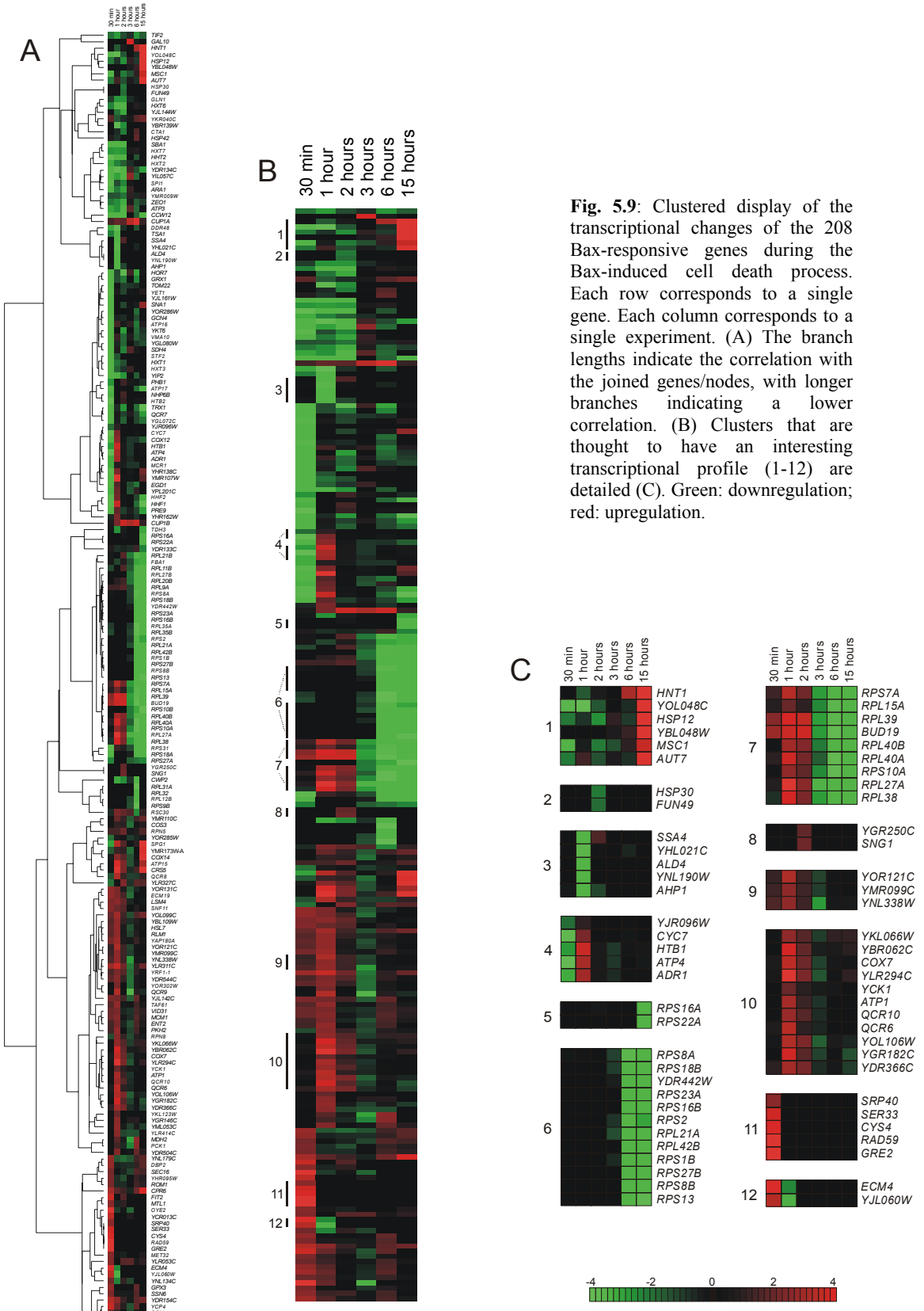

Fig. 5.9: Clustered display of the transcriptional changes of the 208 Bax-responsive genes during the Bax-induced cell death process. Each row corresponds to a single gene. Each column corresponds to a single experiment. (A) The branch lengths indicate the correlation with the joined genes/nodes, with longer branches indicating a lower correlation. (B) Clusters that are thought to have an interesting transcriptional profile (1-12) are detailed (C). Green: downregulation; red: upregulation.

pattern via a hierarchical clustering algorithm (Eisen *et al.*, 1998). It is assumed that genes with similar expression behaviour are likely to be related functionally (Chu *et al.*, 1998). Clustering of this gene expression data allowed some of the affected genes to be grouped by functionality (Fig. 5.9C). Clusters 6 and 7 for instance almost completely consisted of ribosomal protein transcripts. But also the opposite could be found: transcripts placed together did not necessarily group into the same cellular function (e.g. clusters 3, 4 and 11). This could be regarded as the Bax-induced transcriptional changes actually overruling our present functional classification, thereby adding a new functionality level to these genes. On the other hand, the cellular profiling could be blurred due to heterogeneous responses of cells within a clonal population. Particularly, Attfield and colleagues (Attfield *et al.*, 2001) reported the heterogeneity of the heat-shock induced stress response – and the correlated heat stress resistance – within cells of a given clonal population. This heterogeneity may flatten strong transcriptional responses seen at the single-cell level, possibly explaining why genes with seemingly unrelated cellular functions are observed to respond similarly to the Bax protein induction.

The validation of the expression profiles by means of an independent transcript quantification method is a logical next step in the information extraction process. As will be demonstrated in Chapter 6, GeneFilter® macroarrays have very little potential for correct profiling. Therefore, the Bax-responsive genes presented in this chapter should be used with great care only.

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Chapter 6

Search for unique Bax-responsive genes (I)

One could imagine that the transcriptional responses of *S. cerevisiae* to the mammalian pro-apoptotic Bax protein contain a Bax-specific part, leading to the generation or accumulation of reactive oxygen species, and a general pathway of oxidative stress-induced cell death. In the present chapter, a macroarray profiling was used to compare the transcriptional responses to Bax protein expression with the responses elicited by an oxidative treatment of the same final toxicity. The subtraction of the transcriptional effects of the Bax protein with the effects caused by the oxidative treatment was performed in order to determine whether Bax-associated responses are different from those elicited by oxidative stress.

1. Transcriptional changes due to H_2O_2 treatment

For comparison of Bax-induced with oxidative stress-induced cell death, we optimised the H_2O_2 dose so that, under the same growth conditions, a 1-h treatment would be as lethal as 1 hour of Bax induction. Yeast cells were challenged with increasing concentrations of H_2O_2 and the percentage of cell death after 1 hour was determined by clonogenic survival assay. Incubation in 0.1 mM H_2O_2 for 1 hour resulted in the same level of mortality observed after 1 hour of Bax induction (Fig. 6.1A). Subsequently, a yeast culture containing INVSc1::pSCTyGAL1-L cells was grown to mid-log phase in minimal selective SD-medium and then switched to minimal selective SG-medium containing 0.1 mM H_2O_2 for 1 hour. Total RNA was extracted from the cells at this time point and used for GeneFilter® experiments. The hybridisation images are presented in Fig. 6.1B. The raw data were normalised to allow comparison between the experimental datasets, as specific activity of the probe and exposure time cannot be exactly reproduced. The procedure divides the raw value for each spot by the sum of values for all spots and then multiplies by the number of elements in the macroarray. This normalisation against all data points finally reports normalised values, the intensity of which thus represent the proportion of total expression.

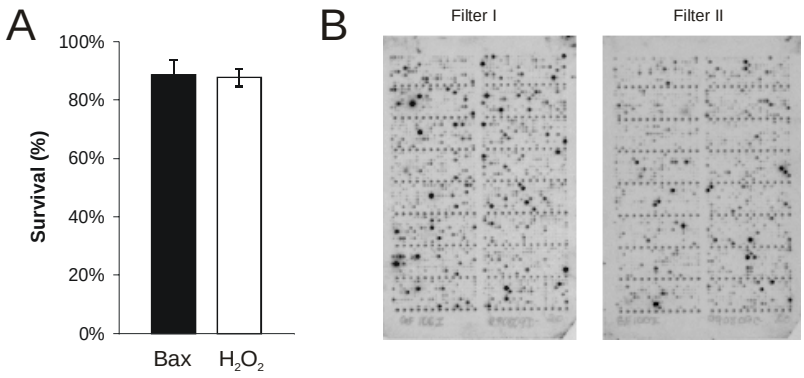


Fig. 6.1: Adjustment of Bax protein expression and H_2O_2 treatment to the same final toxicity. (A) Comparison of the clonogenic survival of wild type INVSc1 cells after a 1-hour Bax expression or a 1-hour treatment with 0.1 mM H_2O_2. (B) Images of GeneFilter® hybridisation for the sample treated with 0.1 mM H_2O_2 for 1 hour. The GeneFilter® macroarray contains 6144 open reading frames spotted on two nylon membranes (Filter I and Filter II). For details concerning GeneFilter® organisation, please refer to Fig. 5.3.

For the investigated time point, the expression value of each gene in the control versus the H_2O_2-treated samples was compared in a scatter plot (Fig. 6.2A). Genes with equal expression values fall along the diagonal. Genes that are differentially expressed fall off the diagonal, the deviation of which reflecting the difference in the expression of that given gene. In addition, a similar scatter plot was drawn for comparing the H_2O_2-treated and the Bax-induced samples [The latter data set has been described and analysed in Chapter 5] (Fig. 6.2B). Points lying above the upper or below the

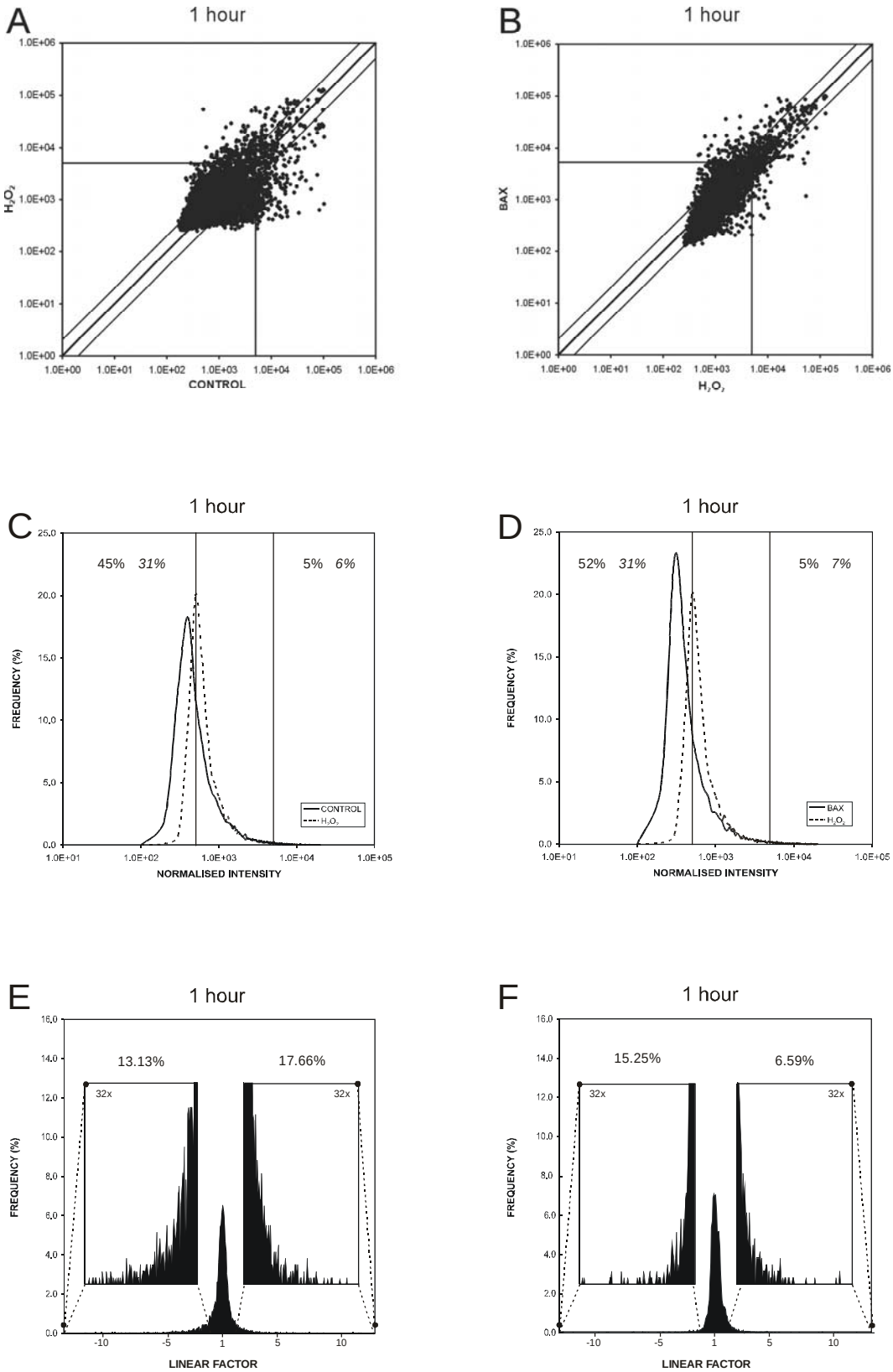


Fig. 6.2: Analysis of the normalised hybridisation signals. (A) Scatter plot for control versus H_2O_2-treated cells. (B) Scatter plot for H_2O_2-treated cells versus cells with induced Bax expression. (C) Frequency distribution for control cells (full) compared to H_2O_2-treated cells (dashed). (D) Frequency distribution for Bax-treated cells (full) versus H_2O_2-treated cells (dashed). (E) Expression level changes due to H_2O_2 treatment compared to control. (F) Expression level changes due to Bax expression compared to H_2O_2 stimulus. For details concerning data representation: see Fig. 5.5 to Fig. 5.7.

lower diagonal parallel represent genes for which expression due to Bax was 2-fold higher or 2-fold lower compared to the H_2O_2-treated sample.

We analysed the frequency distribution of the expression levels (signal intensities) of all genes in control cells versus the H_2O_2-treated sample (Fig. 6.2C) and in the Bax-induced versus the H_2O_2-treated samples (Fig. 6.2D). Again, a large amount of hybridisation signals were found to be within background range (intensity ≤ 500), clearly demonstrating a major drawback of macroarray profiling: weakly expressed genes are under-represented in these analyses.

The hybridisation signal changes of all genes due to the H_2O_2 treatment are depicted in Fig. 6.2E, which represents an overall picture of the changes induced by oxidative stress. The zoom-in boxes display the curve tails; more particularly at the regions were the expression factors change from -2 till -12 (left) and from +2 till +12 (right). Although the curve still centres on factor 1, more genes are observed to change here in comparison with a 1-hour Bax induction (See Fig. 5.7, upper right panel). Indeed, more genes are upregulated (17.66% instead of 6.44%) within the +2 → +12 range and even the subtle changes (less than 2-fold) are more represented. The hybridisation signal changes of all genes due to Bax expression, compared with the H_2O_2 treatment, are depicted in Fig. 6.2F. Although the curve centres on factor 1, more genes are observed to subtly change (less than 2-fold) in comparison with a 1-hour Bax induction versus the control (See Fig. 5.7, upper right panel). In fact, Fig 6.2E and Fig 6.2F represent a lateral view of the data cloud along the diagonal of the corresponding scatter plots (Fig. 6.2A,B).

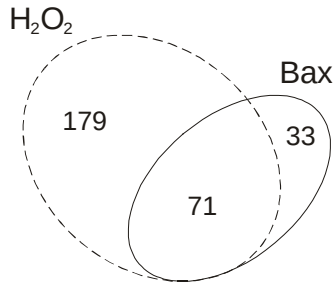


Fig. 6.3: Venn diagram illustrating the Bax-elicited and H_2O_2-elicited transcriptional responses to have responsive genes in common. The non-shared transcriptional changes are far more elaborate upon H_2O_2 treatment.

The criterion to define a gene as differentially expressed was essentially the same as used before (See Chapter 5). Using these cut-offs, the mRNA level of a total of 250 genes demonstrated to change significantly after a 1-hour H_2O_2 treatment [128 up: 51%: 122 down: 49%]. Fig. 6.3 depicts the distribution of these H_2O_2-responsive genes compared to the Bax-responsive data set.

2. Identification of specific Bax-responsive genes

The changes in normalised spot intensity due to 1 hour of Bax induction and due to the H_2O_2 stimulus were compared, whilst focusing on the 104 genes affected by a 1-hour Bax expression (See Chapter 5). Genes only responding to the 1-hour H_2O_2 treatment were not the scope of this study, as others have already extensively studied the H_2O_2 stimulus (Jamieson *et al.*, 1994; Godon *et al.*, 1998; Gasch *et al.*, 2000; Causton *et al.*, 2001). The Bax-specific gene expression changes within the pool of Bax-responsive genes were subtractively selected, choosing only the Bax-responsive genes

for which the normalised signal intensity after Bax induction was at least two-fold more manifest or with a different tendency compared to the corresponding signal intensities after oxidative stress (Fig. 6.4). Although Fig. 6.3 apparently depicts 33 Bax-specific genes, only 23 Bax-responsive genes were observed to have a Bax-specific transcriptional response based on this criterium. (Fig. 6.5) (Table 6.1).

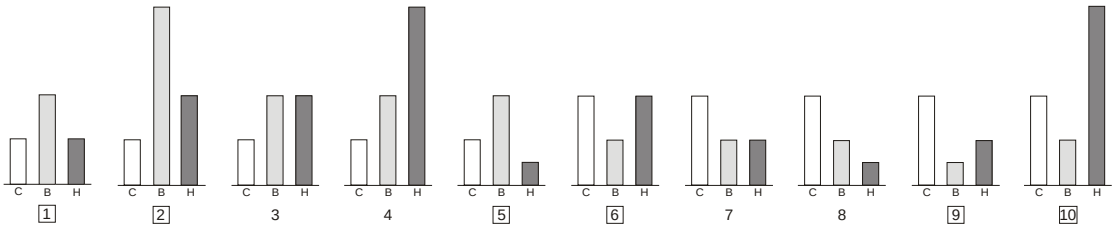


Fig. 6.4: Schematic representation of all pair wise combinations of the Bax-induced and H_2O_2-induced transcriptional response for a certain Bax-responsive gene. C: control; H: H_2O_2 treatment; B: Bax expression. In case the Bax-response was at least two-fold more manifest compared to the H_2O_2-elicited effect, as depicted in diagram 1, 2, 6 and 9, or with a different tendency, as depicted in diagram 5, and 10, the gene was designated to have a Bax-specific transcriptional response.

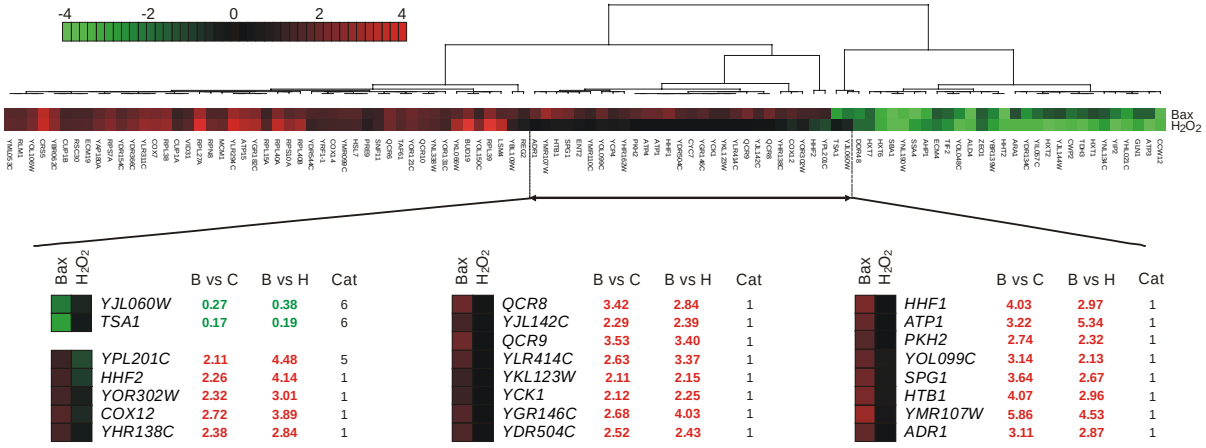


Fig. 6.5: Comprehensive scheme illustrating the comparison between the Bax-response and oxidative stress response for the Bax-responsive genes known to be affected by 1-hour of Bax expression. Candidate genes were dissected based on a Bax-induced expression level change that was more manifest or with a different tendency compared to the H_2O_2 stimulus. The figure zooms in on the Bax-specific genes. These genes demonstrated an expression level change that was at least 2-fold more manifest upon Bax expression than the corresponding H_2O_2-elicited change (categories 1 and 6) or with a different tendency (category 5). The other categories (2, 9 and 10) were not present. B vs C: ratio of signal intensity upon Bax expression compared to the control. B vs H: Bax-elicited versus H_2O_2-elicited signal intensity. Red: upregulation; green: downregulation. Cat: category, classification as depicted in Fig. 6.4.

The genes out of these 23 that are predicted to have an effect on the Bax-sensitivity when knocked-out are *ATP1*, *QCR8*, *QCR9* and *COX12* (Matsuyama *et al.*, 1998; Harris *et al.*, 2000). The genes *ATP3*, *ATP4*, *ATP15*, *ATP16*, *ATP17*, *QCR6* and *QCR10* present in the list of Bax-responsive genes (See Table 5.1), seem to be part of a shared response and it would be interesting to determine whether in the corresponding deletion strains the Bax-sensitivity and the ROS-sensitivity are

comparably modulated. In the case of the *COX* genes, the *COX12* response turned out to be Bax-specific while *COX7* and *COX14* responses are shared between both triggers. It remains to be seen whether the identification of the *ATP1*, *QCR8*, *QCR9* and *COX12* transcriptional changes as Bax-specific makes any sense, since the Bax-specific transcriptional modulation of one subunit of a multiprotein complex is likely not to make biological difference within a shared modulation of other subunits within these protein complexes. The same remark can be given for *HTB1*, *HHF1* and *HHF2*: the upregulation of one part of a dimer (H2B or H4) is likely not to be that destructive, as both parts (H2A and H2B or H3 and H4) need to be upregulated first. Thus, the Bax-specific functions encoding proteins not involved in heterodimeric or multimeric complexes are favoured because their transcriptional changes are most likely to have a biological effect.

ORF	Gene	Description	Bax/Ctrl	Bax/H_2O_2
Amino-acid metabolism				
YOR302W	*YOR302W*	Arginine attenuator peptide, regulates mRNA translation of *CPA1*	2.32	3.01
Cell stress				
YML028W	*TSA1*	Thioredoxin peroxidase, abundant cytoplasmic antioxidant protein	0.17	0.19
Chromatin/chromosome structure				
YBR009C	*HHF1*	Histone H4, stoichiometry of H2 to H3-H4 important for chrom. stability	4.03	2.97
YDR224C	*HTB1*	Histone H2B, overproduction of H2 dimer causes chrom. instability	4.07	2.96
YNL030W	*HHF2*	Histone H4, stoichiometry of H2 to H3-H4 important for chrom. stability	2.26	4.14
Energy generation				
YBL099W	*ATP1*	Alpha subunit of the F_1-ATP synthase	3.22	5.34
YGR183C	*QCR9*	Subunit 9 of ubiquinol cytochrome c reductase complex	3.53	3.40
YJL166W	*QCR8*	Subunit 9 of ubiquinol cytochrome c reductase complex	3.42	2.84
YLR038C	*COX12*	Subunit VIb of cytochrome c oxidase	2.72	3.89
Signal transduction				
YHR135C	*YCK1*	Casein kinase I, phosphorylation of plasma membrane H^+-ATPase	2.12	2.25
YOL100W	*PKH2*	Serine/threonine protein kinase, phosphorylates Pkc1p *in vitro*	2.74	2.32
Transcription factor				
YDR216W	*ADR1*	Zinc-finger transcription factor, regulation of peroxisomal genes	3.11	2.87
Unknown				
YDR504C	*YDR504C*	Protein of unknown function	2.52	2.43
YGR146C	*YGR146C*	Protein of unknown function	2.68	4.03
YGR236C	*SPG1*	Protein of unknown function	3.64	2.67
YJL060W	*YJL060W*	Protein of unknown function	0.27	0.38
YJL142C	*YJL142C*	Protein of unknown function	2.29	2.39
YKL123W	*YKL123W*	Protein of unknown function	2.11	2.15
YLR414C	*YLR414C*	Protein of unknown function	2.63	3.37
YMR107W	*YMR107W*	Protein of unknown function	5.86	4.53
YOL099C	*YOL099C*	Protein possibly interacting with Erg3p (ergosterol biosynthesis)	3.14	2.13
YPL201C	*YPL201C*	Protein of unknown function	2.11	4.48
Vesicular transport				
YHR138C	*YHR138C*	Protein possibly involved in vacuolar fusion	2.38	2.84

Table 6.1: Bax-responsive genes selected to have a Bax-specific transcriptional response. Selection was based on (i) a Bax-response at least two-fold up/down compared to the control and (iia) a Bax response at least two-fold more prominent up/down than the up/down H_2O_2 response or (iib) a Bax response up/down with a different tendency compared with the H_2O_2 response (down/up).

We wondered whether these 23 Bax-specific genes would represent a Bax-exclusive pathway, in that the response to Bax protein expression would not be shared with any other type of treatment. Therefore, we checked if one of these 23 genes was known to have less chance to be differentially expressed upon a certain toxic trigger, based on statistical data hereabout in the on-line YMGV (Yeast Microarray Global Viewer) database (http://transcriptome.ens.fr/ymgv/) (Marc *et al.*, 2001). However, the 23 focus genes were not enriched for genes that hardly respond to a certain stressful condition.

3. Phenotypic effect of the Bax-specific genes

3.1 Episomal Bax expression systems

To determine the sensitivity to Bax-induced cell death of the 23 strains knocked-out for one of the genes identified to have a Bax-specific transcriptional response, episomal Bax expression systems on centromeric plasmids were constructed. Centromeric expression systems should allow streamlining the phenotypical validation process, avoiding the control/selection of transformants with the same amount of Bax expression cassettes.

The Bax expression cassette containing the *GAL1* promoter, the mouse Bax-α cDNA and the *FLP1* terminator, was isolated from pSCTyGAL1mBAX-L and inserted into the centromeric vector pRS415, obtaining pRS415GAL1mBAX as an episomal vector for *GAL1*-driven Bax expression.

The stronger a deadly trigger, the more difficult it may be for a single gene to influence the cell death process (Manon *et al.*, 2001). Therefore, another Bax expression vector was constructed, to obtain a lower level of expression of Bax. The cDNA encoding the full-length mouse Bax-α protein was removed from pSCTyGAL1mBAX-L and subcloned into the centromeric vector p415GALL, creating p415GALLmBAX. This placed Bax expression under the control of the *GALL* promoter, a deletion variant of the *GAL1* promoter. Transcriptional induction of the *GALL* promoter is reduced to half of the *GAL1* level, although exhibiting the same galactose regulation (Mumberg *et al.*, 1994).

The *S. cerevisiae* strain BY4742 was transformed with plasmids pRS415, pRS415GAL1mBAX, p415GALL or p415GALLmBAX and the galactose-inducible nature of the Bax protein production was demonstrated by Western analysis (Fig. 6.6A). We observed the *GALL*-driven Bax expression to result in reduced Bax protein levels compared to the *GAL1*-driven system.

Striping cells of BY4742 pRS415GAL1mBAX transformants on semi-solid medium that contained galactose resulted in almost complete inhibition of colony formation, whereas the colony formation on glucose-based medium occurred with about the same efficiency as observed for BY4742 pRS415. However, striping cells of BY4742 p415GALLmBAX transformants on semi-solid medium that contained galactose resulted merely in retardation of colony growth, whereas the colony formation on glucose-based medium occurred with about the same efficiency as observed for BY4742 p415GALL (Fig. 6.6B).

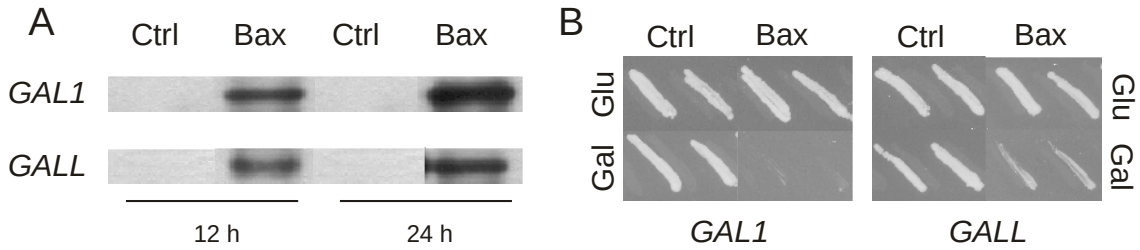


Fig. 6.6: Verification of the episomal Bax expression systems. (A) Western analysis of the induction of Bax protein expression following resuspension in galactose-containing medium for the timepoints indicated. Total protein extracts (30 µg) were subjected to SDS-PAGE, followed by immunoblot analysis with anti-mouse Bax antibody. (B) Striping of control transformants on glucose- or galactose-based medium does not reveal growth differences. For yeast cells transformed with pRS415GAL1mBAX, growth on galactose-containing medium was almost inhibited due to the galactose-inducible nature of the Bax protein production. For yeast cells transformed with p415GALLmBAX, growth on galactose-containing medium was retarded due to the lower levels to which Bax proteins accumulate upon Bax induction via the *GALL* promoter. *GAL1*: pRS415, *GALL*: p415GALL, Ctrl: pRS415 or p415GALL, Bax: pRS415GAL1mBAX or p415GALLmBAX, Glu: glucose-containing medium, Gal: galactose-containing medium.

3.2 Respiratory capacity

To determine whether the genes identified to be unique Bax-responsive could have an effect on mitochondrial physiology, we determined the respiratory competence of the corresponding knockout strains. The 23 knockout strains were ordered from Euroscarf, a European consortium that distributes yeast strains generated during yeast functional analysis projects. All were available as haploid ablations, the *YDR224C* (*HTB1*) single knockout in a diploid background (BY4743) being an exception. The strains were grown in rich YPD-medium, washed thoroughly, resuspended in sterile dH_2O, and spotted in ten-fold dilutions (5000, 500 and 50 cells) on semi-solid medium containing either 2% glucose (YPD) or 3% glycerol (YPGly). Glycerol metabolism, more particular the conversion of glycerol-3-phosphate to dihydroxyacetonephosphate, is known to require mitochondrial respiration. Complete or partial growth retardation in the presence of a respiratory carbon source (glycerol), while growth on a fermentative carbon source is unaffected (glucose), was used as the criterion to define a strain respiratory deficient. We observed 4 out of the 23 knockout strains to have a respiratory deficiency (Δ*QCR9*, Δ*COX12*, Δ*ATP1* and Δ*YDR504C*) (Fig. 6.7).

3.3 Determination of Bax sensitivity via growth assay

The plasmids pRS415, pRS415GAL1mBAX, p415GALL, p415GALLmBAX were transformed to the haploid wild type BY4742, the 22 haploid knockout strains, the diploid wild type BY4743 and the diploid Δ*HTB1* single knockout strain. The transformants were analysed via a growth assay in selective synthetic galactose-containing SG-medium. Selective synthetic SGR-medium medium, containing additional raffinose (2%) as carbon source, was used for knockout strains demonstrating a slow intrinsic growth in SG-medium (respiratory deficient strains and others). For the empty vector transformants, the growth of all knockout strains did not differ from that of the control (BY4742 or

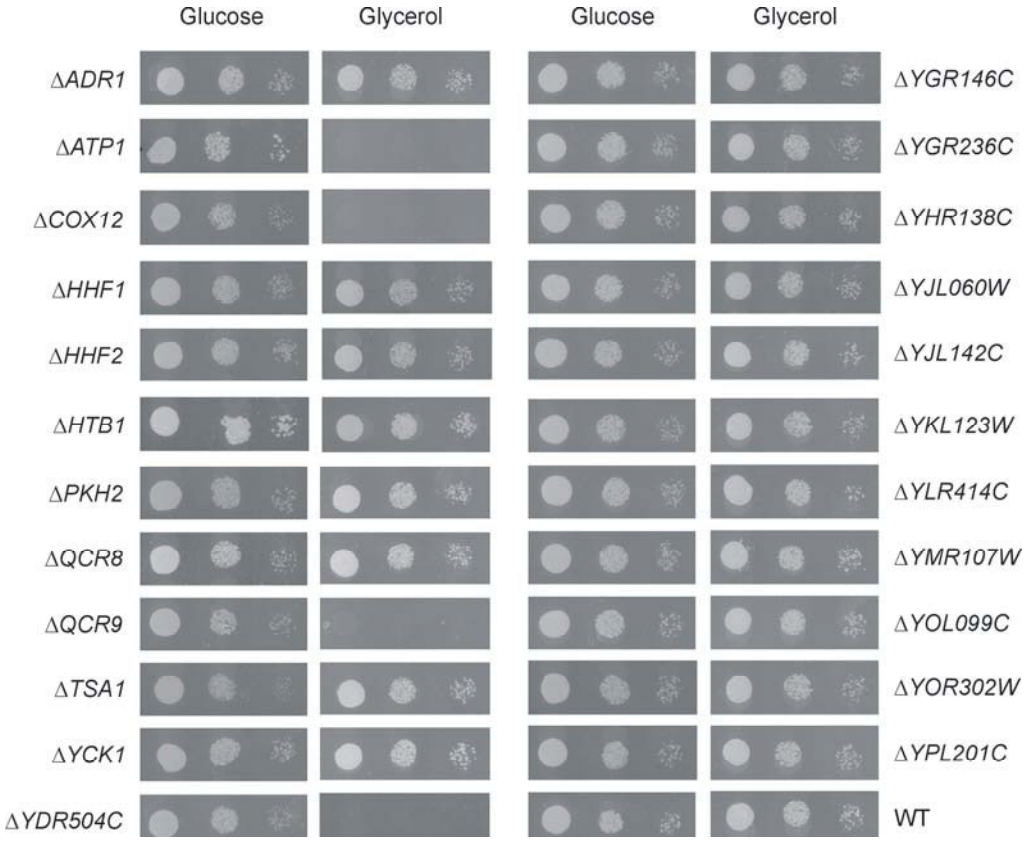


Fig. 6.7: Determination of the respiratory capacity of strains knocked out for one of the 23 genes determined to have a Bax-specific transcriptional response. In case mitochondrial respiration is hampered due to a particular gene knockout, growth on glycerol-containing medium is retarded or completely absent. Glucose: rich YPD medium containing 2% glucose, Glycerol: rich YPGly medium containing 3% glycerol. Ten-fold dilutions were spotted (from left to right: 5000, 500 and 50 cells) and sans were taken after 36 hours (YPD) or 72 hours (YPGly). WT: BY4742 (haploid) and BY4743 (diploid).

BY4743) (Fig. 6.8). Growth characteristics were analysed by fitting to an equation describing logistic growth (Richards, 1959). Growth rate was calculated as the first derivative at the inflection point and growth rate ratio of the Bax-expressing population to the control transformant was calculated for each strain (See Fig 6.9, Fig. 6.10 and Fig. 6.11). An increased growth rate ratio (Bax/control) was indicative for a decreased Bax toxicity level (resistance), while a decreased growth rate ratio (Bax/control) indicated an increased Bax toxicity (sensitivity). When no change was observed, the knockout strain was identified as being as robust as the wild type strain to Bax-induced growth retardation.

Some knockout strains did not show any alteration in their growth rate upon Bax expression (Δ*ADR1*, Δ*HTB1*, Δ*YCK1*, Δ*YJL060W*, Δ*YMR107W*, Δ*YOL099C*, Δ*YOR302W*, Δ*YPL201C*, Δ*YGR236C*) (Fig. 6.9). Bax-sensitivity due to gene knockout was observed in Δ*ATP1*, Δ*COX12*, Δ*YDR504C* and Δ*YJL142C* for both the *GAL1*- and *GALL*- expression systems and in Δ*YGR146C* and Δ*QCR9* for the *GAL1*-driven system only (Fig. 6.10). The absence of *YGR146C* or *QCR9* thus only result in Bax-sensitivity if Bax protein accumulates to a high level. Bax-resistance due to gene

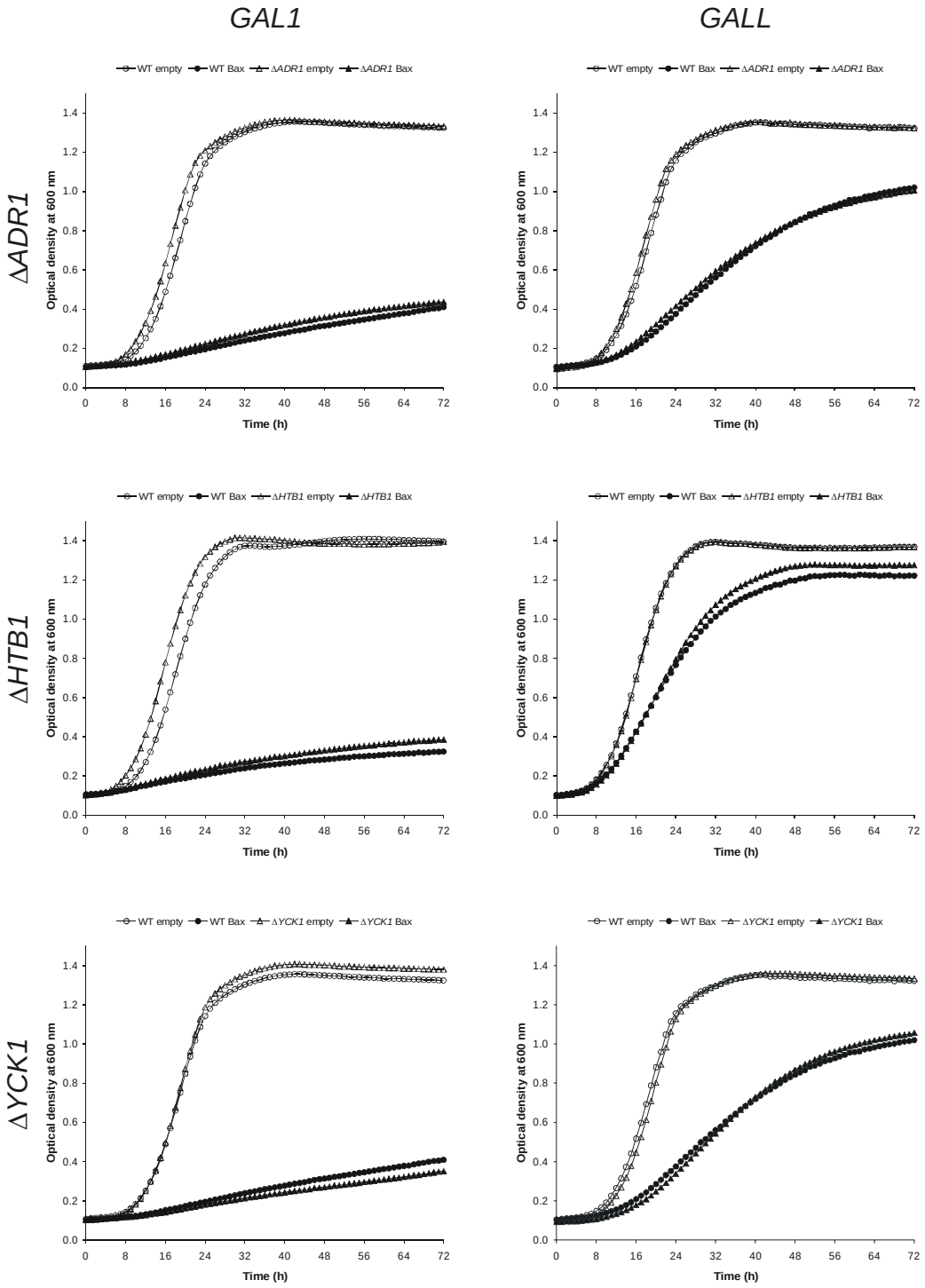
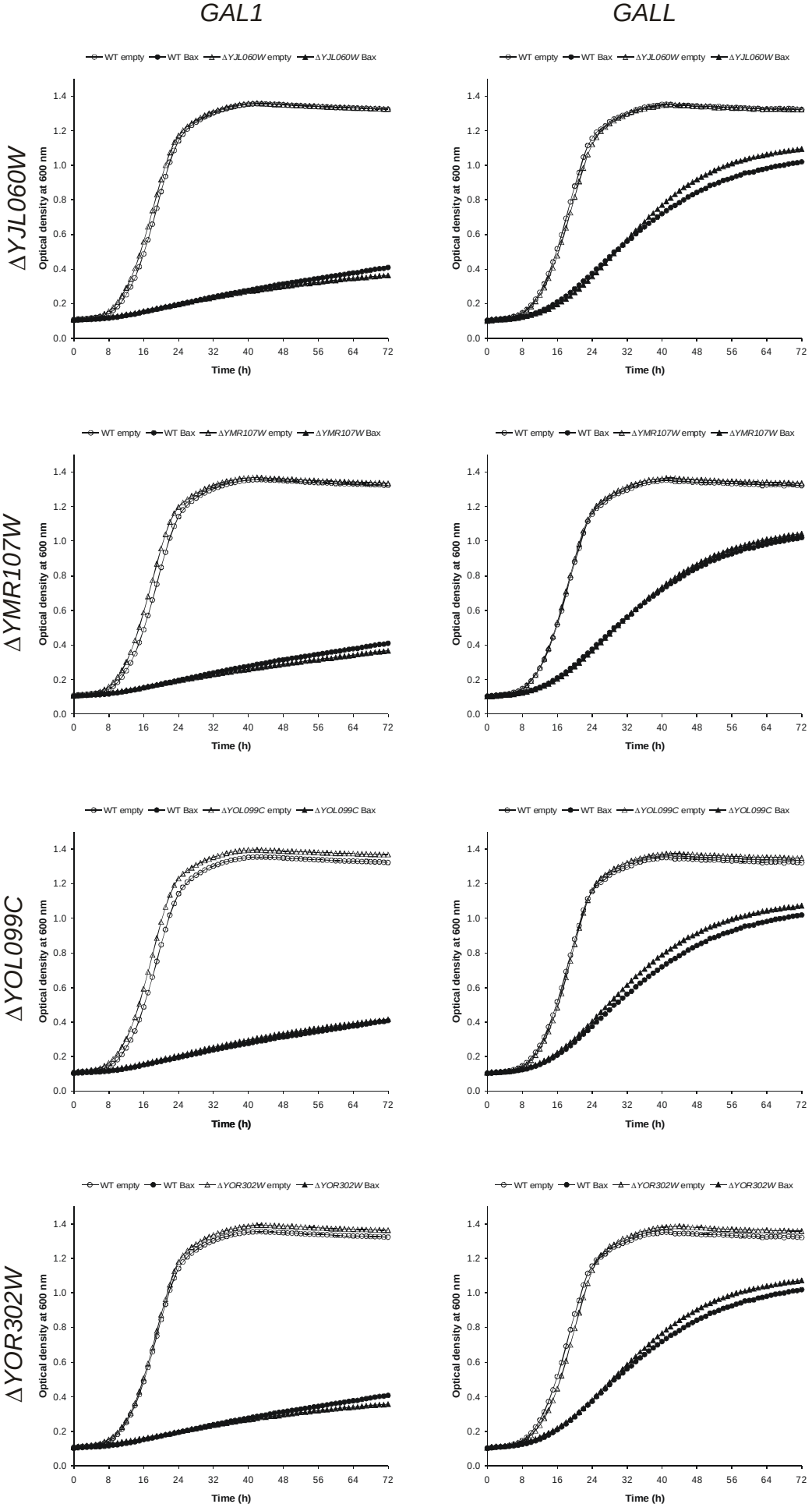
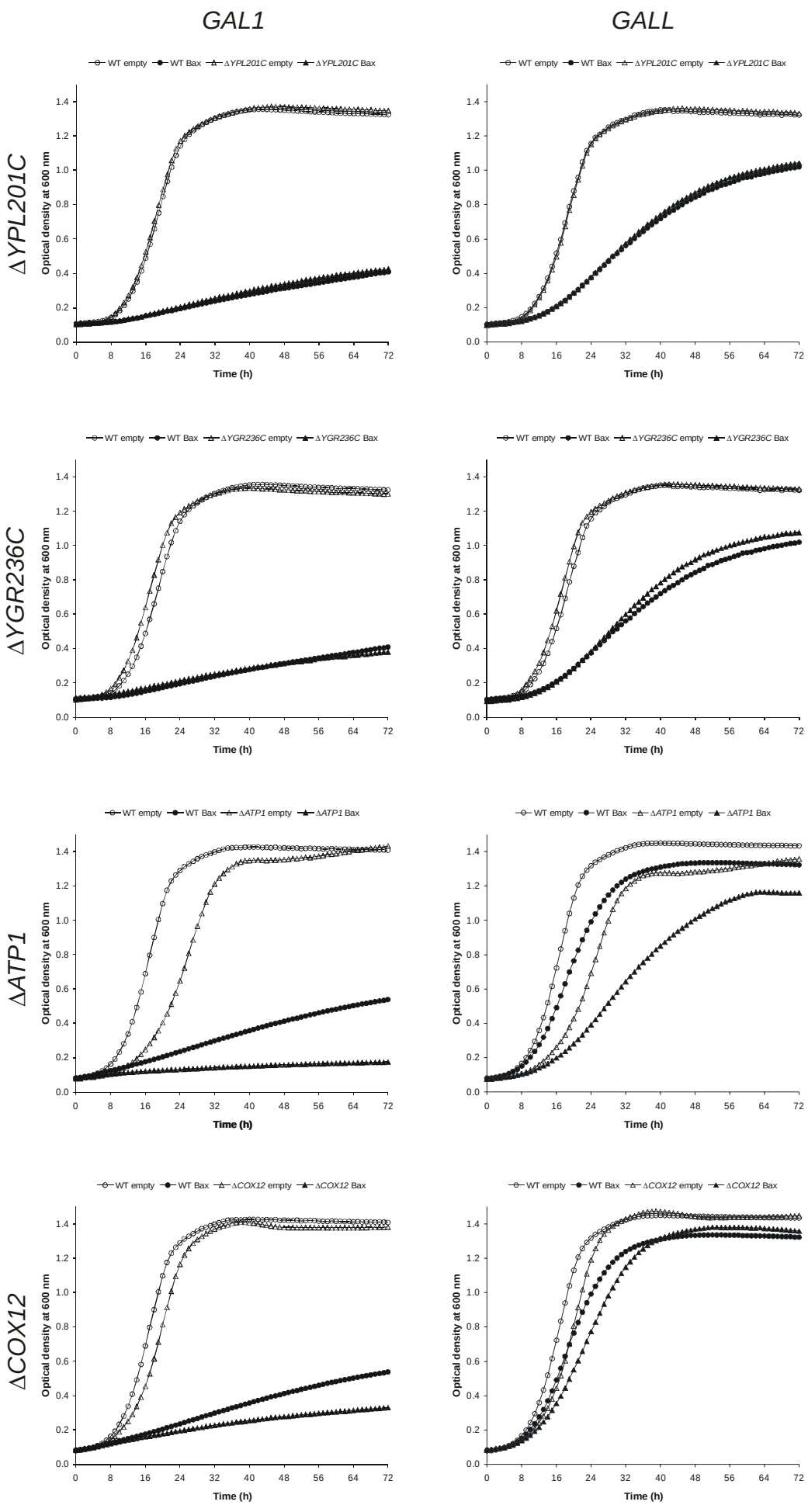
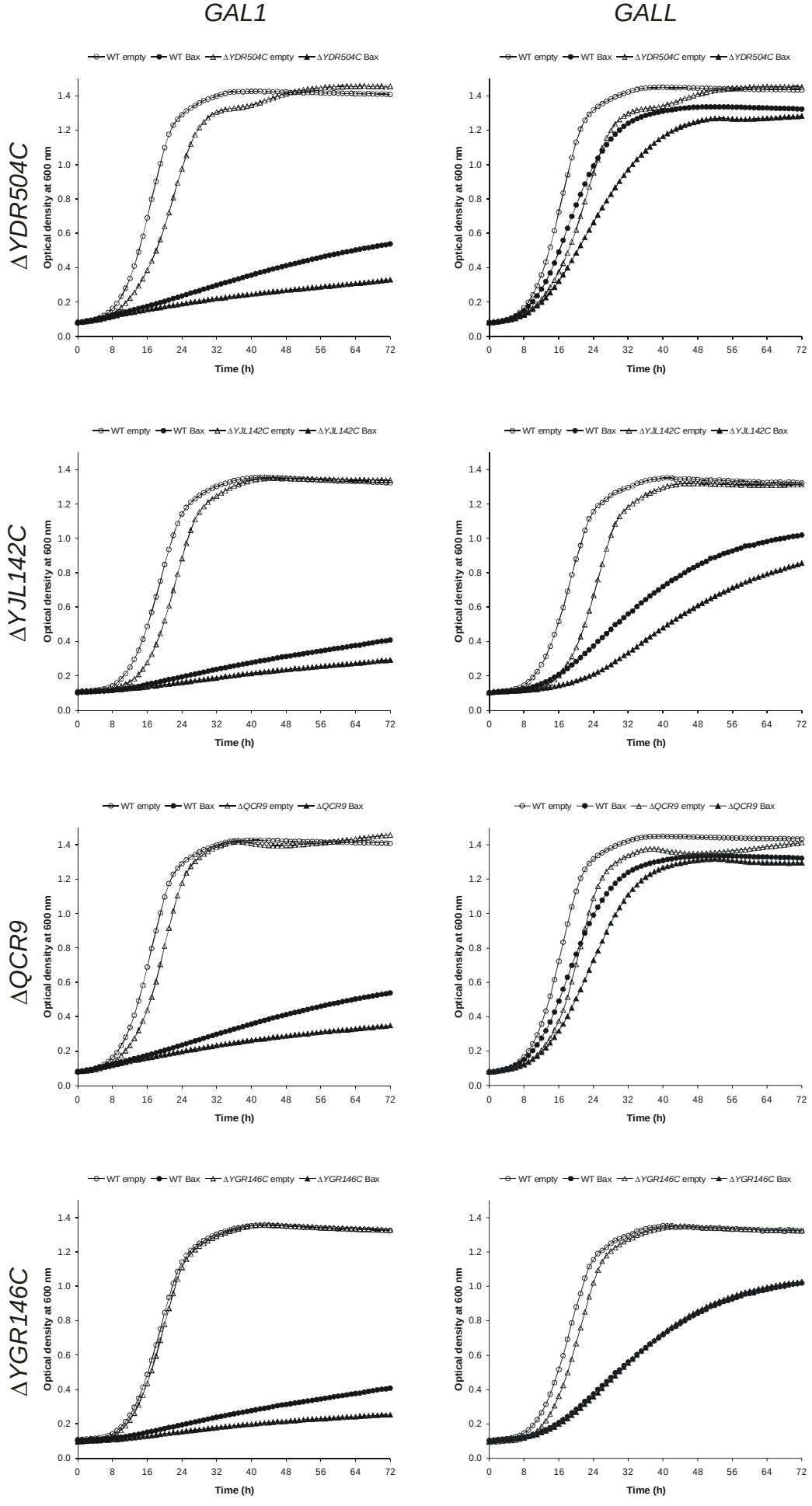
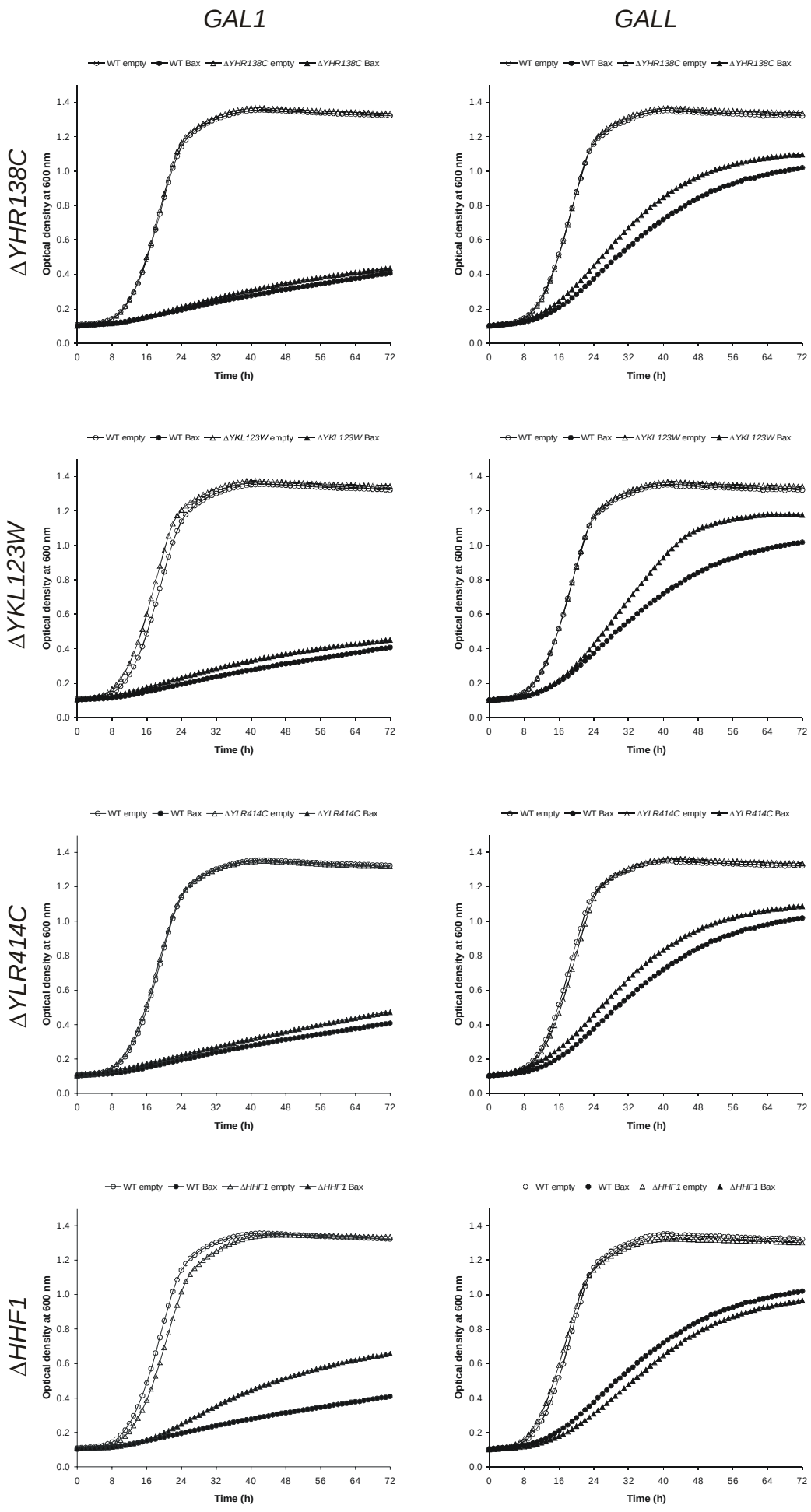


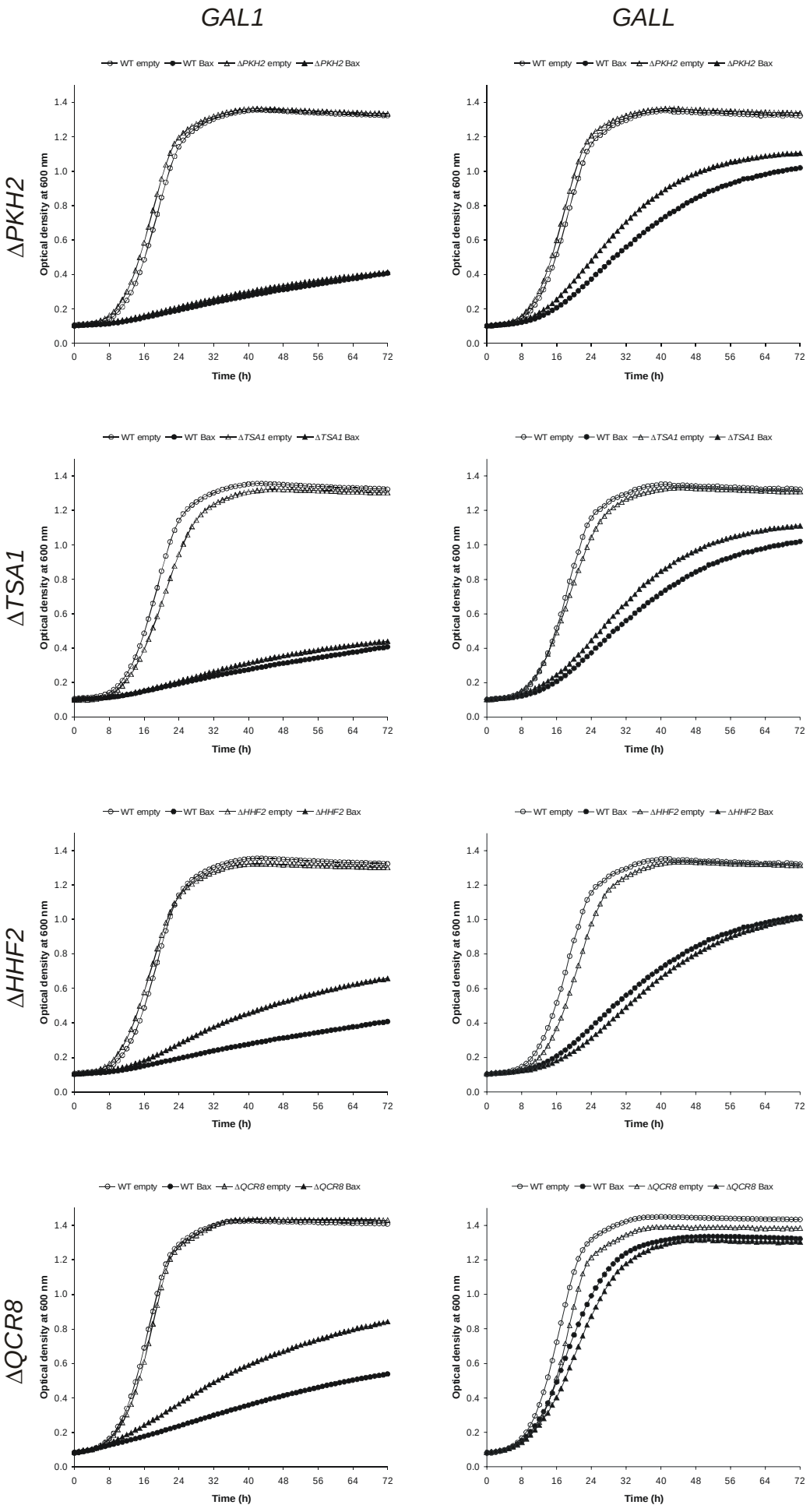
Fig. 6.8: The effect of knocking out one of the 23 genes, thought to be unique Bax-responsive, on Bax-induced growth inhibition. The cells were transformed with pRS415 (empty) or pRS415GAL1mBAX (Bax) to check growth phenotypes in the episomal *GAL1* expression system (indicated with *GAL1*). The cells were transformed with p415GALL (empty) or p415GALLmBAX (Bax) to check growth phenotypes in the episomal *GALL* expression system (indicated with *GALL*). Growth was monitored automatically over 72 hours. Growth was performed in synthetic selective galactose-containing SG-medium, except for Δ*QCR9*, Δ*QCR8*, Δ*COX12*, Δ*YDR504C* and Δ*ATP1*. These strains were grown in synthetic selective SGR-medium (2% galactose + 2% raffinose) due to their respiratory deficiency or slow growth phenotype in SG-medium. The additional carbon source is known not to affect *GAL* promoter induction. WT: BY4742, except for Δ*HTB1* (diploid BY4743). The mean of three independent experiments is shown.


GAL1
GALL
ΔYJL060W
ΔYMR107W
ΔYOL099C
ΔYOR302W
WT empty
WT Bax
ΔYJL060W empty
ΔYJL060W Bax
ΔYMR107W empty
ΔYMR107W Bax
ΔYOL099C empty
ΔYOL099C Bax
ΔYOR302W empty
ΔYOR302W Bax
Optical density at 600 nm
Time (h)


GAL1
GALL
ΔYPL201C
ΔYGR236C
ΔATP1
ΔCOX12
WT empty
WT Bax
ΔYPL201C empty
ΔYPL201C Bax
ΔYGR236C empty
ΔYGR236C Bax
ΔATP1 empty
ΔATP1 Bax
ΔCOX12 empty
ΔCOX12 Bax
Optical density at 600 nm
Time (h)


GAL1
GALL
ΔYDR504C
ΔYJL142C
ΔQCR9
ΔYGR146C
WT empty
WT Bax
ΔYDR504C empty
ΔYDR504C Bax
ΔYJL142C empty
ΔYJL142C Bax
ΔQCR9 empty
ΔQCR9 Bax
ΔYGR146C empty
ΔYGR146C Bax
Optical density at 600 nm
Time (h)


GAL1
GALL
ΔYHR138C
ΔYKL123W
ΔYLR414C
ΔHHF1
WT empty
WT Bax
ΔYHR138C empty
ΔYHR138C Bax
ΔYKL123W empty
ΔYKL123W Bax
ΔYLR414C empty
ΔYLR414C Bax
ΔHHF1 empty
ΔHHF1 Bax
Optical density at 600 nm
Time (h)


GAL1
GALL
ΔPKH2
ΔTSA1
ΔHHF2
ΔQCR8
WT empty
WT Bax
ΔPKH2 empty
ΔPKH2 Bax
ΔTSA1 empty
ΔTSA1 Bax
ΔHHF2 empty
ΔHHF2 Bax
ΔQCR8 empty
ΔQCR8 Bax
Optical density at 600 nm
Time (h)

knockout was observed in Δ*PKH2*, Δ*TSA1*, Δ*YHR138C*, Δ*YKL123W* and Δ*YLR414C* for the *GALL*-expression system. The absence of *PKH2*, *TSA1*, *YHR138C*, *YKL123W* or *YLR414C* thus only result in Bax-resistance if Bax protein accumulates to a lower level (Fig. 6.11). Bax-resistance due to gene knockout was demonstrated in Δ*HHF1*, Δ*HHF2*, and Δ*QCR8* for the *GAL1*-expression system only (Fig. 6.10). In total, 14 out of the 23 knockout strains demonstrated an alteration of their growth rate upon Bax expression in one or both of the Bax expression systems.

3.4 Independent validation of macroarray responses

Array-based expression profiles can be verified by Real-Time Quantitative PCR (Q RT-PCR) (Rajeevan *et al.*, 2001a; Rajeevan *et al.*, 2001b). Therefore, total RNA of Bax-treated, H_2O_2-treated and mock-treated samples (the same pool as for the primary gene expression profiling) was used for first-strand cDNA synthesis and subsequent Q RT-PCR with real-time SYBR Green fluorescence monitoring (Q RT-PCR). As the Q RT-PCR primers (Table 6.2) could not discriminate between first-strand cDNA and contaminating genomic DNA, RNA samples were DNAse-treated in advance.

ORF/Gene	Forward primer	Reverse primer
ADR1	AAGGATTTGCCGAAAAGTACCAA	GCCATCTTATCTAACATGTGACAATGA
ATP1	TTGCATCCAAGATTGCTAGAAAGA	GTTGGAATATAAGCGGAGACATCA
COX12	TTTCCCCAACAAAACCAAACA	AAGACCTTGCACGGAGCAA
HHF1	GCTGTCTTGAAATCCTTCTTGGAAT	GCATAAACAACATCCAAAGAAGTAACA
HHF2	GTCAGAGCCGTCTTGAAATCCT	GCATAAACAACATCCAAAGAAGTAACA
HTB1	AGGCTAGAAAGGAAACATACTCTTCTTACA	AAGAGTTCAAGATAGACATGGACTTTTG
PKH2	GAGGTGACTTATCGGAGTTGCTTACTA	GTTAGATTCGGATTTATCATCGTCTTT
QCR8	CAAGGTATTTTCCATAACGCTGTATTC	CGTTCTTCCACCAGTACCAATAAA
QCR9	CCTTTGTTTTCCAAACTGTATTTGATACT	TGCAGCTATTCGAGCCTTGAC
TSA1	GCCAGTCGGTAGAAACGTTGA	GCAGCACCTGGAGTCCAGTT
YCK1	TGGCGCTCGTTATCAACCA	CCTTATGAGAAGCAGCTAGACTTGAATTA
YDR504C	GTTGTTTACCTATTTTCCTGCCAATT	TTTCTCCCTGTGAAGTTTTCATATAGAA
YGR146C	ACATCTCCACAGAAAACGAACATTAAT	CAATCTTCGGAGCAATATAACTTATTTG
YGR236C	TGATGGATGATGATGAAGTAACGTATT	GAAAACAAAATGCAAAGAACATAAATG
YHR138C	CCAAATTTTGGACGGTCTGAA	CGGTTTAGAGCATGAACTTCTGAA
YJL060W	TATTTTGTCCTCGTTGATTTCTCTAAAG	CCAGTGAGAAATGCGAAAATCTT
YJL142C	CAACAAGCGCGTTCAATAAACT	TGGCACCAAGCCGTAATAAAAT
YKL123W	ACGTCAAAGAAACAGAGGCAAAT	ATTGTGTATGCGTAAATTGTAAAAAAAAC
YLR414C	CTCCTCGCATAACATCCAATCAT	CCAGAGCCTCATTGTTGTTGATATT
YMR107W	CAGTTCCGATGTTGAGGACGTTA	CTTAAAAACTCACCTTGCGACATGT
YOL099C	TCAGAAAACGAATTGTGCTTTGA	CGGAAGAGCCATACCTAAAGATCT
YOR302W	AGCTTATCGAACTCTCAATACACCTG	CCAGATGTGGTCAGATATGTAGTCTTG
YPL201C	ATCTACGCACGACCACAAATGT	TCCTAATAGATTCTTGCAGTGGAAAAT
YDR177W	AATATATCATCAGTGACAGGTGCCATT	AGCGCCTGTAGCGAAATCA
YDR399W	GCTGAACAGGCAAAGGCTAAA	TCTGCTTTCTTTGGTTTTTGCTT
YER148W	TGAAGCCGAAAATTGTGTTGTT	GTATATAGCTTCAAAAGCTTGGTAAATTTCT
YFL039W	ATCATTGCTCCTCCAGAAAGAAAGTA	TCGTATTCTTGTTTTGAGATCCACAT

Table 6.2: Characteristics of the primers used to validate the 23 Bax-responsive genes, selected as candidates to have a Bax-specific transcriptional response. Primer sequences are given in 5' to 3' direction.

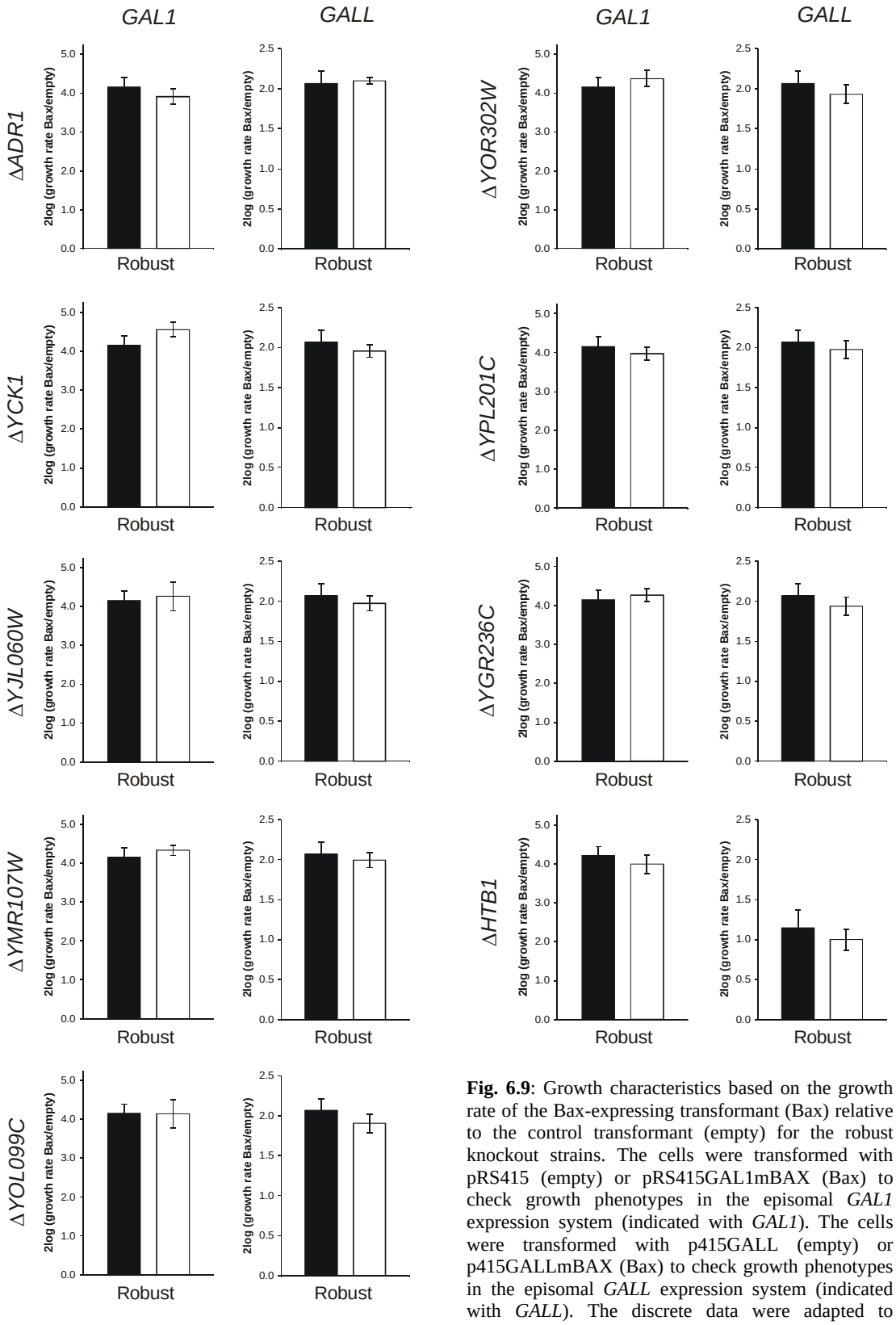


Fig. 6.9: Growth characteristics based on the growth rate of the Bax-expressing transformant (Bax) relative to the control transformant (empty) for the robust knockout strains. The cells were transformed with pRS415 (empty) or pRS415GAL1mBAX (Bax) to check growth phenotypes in the episomal *GAL1* expression system (indicated with *GAL1*). The cells were transformed with p415GALL (empty) or p415GALLmBAX (Bax) to check growth phenotypes in the episomal *GALL* expression system (indicated with *GALL*). The discrete data were adapted to continuity via growth model fitting and the growth rate was calculated as the first derivative at the growth curve's inflection point. Error bars represent SD of the mean of at least three independent experiments.

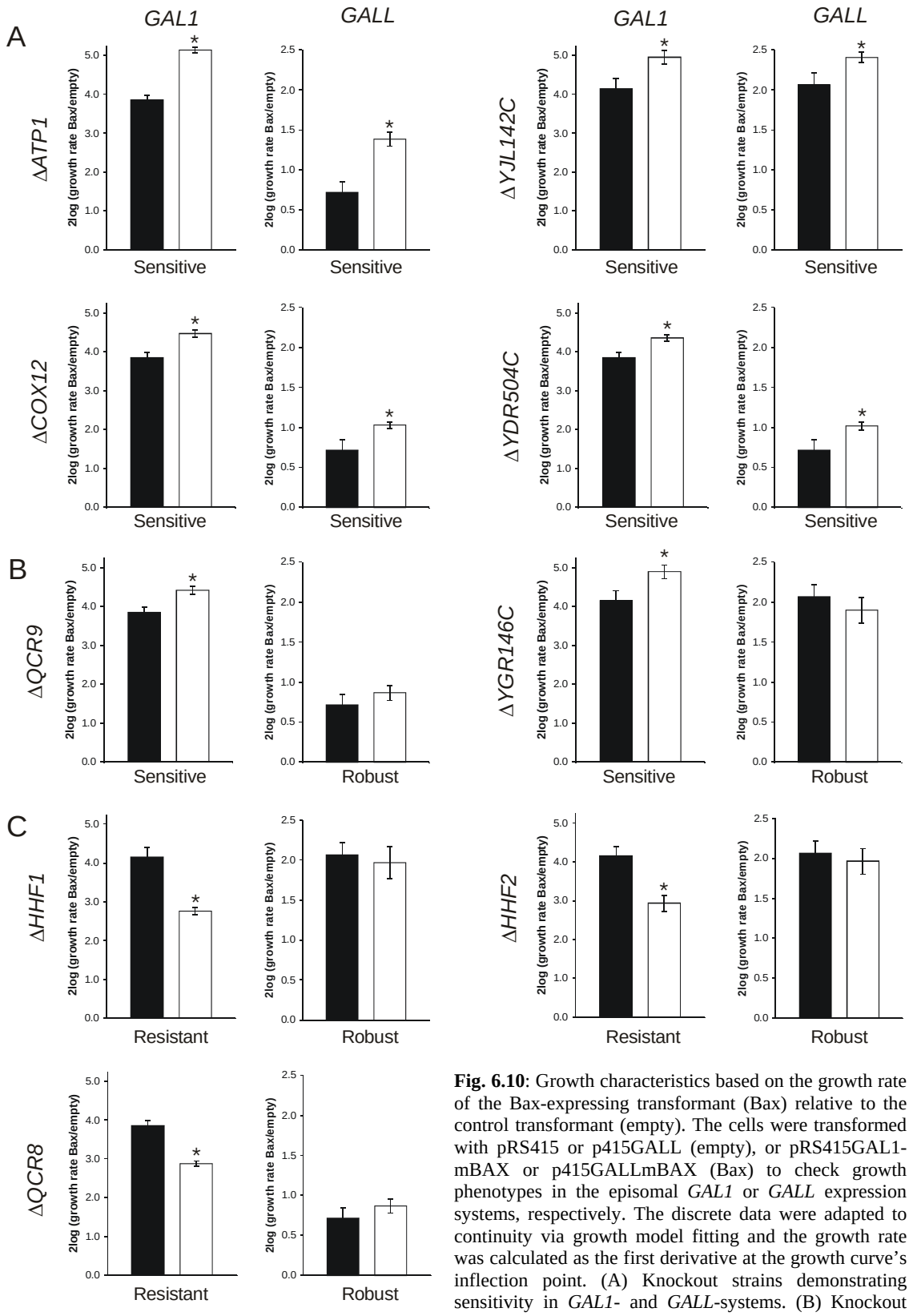


Fig. 6.10: Growth characteristics based on the growth rate of the Bax-expressing transformant (Bax) relative to the control transformant (empty). The cells were transformed with pRS415 or p415GALL (empty), or pRS415GAL1-mBAX or p415GALLmBAX (Bax) to check growth phenotypes in the episomal *GAL1* or *GALL* expression systems, respectively. The discrete data were adapted to continuity via growth model fitting and the growth rate was calculated as the first derivative at the growth curve's inflection point. (A) Knockout strains demonstrating sensitivity in *GAL1*- and *GALL*-systems. (B) Knockout strains sensitive to Bax-induced growth retardation in the *GAL1*-system only. (C) Knockout strains resistant to Bax-induced growth retardation in the *GAL1*-system only. Error bars represent SD of the mean of at least three independent experiments. (*): Significant difference based on the Wilcoxon Rank sum test (α=0.05).

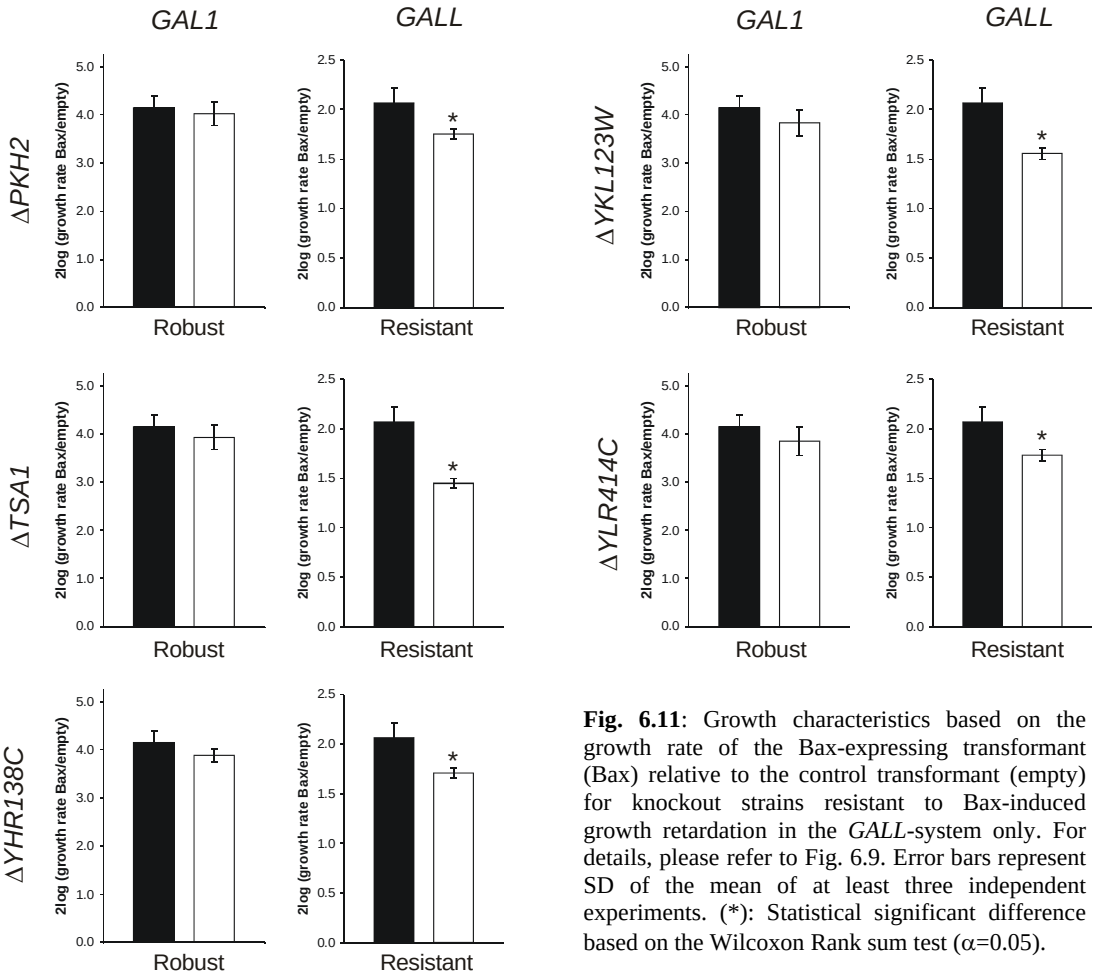


Fig. 6.11: Growth characteristics based on the growth rate of the Bax-expressing transformant (Bax) relative to the control transformant (empty) for knockout strains resistant to Bax-induced growth retardation in the *GALL*-system only. For details, please refer to Fig. 6.9. Error bars represent SD of the mean of at least three independent experiments. (*): Statistical significant difference based on the Wilcoxon Rank sum test (α=0.05).

The expression level of the 23 Bax-responsive genes selected to have a Bax-specific transcriptional response was determined in the control, Bax-induced and H_2O_2-treated samples relative to the control genes *YER148W* and *YDR177W*, for which the transcript levels remained stable upon Bax-expression or H_2O_2 addition (Fig. 6.12). It has been proposed to use indeed at least two types of control genes as internal standards (Dent *et al.*, 1997).

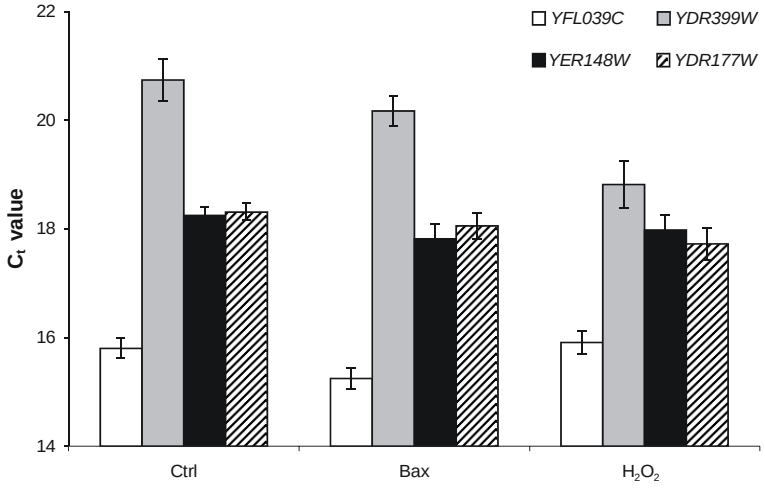


Fig. 6.12: Selection of internal standards (reference genes) for which transcript levels do not change due to Bax expression or H_2O_2 addition. Transcript levels were quantified based on the threshold cycle (C_t value) at which a statistically significant increase in fluorescence was first detected. Two control genes were chosen (*YDR177W* and *YER148W*).

It was observed that only 3 out of the 23 candidates (*YJL060W*, *YLR414C* and *YGR146C*) (13%) could be validated as being Bax-responsive. These three Bax-responsive genes were also validated to have a Bax-specific transcriptional response (Fig. 6.13). The high false positive rate of the GeneFilter® experiment may be attributed to cross-hybridisation, innate experimental variation or intrinsic macroarray errors. Particularly, clone sets used to produce Invitrogen macroarrays were demonstrated to have a 12% well error rate (Halgren *et al.*, 2001).

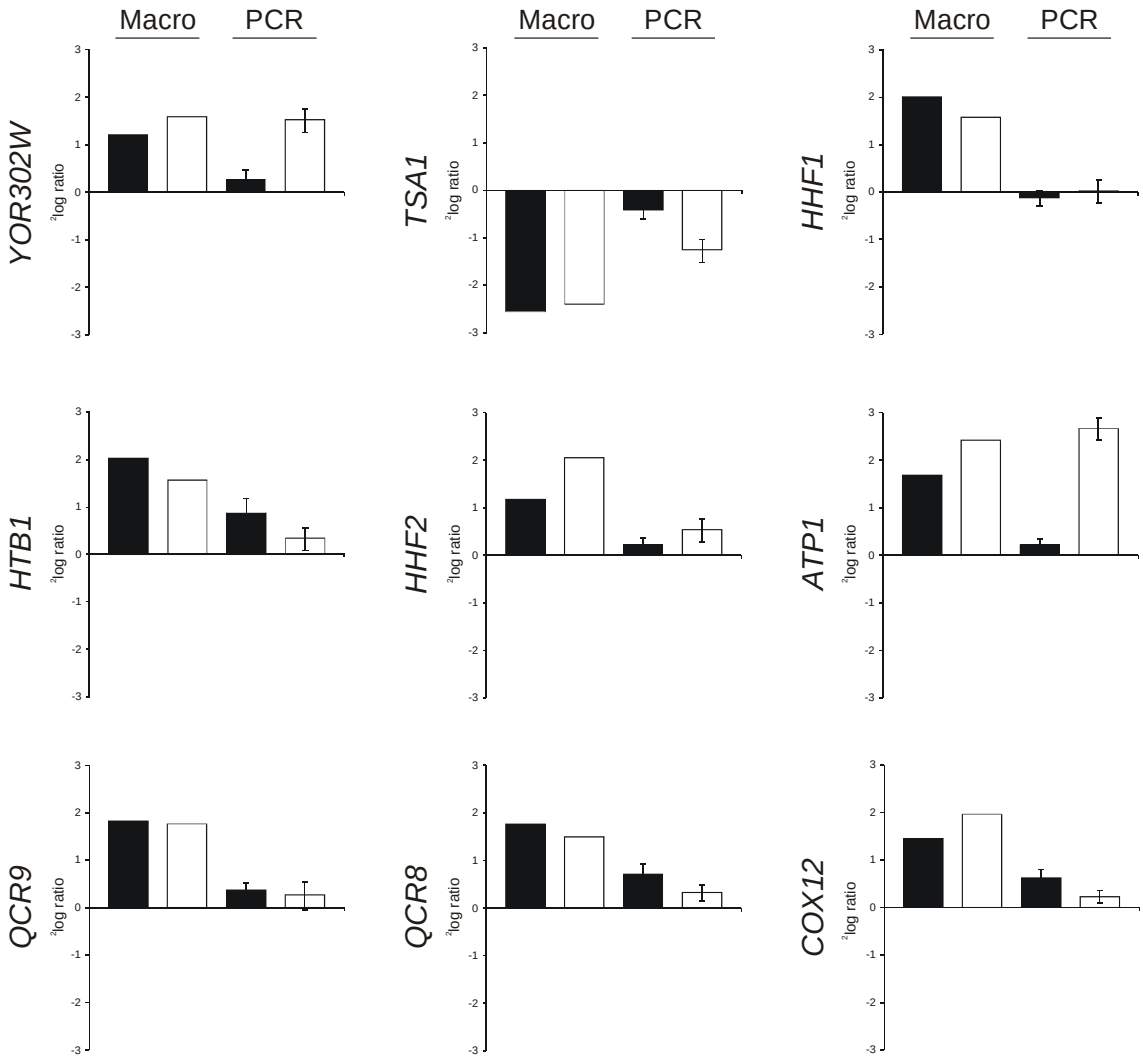
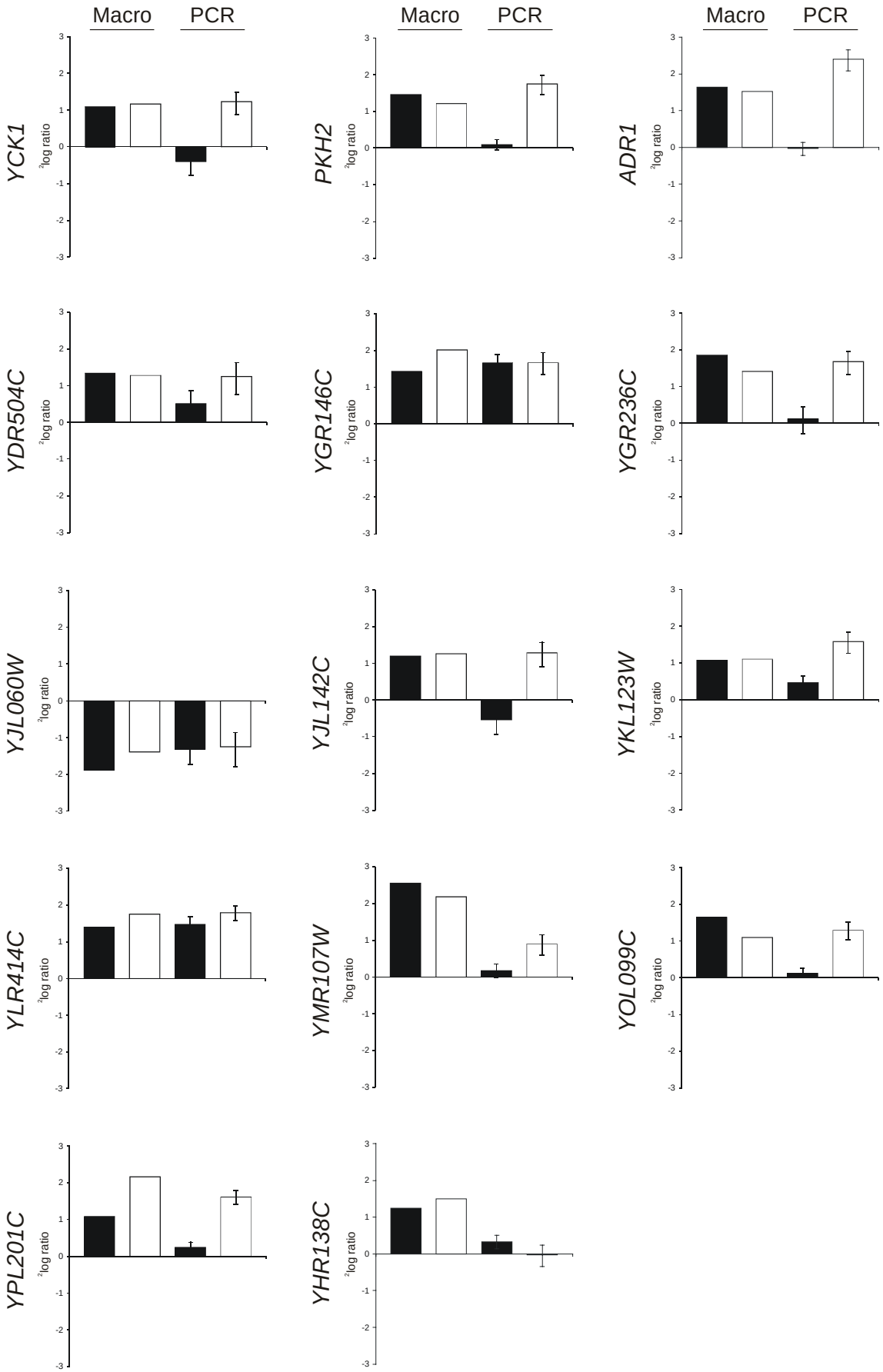


Fig. 6.13: Independent validation of the macroarray results by quantitative real-time PCR. The 23 Bax-responsive genes, thought to have a unique Bax-transcriptional response, were validated using gene-specific primers. Transcriptional levels were normalised to *YER148W* and *YDR177W*, for which mRNA stayed stable upon Bax expression or H_2O_2 treatment. The ratio's (Bax/Ctrl) (black bars) and (Bax/H_2O_2) (white bars) were calculated and depicted as their ^{2}log value. Selection criteria were $|^2\log(\text{Bax/Ctrl})| \geq 1.0$ (Bax-responsive) and $|^2\log(\text{Bax/H}_2\text{O}_2)| \geq 1$ (Bax-specific). Macro: macroarray results; PCR: Q RT-PCR validation results. Error bars represent SD of the mean of three independent experiments.


Macro
PCR
YCK1
PKH2
ADR1
YDR504C
YGR146C
YGR236C
YJL060W
YJL142C
YKL123W
YLR414C
YMR107W
YOL099C
YPL201C
YHR138C
2log ratio

Out of the three genes validated to have a Bax-unique transcriptional response, the knockout strains of two of them (Δ*YLR414C* and Δ*YGR146C*) were demonstrated to have an effect on the Bax-induced growth retardation (See Fig. 6.8, Fig. 6.10B, and Fig. 6.11). Δ*YLR414C* was observed to result in some resistance to Bax-induced growth retardation in the *GALL* Bax-expression system, while Δ*YGR146C* demonstrated some sensitivity to Bax-induced growth retardation in the *GAL1* Bax expression system. Thus, *YGR146C* and *YLR141C* interfere with the Bax-induced growth retardation, influencing directly or indirectly the cell cycle disturbing function of the Bax protein.

3.5 Cell death assays for extending growth phenotypes

Bax expression in *S. cerevisiae* induces growth arrest, which may or may not be followed by mortality (cell death) (Greenhalf *et al.*, 1996). To determine whether *YLR414C* or *YGR146C* also influenced Bax-induced cell death, clonogenic survival assays were performed. Upon transformation of the plasmids pRS415 and pRS415GAL1mBAX to Δ*YLR414C*, transformants were analysed to determine whether the resistance to Bax-induced growth retardation was linked to a decreased cell death rate. The plasmids p415GALL and p415GALLmBAX were transformed to Δ*YGR146C* and for the resulting transformants the potential link between a sensitivity to Bax-induced growth retardation and an increased Bax-induced cell death rate was determined (Fig. 6.14). For the clonogenic survival assay, cells were resuspended in selective synthetic SG-medium and a fixed amount of cells was taken out from these cultures regularly and plated on selective synthetic SD-plates. We observed that neither *YGR146C*, nor *YLR414C* had any influence on cell death induced upon Bax expression.

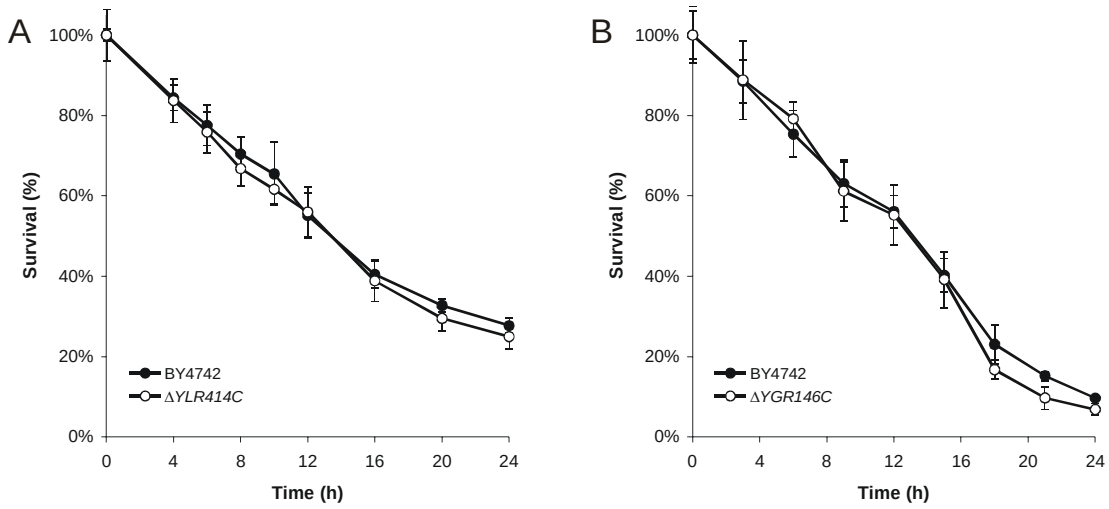


Fig. 6.14: The absence of *YLR414C* or *YGR146C* does not influence the Bax-induced cell death rate. Clonogenic survival was determined by recovering cells at various times from galactose-based medium and plating 1000 cells on glucose-based semisolid medium. For each time point, the percentage of survival was calculated by dividing the number of colonies surviving the toxic treatment (Bax) by the number of colonies appearing after mock-treatment (empty). (A) Cells of BY4742 and Δ*YLR414C* were transformed with the plasmids p415GALL (empty) and p415GALLmBAX (Bax), and recultured in SG-medium to induce the *GALL* promoter. (B) Cells of BY4742 and Δ*YGR146C* were transformed with the plasmids pRS415 (empty) and pRS415GAL1mBAX (Bax), and recultured in SG-medium to induce the *GAL1* promoter. Error bars respresent SD of the mean of two independent experiments.

4. Repeat of the macroarray experiment

To verify whether the high false positive rate of a single macroarray experiment could be diminished by a repeat, we performed the same experiment with another set of GeneFilters® but with the same total RNA (technical repeat). That is, we determined (i) genes responsive to a 1-hour Bax-induction, (ii) genes responsive to a 1-hour treatment with 0.1 mM H_2O_2, and (iii) Bax-responsive genes that have a unique Bax-triggered transcriptional response. We used the same criteria for defining responsive genes and Bax-specific transcriptional responses, as described above.

The expression value of each gene in the control and Bax-induced samples was compared in a scatter plot with an overlay of the first and repeat experiment (Fig. 6. 15A). Similarly, the expression values in the control and H_2O_2-treated samples (Fig. 6.15B) and the expression values in the Bax-induced and H_2O_2-treated samples were overlayed for both experiments (Fig 6.15C). We observed the first and repeat experiment to be different in signal intensities and signal distribution.

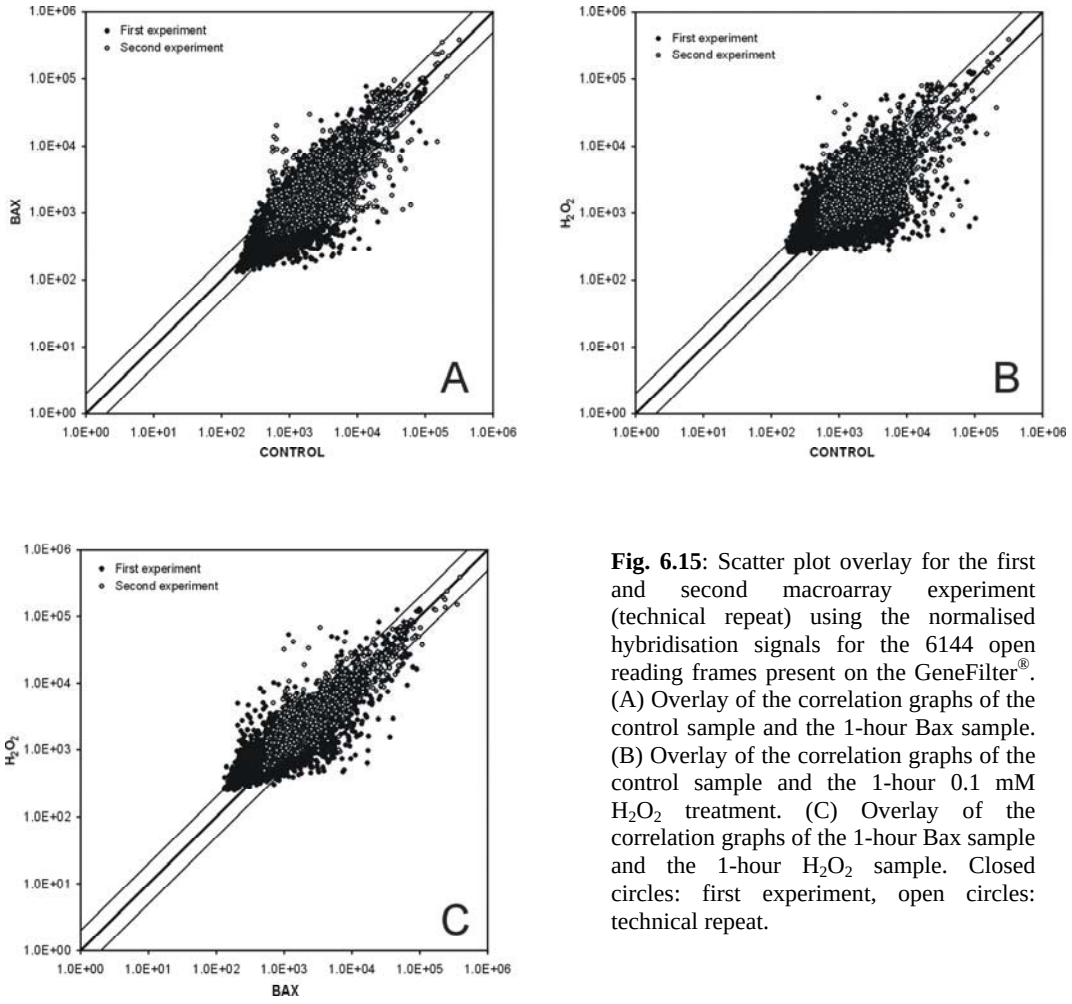


Fig. 6.15: Scatter plot overlay for the first and second macroarray experiment (technical repeat) using the normalised hybridisation signals for the 6144 open reading frames present on the GeneFilter®. (A) Overlay of the correlation graphs of the control sample and the 1-hour Bax sample. (B) Overlay of the correlation graphs of the control sample and the 1-hour 0.1 mM H_2O_2 treatment. (C) Overlay of the correlation graphs of the 1-hour Bax sample and the 1-hour H_2O_2 sample. Closed circles: first experiment, open circles: technical repeat.

Next, the Bax-responsive and H_2O_2-responsive genes genes were determined, now taking into account the results of both data sets. We observed 44 and 28 genes to be Bax-responsive and H_2O_2-

responsive, respectively (Table 6.3 and Table 6.4). Only 8 genes (*YLR294C*, *YOL106W*, *YLR311C*, *TAF61*, *YDR366W*, *YHR145C*, *YKL066W* and *HXT6*) were part of a shared response.

ORF	Gene	Description	Bax/Ctrl
Carbohydrate metabolism			
YBR149W	*ARA1*	Subunit of $NADP^+$-dependent D-arabinose dehydrogenase	0.29
YJR009C	*TDH2*	Glyceraldehyde 3-phosphate dehydrogenase 2	0.29
YOR374W	*ALD4*	Aldehyde dehydrogenase	0.29
YPL061W	*ALD6*	Aldehyde dehydrogenase	0.36
Cell cycle			
YBR133C	*HSL7*	Negative regulator of Swe1 kinase	2.53
Cell stress			
YKL097W-A	*CWP2*	Mannoprotein of the cell wall	0.25
YML100W	*TSL1*	α,α trehalose-phosphate synthase	0.33
YOR031W	*CRS5*	Metallothionein-like protein	6.33
Cell wall maintenance			
YLR110C	*CCW12*	Cell wall mannoprotein	0.08
YNL161W	*CBK1*	Cell wall biosynthesis Ser/Thr kinase	3.79
Energy generation			
YBR039W	*ATP3*	Gamma subunit of the F_1-ATP synthase	0.43
YGR183C	*QCR9*	Subunit 9 of the ubiquinol cytochrome c oxidoreductase complex	3.14
YHR001W-A	*QCR10*	Subunit 10 of the ubiquinol cytochrome c oxidoreductase complex	2.50
YJR121W	*ATP2*	Beta subunit of the F_1-ATP synthase	0.46
YLR395C	*COX8*	Subunit VIII of cytochrome c oxidase	0.29
Protein modification			
YEL036C	*ANP1*	Required for protein glycosylation in the Golgi	2.42
Signal transduction			
YPL089C	*RLM1*	May function downstream of *MPK1* MAP-kinase pathway	2.71
Small molecule synthesis			
YOL143C	*RIB4*	6,7-dimethyl-8-ribityllumazine synthase (DMRL synthase)	0.36
Small molecule transport			
YDR342C	*HXT7*	High-affinity glucose transporter of the major facilitator superfamily	0.27
YDR343C	*HXT6*	High-affinity glucose transporter of the major facilitator superfamily	0.15
YMR011W	*HXT2*	High-affinity glucose transporter of the major facilitator superfamily	0.45
Transcription factor			
YDR145W	*TAF61*	Component of the TAF(II) complex	2.90
YMR043W	*MCM1*	Transcription factor of the MADS box family	2.49
Unknown			
YBR214W	*SDS24*	Protein of unknown function	0.20
YDL124W	*YDL124W*	Protein of unknown function	0.19
YDR134C	*YDR134C*	Protein of unknown function	0.11
YDR154C	*YDR154C*	Protein of unknown function	2.63
YDR366C	*YDR366C*	Protein of unknown function	2.86
YGR182C	*YGR182C*	Protein of unknown function	3.79
YGR236C	*SPG1*	Protein of unknown function	3.50
YHL021C	*FMP12*	Protein found in the mitochondrial proteome	0.27
YHR145C	*YHR145C*	Protein of unknown function	2.78
YIL057C	*YIL057C*	Protein of unknown function	0.25
YJR023C	*YJR023C*	Protein required for cell viability	3.03
YKL054C	*VID31*	Protein of unknown function	2.22
YKL066W	*YKL066W*	Protein of unknown function	2.66
YLR294C	*YLR294C*	Protein of unknown function	4.79
YLR311C	*YLR311C*	Protein of unknown function	3.08
YMR081C	*ISF1*	Protein of unknown function	0.46
YNL134C	*YNL134C*	Protein of unknown function	0.26
YOL048C	*YOL048C*	Protein of unknown function	0.27
YOL053C-A	*DDR2*	Multistress response protein	0.32
YOL106W	*YOL106W*	Protein of unknown function	3.13
YOR121C	*YOR121C*	Protein of unknown function	2.75

Table 6.3: Genes responsive to a 1-hour Bax induction as was determined via a repeated macroarray experiment. The average of both experiments is represented by the Bax/Ctrl ratio. In total, 44 Bax-responsive genes were identified.

ORF	Gene	Description	H_2O_2/Ctrl
Cell stress			
YDR171W	*HSP42*	Heat shock protein, restoration of the cytoskeleton during stress	0.47
YGR209C	*TRX2*	Thioredoxin 2	3.25
YKR066C	*CCP1*	Cytochrome-c peroxidase	4.62
Energy generation			
YMR256C	*COX7*	Subunit VII of cytochrome c oxidase	3.81
Protein degradation			
YBR286W	*APE3*	Vacuolar aminopeptidase Y	0.40
YDL147W	*RPN5*	Non-ATPase subunit of 26S proteasome complex	3.05
RNA modification			
YER112W	*LSM4*	U6 snRNA associated protein of the Sm-like group	5.71
Signal transduction			
YDL079C	*MRK1*	Ser/Thr protein kinase	0.49
Small molecule transport			
YCR010C	*ADY2*	member of the YaaH family of putative transporters	0.40
YDR343C	*HXT6*	High-affinity glucose transporter of the major facilitator superfamily	7.38
Transcription			
YDR253C	*MET32*	Transcriptional regulation of methionine metabolism	8.96
YDR145W	*TAF61*	Component of TAF(II) complex	2.48
Unknown			
YCR097WA	*YCR097WA*	Protein of unknown function	2.93
YDL027C	*YDL027C*	Protein of unknown function	2.36
YDR133C	*YDR133C*	Protein of unknown function	0.25
YDR366C	*YDR366C*	Protein of unknown function	3.54
YGR086C	*YGR086C*	Protein of unknown function	0.37
YGR106C	*YGR146C*	Protein of unknown function	3.50
YHR087W	*YHR087W*	Protein of unknown function	0.29
YHR145C	*YHR145C*	Protein of unknown function	3.64
YKL066W	*YKL066W*	Protein of unknown function	5.03
YKR011C	*TOS5*	Protein of unknown function	2.65
YLR294C	*YLR294C*	Protein of unknown function	7.51
YLR311C	*YLR311C*	Protein of unknown function	4.08
YML132W	*COS3*	Member of the COS family of subtelomerically-encoded proteins	2.21
YOL106W	*YOL106W*	Protein of unknown function	2.98
YOR193W	*YOR193W*	Protein of unknown function	2.23
YPL201C	*YPL201C*	Protein of unknown function	0.31

Table 6.4: Genes responsive to a 1-hour H_2O_2 treatment as was determined via a repeated macroarray experiment. The average of both experiments is represented by the H_2O_2/Ctrl ratio. In total, 28 H_2O_2-responsive genes were identified.

To make sure that errors innate to the macroarray analysis procedure were not responsible for the impossibility to discover genes interfering with Bax-induced cell death, we also analysed the macroarray signals via ArrayAn, the in-house analysis software developed and optimised for macroarray experiments (Boonefaes *et al.*, in preparation). We essentially obtained the same results as with Pathways™, the analysis software provided by the manufacturer (Invitrogen).

Out of the 44 Bax-responsive genes, only *QCR9* and *YGR236C* were observed as candidates for having a Bax-specific transcriptonal response.

Macroarray profiling		Macroarray results		Q RT-PCR results	
ORF	Gene	Bax/Ctrl	H_2O_2/Ctrl	Bax/Ctrl	H_2O_2/Ctrl
YGR236C	*SPG1*	3.50	1.24	1.09	0.34
YGR183C	*QCR9*	3.14	1.23	1.30	1.08

However, these 2 genes could not be validated as having a Bax-specific transcriptional response (See also Fig. 6.13). Thus, although an experiment repeat compressed the list of responsive genes (from 104 to 44 for Bax and from 250 to 28 for H_2O_2), it did not enable to discover the effects of *YLR414C* and *YGR146C* on Bax –induced growth retardation.

5. References

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Chapter 7
Search for unique Bax-responsive genes (II)

The transcriptional responses of *S. cerevisiae* to Bax expression may contain a Bax-specific part, leading to cellular oxidative stress, and a general pathway of oxidative stress-induced cell death. In the present chapter, a microarray profiling was used to compare the transcriptional responses to Bax protein expression with the responses elicited by an oxidative treatment of the same final toxicity. The subtraction of the transcriptional effects of the Bax protein with the effects caused by the oxidative treatment was performed to determine whether Bax-associated responses are different from those elicited by oxidative stress, and if so, whether these differences could explain how Bax expression actually generates a cellular oxidative stress.

Part of this chapter has been included in the manuscript "Expression profiling of *S. cerevisiae* cells after Bax expression and oxidative stress reveals that Old Yellow Enzyme interferes with Bax toxicity", which was submitted to FEMS Yeast Research for publication.

1. Expression profiles after Bax and H_2O_2 triggers

To compare Bax-induced with oxidative stress-induced cell death, both triggers were optimised for comparable mortality under the same growth conditions (See Chapter 6). Total RNA extracted from Bax-treated, H_2O_2-treated or control cells (the same pool as for the macroarray gene expression profiling) was used to prepare cDNA probes labelled with Cy3-dCTP (red) or Cy5-dCTP (green). Typically, a labelling density of 44 pmol CyDye/µg was obtained, corresponding to an incorporation rate of one CyDye molecule every 70 nucleotides. Probes were hybridised to the yeast microarray of 6307 genes spotted as representative oligos. The experiment had a triangular loop design comprising control (green) versus Bax (red), Bax (green) versus H_2O_2 (red), and H_2O_2 (green) versus control (red). Digital images of the fluorescent signals from the hybridised slides were normalised for dye intensity differences by Lowess (LOcally WEighted Scatterplot Smoother) fitting (Yang *et al.*, 2002) (Fig. 7.1). Between-slide normalization was performed via MARAN modelling (Engelen *et al.*, 2003) (R^2=0.93). Inspection of the signals revealed that 75 genes had mRNA steady-state levels that were clearly above background and changed at least 2-fold after 1 hour of Bax induction (Table 7.1).

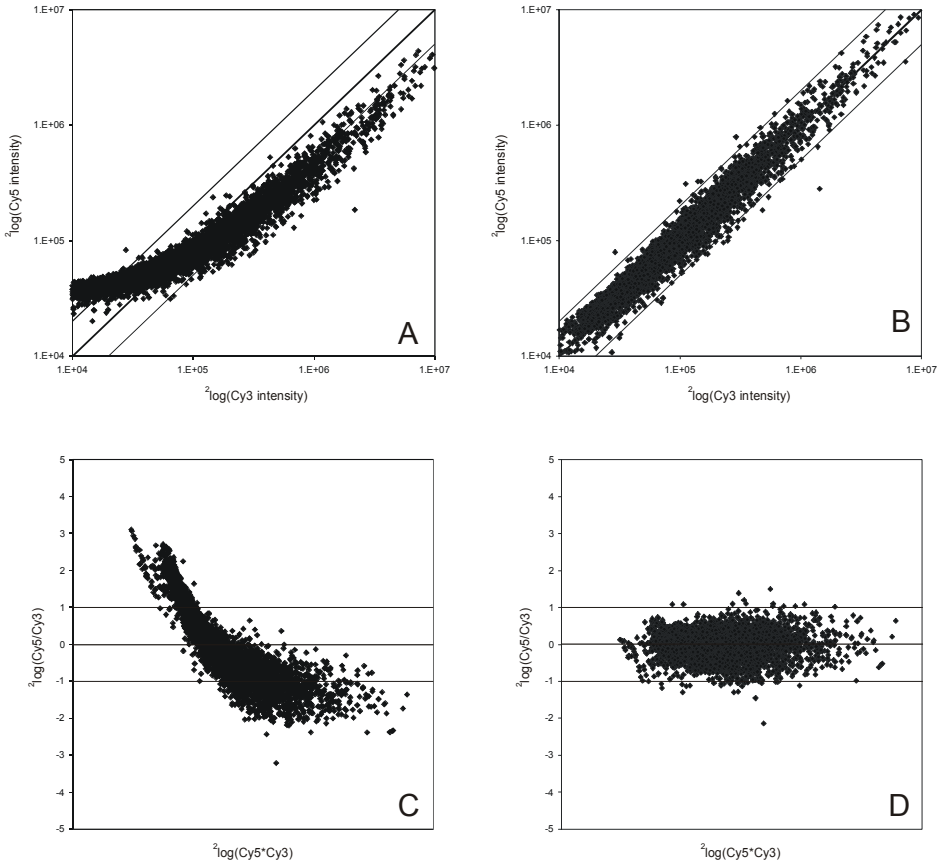

Fig. 7.1: Data normalisation via Lowess fitting for the Bax (Cy3) versus Control (Cy5) microarray hybridisation. (A) Log-log plot of the Cy3 and Cy5 signal intensities as a visualisation of the fold-differences in expression. The central diagonal represents no differentiation; both parallels represent the limits for twofold up- and down-regulation. The measured Cy5 intensity is less than the measured Cy3 intensity. (B) After Lowess fitting, most of the datapoints are within the fold-limit lines. (C) Plot of the log ratios ^{2}log (Cy5/Cy3) against log intensities ^{2}log(Cy5*Cy3) for each element of the microarray reveals systematic intensity-dependent effects in the measured log ratio values. (D) After Lowess fitting, non-linear trends are alleviated.

ORF	Gene	Description	Bax/Ctrl
Carbohydrate metabolism			
YNL117W	*MLS1*	Malate synthase (glyoxylate cycle)	2.09
YBR218C	*PYC2*	Gluconeogenic Pyruvate carboxylase, NADPH regeneration	0.48
YMR169C	*ALD3*	Aldehyde dehydrogenase, induced by oxidative shock	0.40
Cell polarity			
YKR055W	*RHO4*	Ras homolog involved in actin filament organisation	0.37
Degradation			
YMR297W	*PRC1*	Carboxypeptidase Y (Proteinase C) for vacuolar protein catabolism	2.29
YLL039C	*UBI4*	Ubiquitin, transcriptionally induced in stress conditions	2.10
YBR208C	*DUR80*	Urea amidolyase, degrades urea to CO_2 and NH_3	0.50
YBR058C	*UBP14*	Ubiquitin-specific protease, negative regulator of gluconeogenesis	0.49
YLR351C	*NIT3*	Hydrolase acting on carbon-nitrogen (no peptide) bonds	0.48
YGR020C	*VMA7*	Subunit f of vacuolar H^+-ATPase, for vacuolar acidification	0.46
DNA synthesis			
YNL102W	*POL1*	DNA polymerase I alpha subunit p180	2.12
YMR234W	*RNH1*	Ribonuclease H1, involved in DNA replication	2.12
YJR068W	*RFC2*	Replication factor C, involved in cell cycle checkpoint	2.10
Energy generation			
YBR039W	*ATP3*	Gamma subunit of the F_1 sector of mitochondrial F_1F_0 ATP synthase	2.18
YPL078C	*ATP4*	Subunit b of the stator stalk of mitochondrial F_1F_0 ATP synthase	2.16
YGL256W	*ADH4*	Alcohol dehydrogenase type IV for glucose fermentation	2.00
YGR008C	*STF2*	Stabilizing factor of the F_1F_0 ATP synthase	0.49
YEL039C	*CYC7*	Cytochrome c isoform 2 involved in mitochondrial electron transport	0.48
Intracellular transport			
YPL094C	*SEC62*	Membrane component of ER protein cotranslocation apparatus	2.16
YBL102W	*SFT2*	Protein involved in Golgi to endosome transport	2.06
YMR195W	*ICY1*	Protein involved in nuclear transport	0.47
YPL250C	*ICY2*	Protein involved in nuclear transport	0.46
YJR058C	*APS2*	Protein involved in vesicle-mediated transport	0.46
YNL036W	*NCE103*	Endogenous substrate for nonclassical export	0.44
YGR142W	*BTN2*	Protein involved in intracellular protein transport	0.40
YER103W	*SSA4*	Membrane component of ER protein cotranslocation apparatus	0.32
Lipid metabolism			
YGR157W	*CHO2*	Phosphatidyl-ethanolamine N-methyltransferase	0.47
YPL171C	*OYE3*	NADPH dehydrogenase, possibly involved in sterol metabolism	0.45
Protein modifications			
YER123W	*YCK3*	Protein involved in amino acid phosphorylation	2.27
YKL035W	*UGP1*	UTP-glucose-1-phosphate uridylyltransferase for glycosylation	0.47
YGR161C	*RTS3*	Protein involved in amino acid dephosphorylation	0.36
Protein synthesis			
YOL077C	*BRX1*	Essential protein required for biogenesis of the 60S ribosomal subunit	2.55
YOL040C	*RPS15*	Protein component of the 40S ribosomal subunit	0.50
YJL189W	*RPL39*	Protein component of the 60S ribosomal subunit	0.45
YER035W	*EDC2*	RNA-binding protein, activates mRNA decapping	0.39
Small molecule transport			
YDR342C	*HXT7*	Glucose, fructose and mannose transporter activity	2.06
YDR040C	*ENA1*	P-type ATPase sodium pump for sodium ion transport	0.50
YMR011W	*HXT2*	Glucose, fructose and mannose transporter activity	0.38
YJR049C	*UTR1*	Protein involved in iron homeostasis	0.37
YHR094C	*HXT1*	Glucose, fructose and mannose transporter activity	0.34
Stress response			
YHR106W	*TRR2*	Mitochondrial thioredoxin reductase, response to oxidative stress	0.46
YDR258C	*HSP78*	Protein involved in folding of some mitochondrial proteins	0.46
YBR203W	*COS111*	Protein required for resistance to the antifungal ciclopirox olamine	0.45
YKR013W	*PRY2*	Protein similar to pathogen related proteins	0.41
YPL152W	*RRD2*	Protein responding to osmotic stress	0.38
Transcription factor			
YMR270C	*RRN9*	RNA polymerase I upstream activator subunit	2.15
YGL209W	*MIG2*	Involved in repression of invertase expression by high glucose	0.47
YFL021W	*GAT1*	Transcriptional activator involved in nitrogen catabolite repression	0.43
YGL035C	*MIG1*	Transcription factor involved in glucose repression	0.42
YML099C	*ARG81*	Regulator of arginine-responsive genes	0.41

Table 7.1: Classification of the genes responsive to a 1-hour Bax induction, as was determined via microarray transcriptional profiling. Data are represented by the mean of two experiments. In total, 75 Bax-responsive genes were identified.

ORF	Gene	Description	Bax/Ctrl
Unknown			
YPR002C-A		Protein of unknown function	2.80
YDR525W-A	*SNA2*	Protein of unknown function	2.65
YDR396W		Protein required for cell viability	2.63
YKL102C		Protein of unknown function	2.39
YMR046W-A		Protein of unknown function	2.32
YGL029W	*CGR1*	May contribute to compartmentalization of nucleolar constituents	2.25
YNL156C	*NSG2*	Potential homolog of mammalian Insig 1	2.16
YDR034C-A		Protein of unknown function	2.11
YMR118C		Protein of unknown function	2.10
YBL043W	*ECM13*	Protein of unknown function	2.09
YGR122C-A		Protein of unknown function	2.07
YDR051C		Protein of unknown function	2.06
YBR056W		Protein of unknown function	2.04
YPL282C		Protein of unknown function	2.03
YDR033W	*MRH1*	Membrane protein related to Hsp30p	2.00
YPR091C		Protein of unknown function	0.50
YNL134C		Protein with alcohol dehydrogenase ($NADP^+$) activity	0.50
YIL127C		Protein of unknown function	0.49
YLR108C		Protein of unknown function	0.49
YKR075C		Protein of unknown function; expression regulated by Rgt1	0.48
YDR070C	*FMP16*	Found in the mitochondrial proteome	0.46
YFL062W	*COS4*	Subtelomerically-encoded protein of unknown function	0.45
YBR285W		Protein of unknown function	0.44
YER085C		Protein of unknown function	0.43
YNR034W-A		Protein of unknown function	0.42

Next, the changes in normalised spot intensity due to Bax expression and due to H_2O_2 treatment were compared, focusing on the 75 Bax-responsive genes. Out of these genes, we could dissect 9 genes for which the mRNA expression level changed to a higher degree (at least two-fold) with Bax expression than with H_2O_2 treatment (*HXT7, PRC1*, *MLS1*, *CYC7*, *STF2, BTN2*, *YIL127C*, *OYE3*, *ICY2*). We also identified one gene for which the transcriptional level was downregulated by Bax but upregulated by H_2O_2 (*RPL39*) (Table 7.2). These 10 genes were designated as candidates to have a Bax-specific transcriptional response. Remarkably, none of these 10 genes were revealed by a comparable macroarray analysis (See Table 6.1, Chapter 6).

Microarray profiling		Microarray results		Q RT-PCR results	
ORF	Gene	Bax/Ctrl	H_2O_2/Ctrl	Bax/Ctrl	H_2O_2/Ctrl
YDR342C	*HXT7*	2.06	0.68	1.02	0.37
YMR297W	*PRC1*	2.29	0.75	1.81	0.56
YNL117W	***MLS1***	2.09	0.82	**5.75**	1.18
YEL039C	*CYC7*	0.48	1.15	0.79	1.25
YGR008C	*STF2*	0.49	1.09	0.88	0.22
YGR142W	***BTN2***	0.40	1.22	**0.49**	1.45
YIL127C		0.49	1.20	1.89	3.39
YJL189W	*RPL39*	0.45	2.31	1.75	2.48
YPL171C	***OYE3***	0.45	1.85	**0.37**	1.50
YPL250C	***ICY2***	0.46	0.94	**0.42**	2.02

Table 7.2: Bax-responsive genes selected to have a Bax-specific transcriptional response via microarray profiling. Selection was based on (i) a Bax-associated response at least two-fold up/down compared to Control (Ctrl), and (iia) a Bax-response at least two-fold more manifest up/down compared to the up/down H_2O_2 response or (iib) a Bax response up/down with a different tendency compared with the H_2O_2 response (down/up). The profiling results are compared with the independent Q RT-PCR data. Out of the 10 candidates, 4 were finally identified as having a Bax-specific transcriptional response. The mean of at least two independent experiments is shown.

The results of the microarray analysis were verified by a real-time PCR procedure. Total RNA of the Bax-treated, H_2O_2-treated and control samples (the same pool as for the primary gene expression profiling) was converted to first-strand cDNA, followed by quantitative PCR with real-time SYBR Green fluorescence monitoring (Q RT-PCR) using the primers depicted in Table 7.3.

ORF/Gene	Forward primer	Reverse primer
BTN2	CCAGTGAGCTATTATCCAGAATGTAAA	GCATACCTCAAAGGTTCTGAACAA
CYC7	AAACGAGGTGTCAGCAGTGTCATA	CTTTACCTGACCTGAATGTCTACCAA
HXT7	GGCTGTTGGTTTGAGTGACTCTTT	CGACCATATCTCTCAACAACGTAAA
ICY2	GAGTCAGAATGCACGCACACTAA	GCGGCGTTGCCGTTAA
MLS1	GAATGCTCCTGTGAACACCTATATGA	TGATCGCGTCGTAGAGATTAACTT
OYE3	CGCCGATGGTGTAGAAATTCAT	CGATCGTTCCGCCGTATT
PRC1	ACGTTTACGATATCAGGAAGGATTGT	AGCTTCTTTGACGTAGTCCTGGTT
RPL39	GGCTGCTCAAAAGTCTTTCAGAAT	TCCAGTTTCTTCTCTTAGCGTTGTAA
STF2	GGTAAGCCAGGCGATGAGATT	TTGTTCATGCGATTGCAAATTT
YIL127C	AGACAGGAGTAAAAGAAGAAGGTTCAAA	CCGGTGTCAGACCAGGATATCTAT
YDR177W	AATATATCATCAGTGACAGGTGCCATT	AGCGCCTGTAGCGAAATCA
YER148W	TGAAGCCGAAAATTGTGTTGTT	GTATATAGCTTCAAAAGCTTGGTAAATTTCT

Table 7.3: Characteristics of the primers used to verify the Bax-specific transcriptional responses of the 10 candidate genes. Primer sequences are given in 5' to 3' direction.

The level of expression of the 10 Bax-specific candidate genes was determined in the control, Bax-induced and H_2O_2-treated samples relative to the genes *YER148W* and *YDR177W*, for which the transcript levels remained stable upon Bax-expression or H_2O_2 addition (See Chapter 6). Four of the 10 candidate genes (*MLS1*, *OYE3*, *ICY2* and *BTN2*) were confirmed to have a Bax-specific transcriptional response (Table 7.2) (Fig. 7.2).

2. Phenotypic effect of the Bax-specific genes

2.1 Respiratory capacity

To determine whether the four genes, validated to have a Bax-specific transcriptional response, could have an effect on mitochondrial physiology, the respiratory competence of the corresponding knockout strains was analysed. The 4 knockout strains were ordered from Euroscarf, a European consortium distributing yeast strains generated during various yeast functional analysis projects. All were available as haploid ablations. The strains were grown in rich YPD-medium, washed thoroughly, resuspended in sterile dH_2O, and spotted on semi-solid medium containing either 2% glucose (YPD) or 3% glycerol (YPGly) in ten-fold dilutions (5000, 500 and 50 cells). Glycerol metabolism, more particular the conversion of glycerol-3-phosphate to dihydroxyacetonephosphate, required mitochondrial respiration. None of the 4 knockout strains demonstrated any respiratory deficiency (Fig. 7.3).

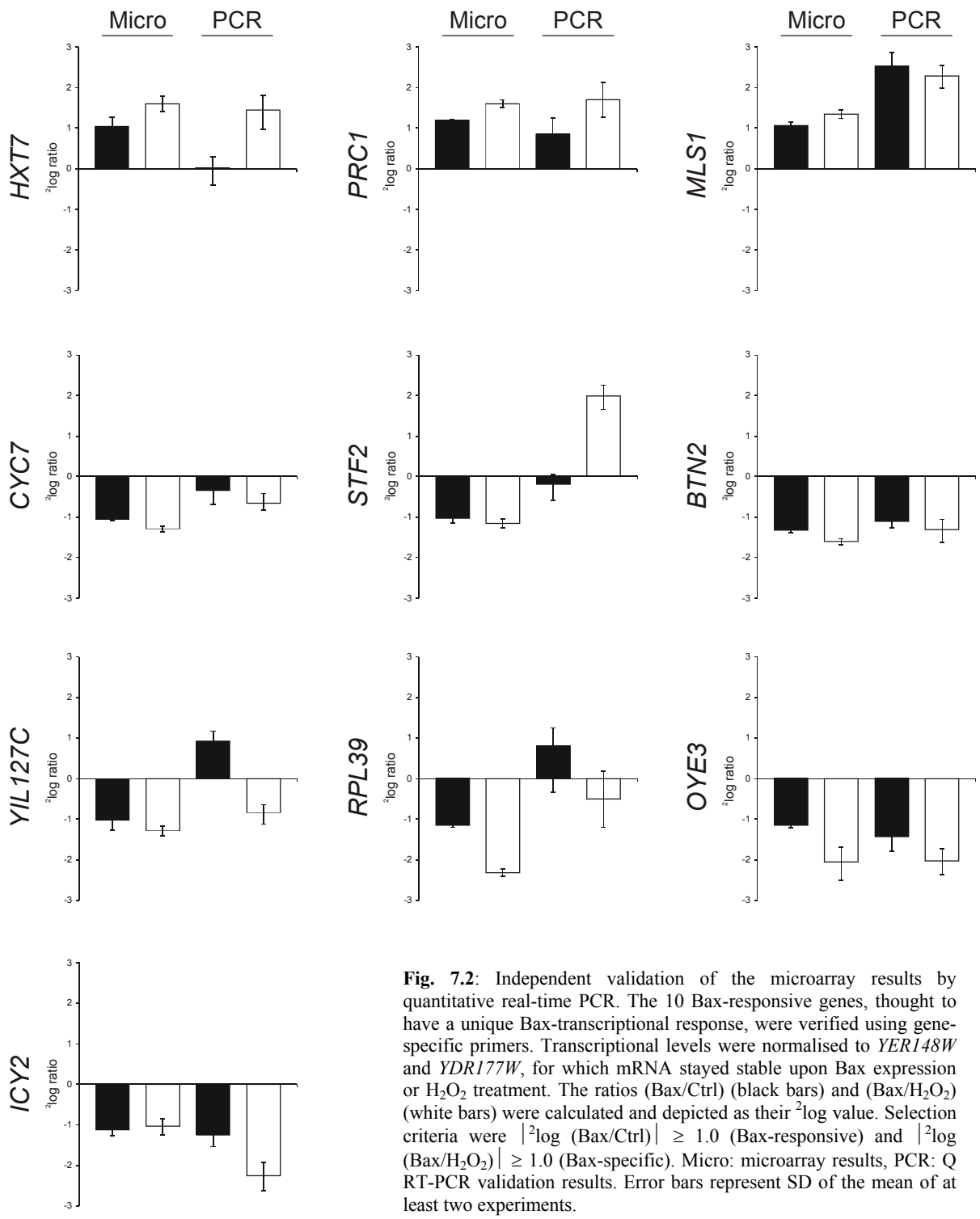

Fig. 7.2: Independent validation of the microarray results by quantitative real-time PCR. The 10 Bax-responsive genes, thought to have a unique Bax-transcriptional response, were verified using gene-specific primers. Transcriptional levels were normalised to *YER148W* and *YDR177W*, for which mRNA stayed stable upon Bax expression or H_2O_2 treatment. The ratios (Bax/Ctrl) (black bars) and (Bax/H_2O_2) (white bars) were calculated and depicted as their ^{2}log value. Selection criteria were $|^2\log(\text{Bax/Ctrl})| \geq 1.0$ (Bax-responsive) and $|^2\log(\text{Bax/H}_2\text{O}_2)| \geq 1.0$ (Bax-specific). Micro: microarray results, PCR: Q RT-PCR validation results. Error bars represent SD of the mean of at least two experiments.

2.2. Determination of Bax sensitivity via growth assay

To determine the Bax sensitivity of the 4 strains knocked out for one of the genes identified to have a Bax-specific transcriptional response, episomal Bax expression systems on centromeric plasmids were constructed (See Chapter 6). The plasmids pRS415, pRS415GAL1mBAX, p415GALL and

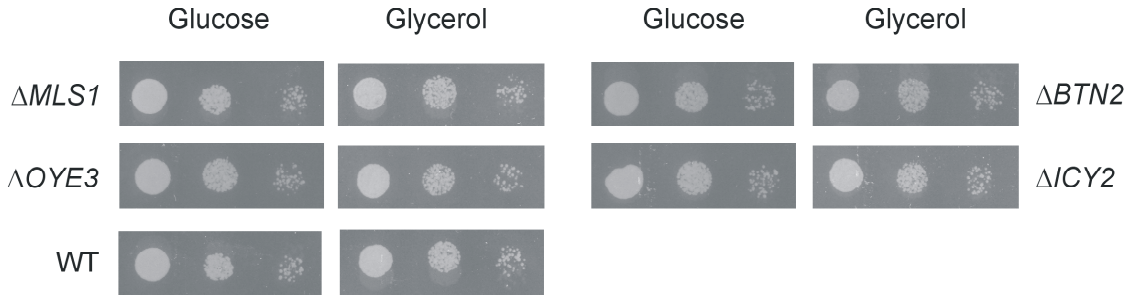

Fig. 7.3: Determination of the respiratory capacity of strains knocked out for one of the four genes verified to have a Bax-specific transcriptional response. In case mitochondrial respiration is hampered, due to a particular gene knockout, growth on glycerol-containing medium is retarded or completely absent. Glucose: rich YPD medium, Glycerol: rich YPGly medium containing 3% glycerol. Ten-fold dilutions were spotted (left to right: 5000, 500 and 50 cells) and scans were taken after 36 h (YPD) or 72 h (YPGly). WT: growth characteristics of BY4742.

p415GALLmBax were transformed into BY4742 and the 4 knockout strains (Δ*MLS1*, Δ*BTN2*, Δ*OYE3* and Δ*ICY2*), and their transformants were analysed by a growth assay in selective synthetic SG-medium (Fig. 7.4). For the empty vector and pRS415GAL1mBAX transformants, the growth of all four knockout strains did not differ from that of the control BY4742. For the p415GALLmBAX transformants, the growth rate of Δ*OYE3* was higher than that of BY4742, but none of the other 3 knockout strains demonstrated any change. Besides visual inspection of the growth curves, the data were fitted to an equation describing logistic growth (Richards, 1959). Growth rate was calculated as the first derivative at the inflection point, and the growth rate ratio of the Bax-expressing population to the control transformant was calculated for each strain (Fig. 7.5). The absence of *OYE3* resulted in a 19.5% (SD: 4.0%) improvement in the growth rate ratio of the Bax-expressing population.

2.3 Determination of cell death sensitivity

2.3.1 Assay for Bax-induced cell death

A clonogenic survival assay was performed to determine whether the growth improvement observed in Bax-expressing Δ*OYE3* transformants (compared to Bax-expressing BY4742 cells) was linked to a decreased cell death rate. Therefore, BY4742 and Δ*OYE3*, both transformed with p415GALL-mBAX, were resuspended in selective synthetic SG-medium for induction of Bax expression, and a fixed number of cells, based on measurement of OD_{600}, was plated on selective synthetic SD-plates at regular intervals. The absence of *OYE3* decreased the rate of Bax-induced cell death (Fig. 7.6), although this protective effect more or less disappeared after 20 h of Bax induction. The maximal cell death diminution was 18.0% (SD: 4.5%), which is in agreement with the results obtained by growth assay.

Next, Bax protein expression in both BY4742 and Δ*YPL171C* was verified by Western blot (Fig. 7.7), to ascertain that the observed effect was not due to a decrease in Bax protein level. The Bax protein levels were found to be identical in both strains.

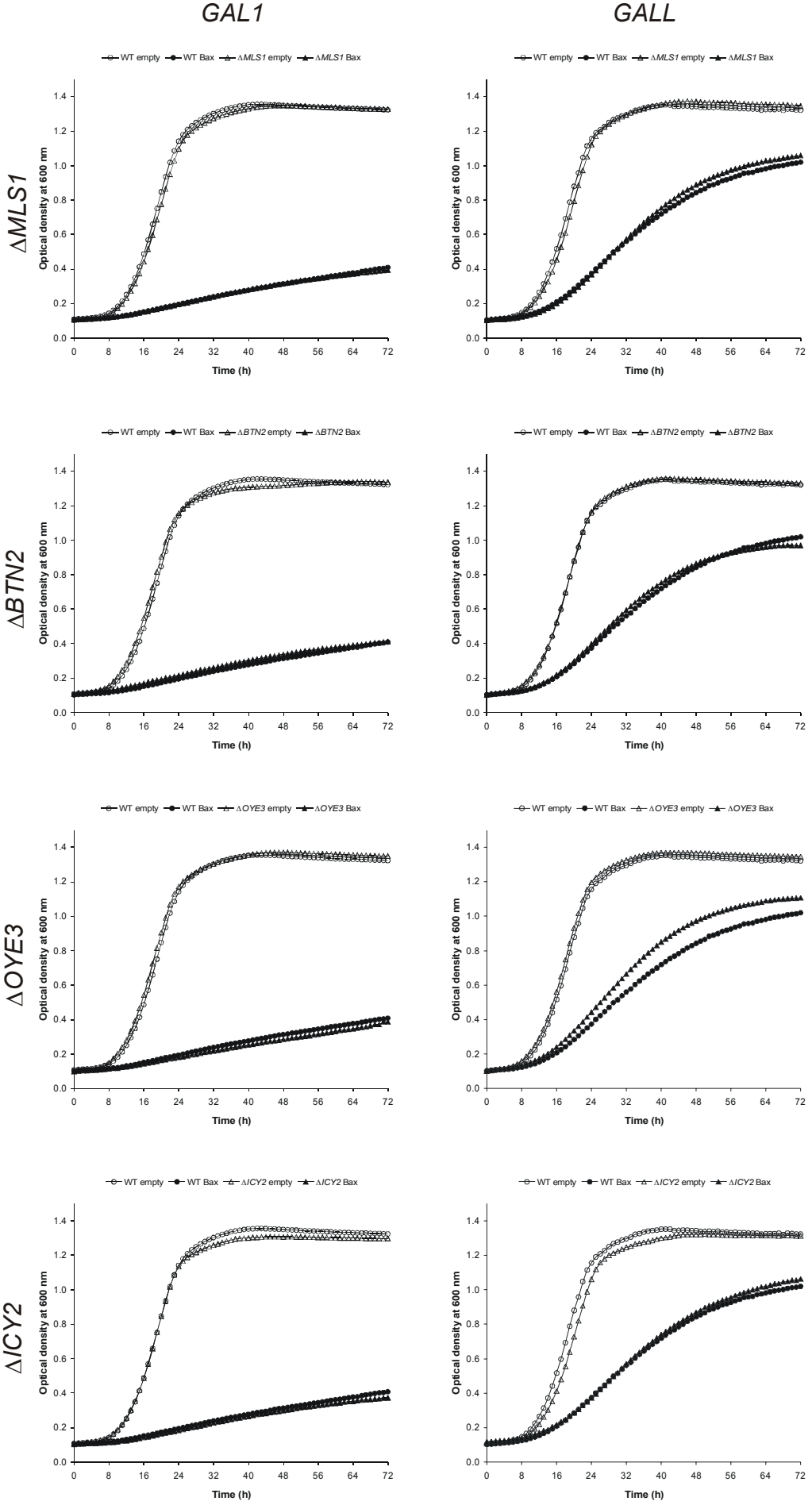
GAL1
GALL
ΔMLS1
ΔBTN2
ΔOYE3
ΔICY2
WT empty
WT Bax
ΔMLS1 empty
ΔMLS1 Bax
ΔBTN2 empty
ΔBTN2 Bax
ΔOYE3 empty
ΔOYE3 Bax
ΔICY2 empty
ΔICY2 Bax
Optical density at 600 nm
Time (h)

Fig. 7.4: The effect of knocking out one of the 4 genes, validated to have a Bax-specific transcriptional response, on Bax-induced growth inhibition. The cells were transformed with pRS415 (empty) or pRS415GAL1mBAX (Bax) to check growth phenotypes in the episomal *GAL1* expression system (indicated with *GAL1*). The cells were transformed with p415GALL (empty) or p415GALLmBAX (Bax) to check growth phenotypes in the episomal *GALL* expression system (indicated with *GALL*). Growth was performed in synthetic selective SG-medium and monitored automatically over 72 hours. WT: BY4742. The mean of three independent experiments is shown.

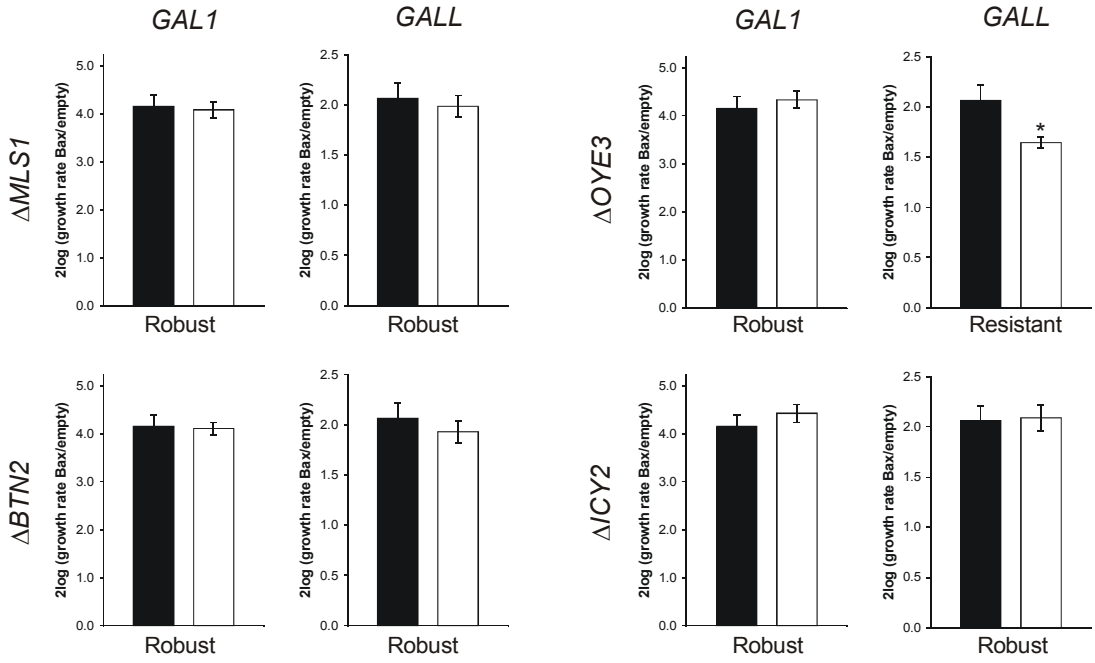

Fig. 7.5: Growth characteristics based on the growth rate of the Bax-expressing transformant (Bax) relative to the control transformant (empty) for the strains knocked out in one of the genes with a Bax-specific transcriptional response. The cells were transformed with pRS415 (empty) or pRS415GAL1mBAX (Bax) to check growth phenotypes in the episomal *GAL1* expression systems (indicated with *GAL1*). The cells transformed with p415GALL (empty) or p415GALLmBAX (Bax) to check growth phenotypes in the episomal *GALL* expression system (indicated with *GALL*). The discrete data were adapted to continuity via growth model fitting and the growth rate was calculated as the first derivative at the growth curve's inflection point. Error bars represent SD of the mean of three independent experiments. (*) Statistical significant difference based on the Wilcoxon Rank sum test (α=0.05).

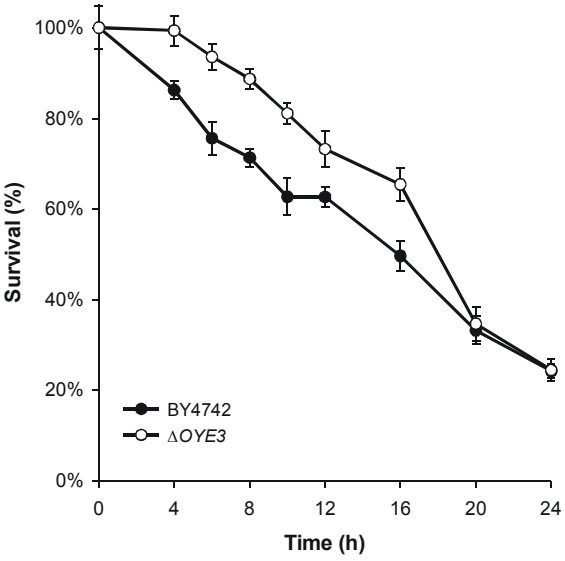

Fig. 7.6: The absence of *OYE3* attenuates Bax-induced cell death. The cells were transformed with the plasmids p415GALLmBAX or p415GALL (empty), grown in glucose-based medium, and recultured in galactose-based medium to induce protein expression from the *GALL* promoter. Clonogenic survival was determined by recovering cells at various times from the galactose-based medium and plating an estimated 1000 cells on glucose-based semisolid medium. For each time point, the percentage survival was calculated with reference to the mock-treatment (empty). Error bars represent SD of the mean of three independent experiments.

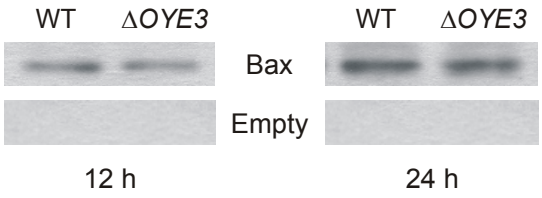

Fig 7.7: Bax protein expression in unaffected in yeast cells knocked out for *OYE3*. Wild type (BY4742) and Δ*OYE3* were transformed with the plasmids p415GALLmBAX (Bax) or p415GALL (empty), grown in glucose-based medium, and recultured in galactose-based medium for an additional period of 12 or 24 hours. Total protein extracts were subjected to SDS-PAGE and immunoblot analysis, using a monoclonal antibody against murine Bax.

2.3.2 Assay for sugar-induced cell death

The addition of sugars, like glucose, induces an apoptosis-like cell death in *S. cerevisiae* in the absence of other additional growth-supporting nutrients (Granot and Dai, 1997; Granot *et al.*, 2003). To determine whether the absence of *OYE3* also attenuated Sugar-Induced Cell Death (SICD), we resuspended stationary phase cells of BY4742 and Δ*OYE3* in pure 2% glucose. SICD was assayed by clonogenic survival (Fig. 7.8A) and by flow cytometry based on PI (Propidium Iodide) exclusion (Fig. 7.9). We observed the absence of *OYE3* to decrease the SICD rate.

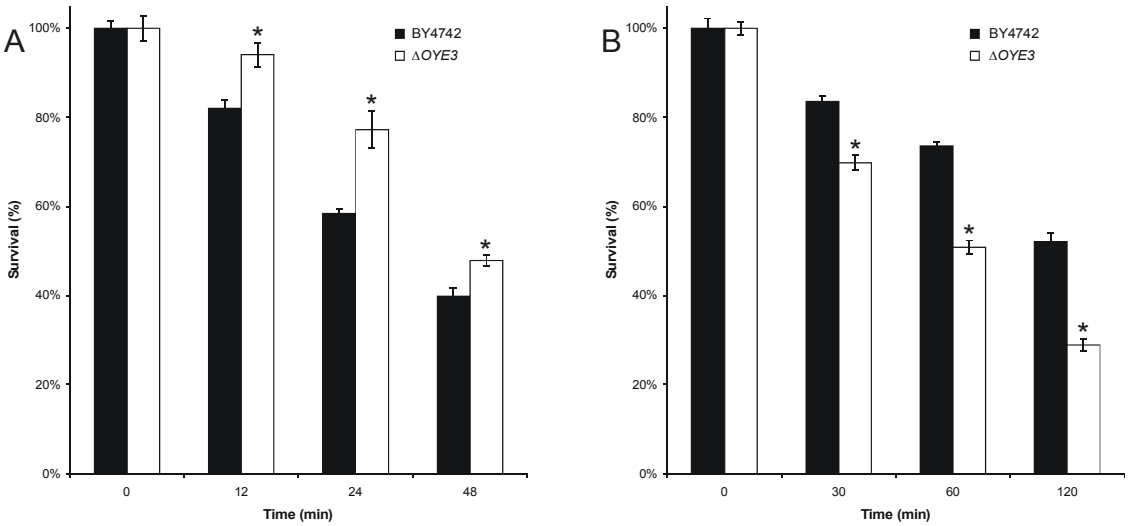

Fig 7.8: The absence of *OYE3* influences the rate of sugar-induced and H_2O_2-induced cell death. (A) Cells of BY4742 or Δ*OYE3* were resuspended in pure 2% glucose and a fixed amount of cells were recovered at various times. Clonogenic survival was determined by the percentage of surviving colonies on synthetic semi-solid medium with reference to the mock-treatment (dH_2O). The absence of *OYE3* attenuates sugar-induced cell death. (B) Cells of BY4742 or Δ*OYE3* were grown in synthetic SD-medium to early logarithmic phase and treated with 0.5 mM H_2O_2. Cells were recovered at various times and their clonogenic survival determined. The absence of *OYE3* leads to a sensitivity towards H_2O_2. Data are representative of at least two independent experiments. (*) Statistical significant difference based on the Wilcoxon Rank sum test (α=0.05).

2.3.3 Assay for hydrogen peroxide-induced cell death

The addition of low concentrations of H_2O_2 leads to apoptosis-like yeast cell death (Madeo *et al.*, 1999). To determine whether the absence of *OYE3* influences the rate of H_2O_2-induced cell death, we resuspended cells of BY4742 and Δ*OYE3* in synthetic SD-medium supplemented with 0.5 mM H_2O_2. Clonogenic survival was determined by recovering cells at various times and plating an

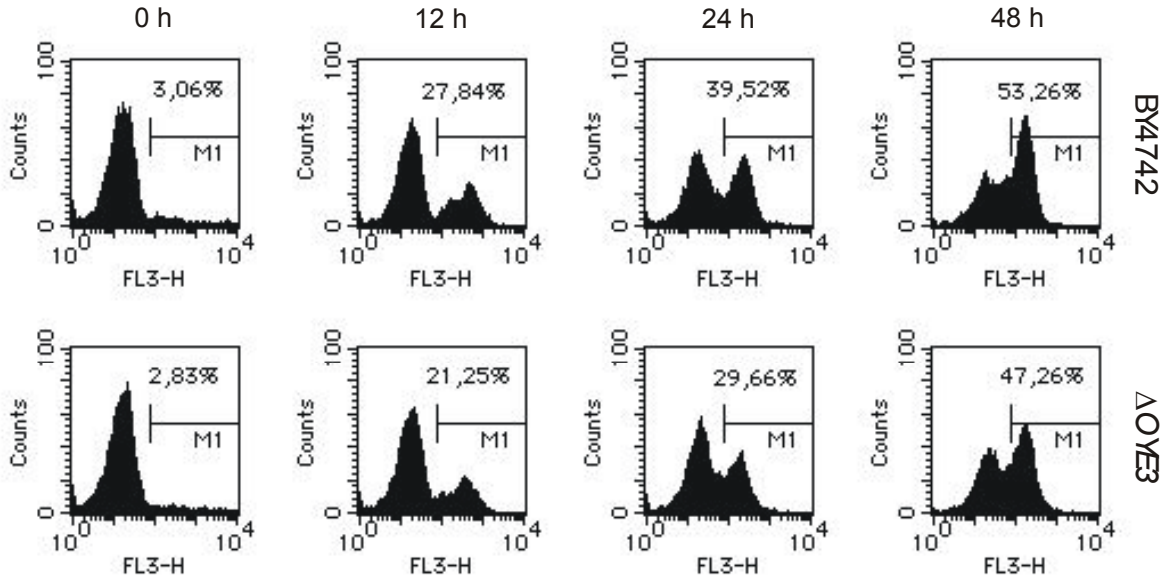

Fig. 7.9: The absence of *OYE3* influences the rate of sugar-induced cell death as determined via exclusion of the membrane-impermeable propidium iodide. Cells of BY4742 or Δ*OYE3* were resuspended in pure 2% glucose and cells were recovered at various times. Cells were analysed for dye exclusion via flow cytometry. For each analysis, 10,000 events were collected.

estimated 1000 cells on synthetic semisolid SD-medium. We observed the absence of *OYE3* actually to increase the rate of H_2O_2-induced cell death (Fig. 7.8B), which is opposite to the observed effect on Bax-induced (See Fig. 7.6) and sugar-induced cell death (Fig. 7.8A). The different phenotypical outcome of Δ*OYE3* upon oxidative stress compared to the Bax trigger was seen as a reflection of the 1.50-fold upregulation of the *OYE3* mRNA following H_2O_2 treatment, compared to 2.70-fold downregulation due to Bax expression.

2.4 Molecular investigation of the Δ*OYE3* cell death effect

2.4.1 Correlation of Bax- and H_2O_2-induced cell death with a drop in NADPH

The gene *OYE3* encodes Old Yellow Enzyme Oye3, an NADPH dehydrogenase the physiological substrate of which is presently unknown. Therefore, we wondered whether an increased NADPH level due to the absence of an NADPH converting enzyme in BY4742 Δ*OYE3* could be responsible for the decreased cell death rate of Bax-expressing cells.

BY4742 and BY4742 Δ*OYE3* yeast cells transformed with p415GALLmBAX were grown in selective synthetic SD-medium. Samples were taken prior to the carbon switch (0 hours) and at three additional time points (6, 12 and 24 h) after resuspension in selective synthetic SG-medium. Extracts were prepared, normalised for protein content, and analysed for intracellular NADPH based on the absorbance of reduced pyridine nucleotides at 340 nm. The decrease in absorbance upon enzymatic conversion of NADPH to $NADP^+$ is indicative of the NADPH level.

We observed that both BY4742 and Δ*OYE3* had the same initial NADPH levels (Fig. 7.10A). Thus, the decreased rate of Bax-induced cell death in the absence of *OYE3* could not be attributed to an intrinsically augmented redox buffering capacity. Further, NADPH levels were shown to decrease during Bax-induced cell death (Fig. 7.10A), though this NADPH drop was less pronounced in Δ*OYE3* compared to BY4742. Next, we wanted to determine whether the absence of *OYE3* had an influence on the rate of NADPH decrease following treatment with 0.5 mM H_2O_2. As expected, NADPH levels decreased during H_2O_2-induced cell death (Fig. 7.10B). However, contrary to the effect observed during Bax-induced cell death, the absence of *OYE3* resulted in a more prominent drop in NADPH level.

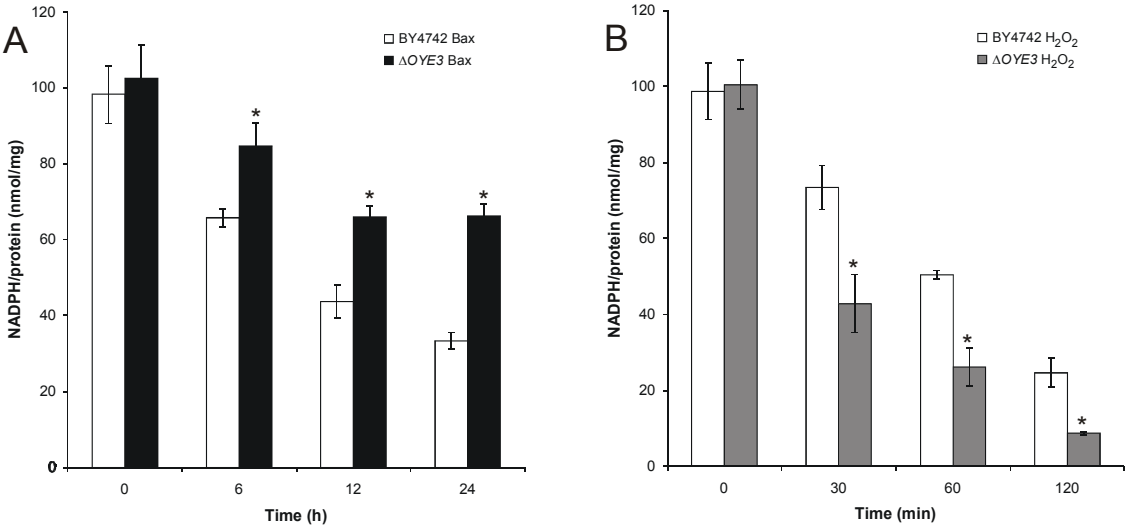

Fig. 7.10: The drop in NADPH level is proportional to the extent of Bax-induced or H_2O_2-induced cell death. (A) Wild type BY4742 and Δ*OYE3* cells were transformed with the plasmid p415GALLmBAX (Bax), grown in glucose-based medium, and recultured in galactose-based medium. Cells were recovered at various times and their NADPH levels determined. The decrease in NADPH level, seen during Bax-induced cell death in wild type, is clearly less pronounced in the strain knocked out for *OYE3*. (B) Wild type and BY4742 cells were grown in glucose-based medium. Following the addition of H_2O_2 to a final concentration of 0.5 mM, cells were recovered at various times and their NADPH levels determined. The decrease in NADPH level, seen during H_2O_2-induced cell death in wild type, in clearly more pronounced in the strain knocked out for *OYE3*. (*) Statistical significant difference based on the Wilcoxon Rank sum test (α=0.05).

2.4.2 Lipid peroxidation during Bax-induced cell death is not present in Δ*OYE3*

The decrease in rate of Bax-induced cell death and the increase in the rate of H_2O_2-triggered cell death, upon deletion of *OYE3*, suggested that the absence of Oye3 may in some way hamper the generation of oxygen stress, thereby providing a protective mechanism against Bax-induced cell death. This led us to investigate the extent of oxidative damage during Bax-induced cell death in Δ*OYE3* compared to wild type.

BY4742 p415GALLmBAX transformants were grown in selective synthetic SD-medium and reference samples were taken prior to the carbon switch (0 hours). Upon resuspension in selective synthetic SG-medium, cells were harvested at two additional time points (12 and 24 h). Samples were assayed for oxidative damage to proteins, mitochondrial DNA and lipids. Oxidative protein

damage was determined by an immunoassay using an anti-DNP antibody following the reaction of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH). Mitochondrial DNA damage was determined based on the extent by which the amplification of a large (error-prone) amplicon of a mitochondrial gene was hampered relative to a short amplicon of that gene. Neither oxidative protein damage (Fig. 7.11A) nor oxidative DNA damage (Fig. 7.11B) could be detected following Bax induction, indicating that either these molecules are not damaged by the oxidative stress accompanying Bax expression, or else that they are rapidly and efficiently repaired/removed.

Lipid peroxidation, however, was found to be associated with Bax-induced cell death in BY4742 (Fig. 7.12A), but we could not observe any lipid peroxidation in Δ*OYE3* during Bax-induced cell death (Fig. 7.12A). These findings implicate Old Yellow Enzyme Oye3, directly or indirectly, in Bax-induced lipid peroxidation. Nevertheless, lipid peroxidation is unlikely to be the only determinant of Bax-induced cell death, as the absence of *OYE3* delayed but did not fully protect against Bax-induced cell death (Fig. 7.6).

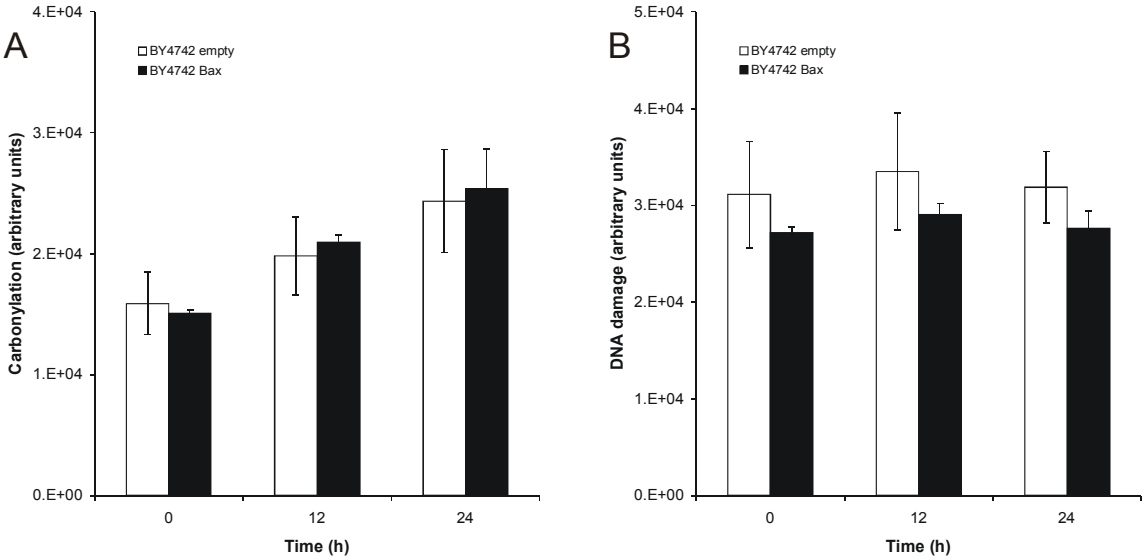

Fig. 7.11: Cellular proteins and mitochondrial DNA do not accumulate oxidative damage during Bax-induced cell death. Yeast cells were transformed with the plasmids p415GALLmBAX (Bax) or p415GALL (empty), grown in glucose-based medium, and recultured in galactose-based medium. Cells were recovered at various times. (A) The extracted proteins were dot blotted and immunoassayed to determine their carbonyl content. (B) The extracted DNA was subjected to a quantitative polymerase chain reaction, and the oxidative mitochondrial DNA damage was measured as the degree by which the amplification of a large (error-prone) amplicon was hampered relative to a short (error-resistant) amplicon of a mitochondiral gene. Data are representative of at least two independent experiments.

2.4.3 Lipid peroxidation during H_2O_2-induced cell death is more prominent in Δ*OYE3*

To evaluate the generality of the effect the Oye3 on lipid peroxidation, we checked whether the absence of *OYE3* could protect against H_2O_2-induced lipid peroxidation. BY4742 and Δ*OYE3* cells were treated with 0.5mM, 1.0 mM or 2.0mM H_2O_2 for 1 hour, after which lipid peroxidation was determined. As expected, we observed a gradual increase in lipid peroxidation with increasing H_2O_2

concentrations in both yeast strains (Fig 7.12B). However, the extent of oxidative lipid damage was actually higher in BY4742 Δ*OYE3*, in line with the increased H_2O_2-sensitivity and the more severe NADPH drop upon H_2O_2 addition compared to the wild type (See Fig. 7.8B and Fig. 7.10B).

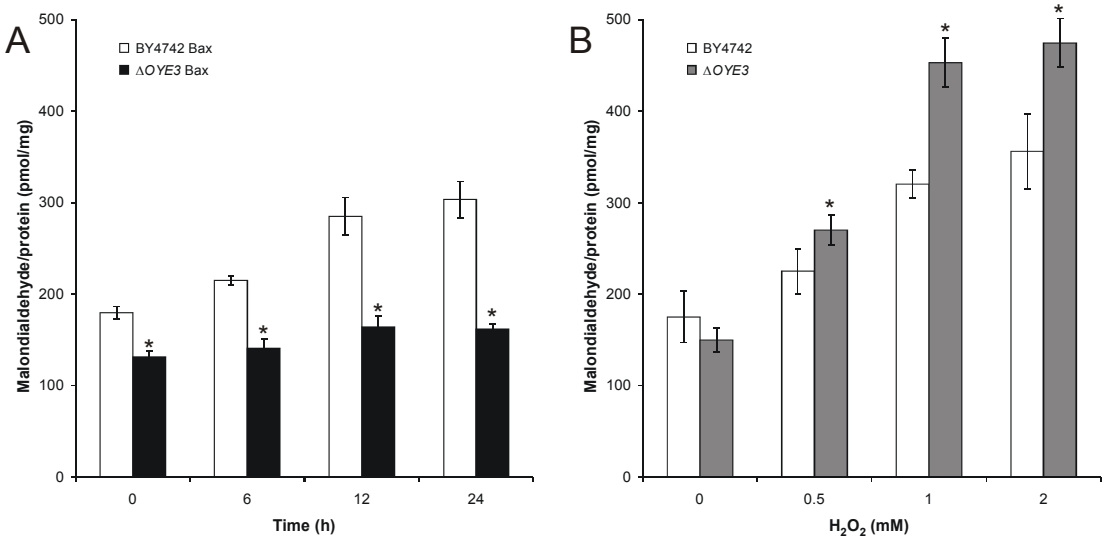

Fig. 7.12: The amount of thiobarbituric acid-reactive species increases during Bax-induced or H_2O_2-induced cell death, the extent of which is influenced by the absence of *OYE3*. (A) Wild type BY4742 and Δ*OYE3* cells were transformed with the plasmid p415GALLmBAX, grown in glucose-based medium, and recultured in galactose-based medium. Cells were recovered at various times and assayed for lipid peroxidation breakdown products. The increase in thiobarbituric acid-reactive species, seen during Bax-induced cell death in wild type, is completely absent for the strain knocked out for *OYE3*. (B) Wild type BY4742 and Δ*OYE3* cells were grown in glucose-based medium. Cells were recovered 1 h following the addition of various concentrations of H_2O_2 and assayed for lipid peroxidation breakdown products. The increase in thiobarbituric acid-reactive species, seen during H_2O_2-induced cell death in wild type, is more pronounced in the strain knocked out for *OYE3*. Data are representative of at least three independent experiments. (*) Statistical significant difference based on the Wilcoxon Rank sum test (α=0.05).

2.4.4 Ergosterol levels are increased in the *OYE3* knockout

In an attempt to explain the above-mentioned results, one could presume Oye3 to have another cellular function besides its NADPH dehydrogenase activity for antioxidant defence. As Oye3 was hypothesed to be involved in sterol metabolism (Stott *et al.*, 1993; Niino *et al.*, 1995; Vaz *et al.*, 1995), we explored this possibility.

BY4742 and Δ*OYE3* cells were grown in synthetic SG-medium to logarithmic phase and ergosterol was quantified based on its non-saponificable nature. Sterol extracts were normalised for wet cell pellet weight and scanned spectrophotometrically between 200 nm and 300 nm. (Fig. 7.13A). Based on the contribution of ergosterol and the late sterol intermediate 24(28)-dehydroergosterol (Fryberg *et al.*, 1973) to the four-peaked curve (Breivik and Owades, 1957; Arthington-Skaggs *et al.*, 1999), it became clear that deletion of *OYE3* increased ergosterol levels by 31.5% (SD: 0.14%). To the best of our knowledge, this is the first report demonstrating the involvement of Old Yellow Enzyme Oye3 in ergosterol metabolism via experimental verification. However, whether Oye3 inhibits ergosterol biosynthesis or accelerates ergosterol breakdown awaits further exploration.

Recently, increased cholesterol levels were found in mice with a shorter life span (Ishigami *et al.*, 2004). However, although Δ*OYE3* was observed to have higher ergosterol levels, its replicative life span was unaffected (Fig. 7.13B).

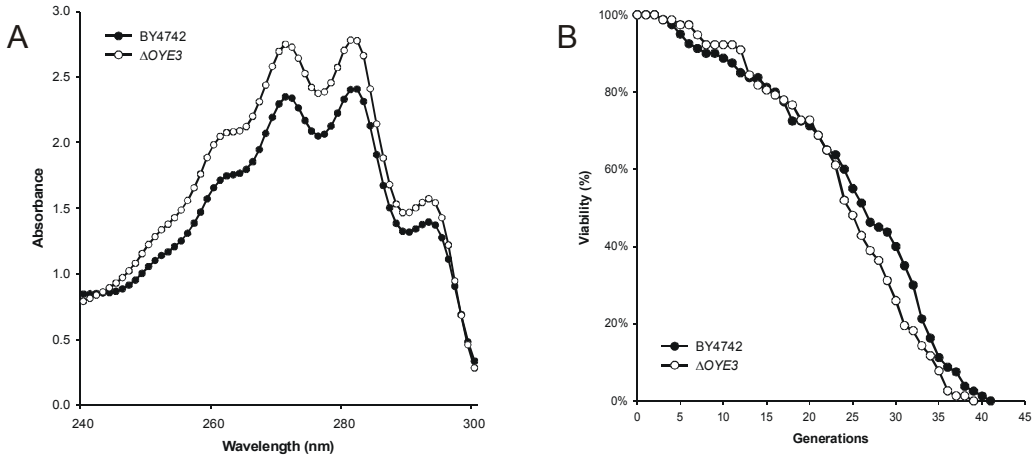

Fig. 7.13: In the absence of *OYE3*, ergosterol levels are increased without any effect on life span. (A) Wild type BY4742 and Δ*OYE3* cells were grown in galactose-based medium. Sterols were extracted and their UV spectrophotometric profiles between 240 and 300 nm determined. The peak at 281.5 nm is solely attributed to ergosterol. The absence of *OYE3* resulted in an increased ergosterol level. (B) Wild type BY4742 and Δ*OYE3* cells were grown in galactose-based medium and their replicative life span determined. The knockout of *OYE3* had no effect on the mean life span. Data are represented by the mean of at least two independent experiments.

3. References

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Chapter 8

Discussion and future prospects

Ectopic overexpression of the mammalian pro-apoptotic protein Bax in *Saccharomyces cerevisiae* induces growth arrest, followed by cell death with an apoptosis-like phenotype (Greenhalf *et al.*, 1996; Ligr *et al.*, 1998). Bax-induced cell death requires mitochondrial respiration (Matsuyama *et al.*, 1998; Priault *et al.*, 1999; Harris *et al.*, 2000) and is associated with the generation of Reactive Oxygen Species (ROS) (Madeo *et al.*, 1999), the accumulation of which may be necessary for Bax-induced cell death to occur (Madeo *et al.*, 1999; Kissova *et al.*, 2000). Generally, conditions that induce growth arrest and/or cell death are associated with augmented ROS levels (Davidson *et al.*, 1996; Aguilaniu *et al.*, 2001; Helmerhorst *et al.*, 2001; Wunder *et al.*, 2004), indicating oxidative stress not to be uniquely associated with Bax toxicity. Apoptosis-like cell death can also be induced by H_2O_2 treatment (Madeo *et al.*, 1999), but it is currently unknown to what extent the Bax-induced and H_2O_2-induced cell death pathway are differential. Particularly, the molecular description of the mechanism by which Bax proteins induce an oxidative stress condition is challenging.

The kinetics of the transcriptional response of *S. cerevisiae* to Bax expression was studied to unravel Bax toxicity at the molecular level (Chapter 5). Contradictory to observations during apoptosis (Nadano and Sato, 2000), RNA extracted at several time points during Bax induction did not reveal any rRNA degradation (data not shown). This observation is in accordance with the recent finding that the structural identity of rRNA is contained during caspase-independent cell death (Saelens *et al.*, submitted). Next, transcriptional profiling at several time points identified 208 Bax-responsive genes, relative to untreated control, via the standard two-fold approach. Besides this *ad hoc* thresholding procedure, other computational methods could have been better suited for the identification of differential and coordinated gene expression (Pan, 2002). Further, the expression of a non-toxic Bax protein variant still demonstrating mitochondrial localisation (Oliver *et al.*, 2000) could have been a better reference. However, half of the Bax-responsive genes demonstrated an early response, suggesting they may encode *causal* factors for Bax-induced cell death to occur. Most of the downregulations were seen in later points of Bax induction, suggesting they could reflect changes as the *consequence* of the cell death process. Due to the enormous diversity of the cellular functions affected by Bax induction, we could neither map the biochemical processes associated with Bax-induced cell death, nor distinguish causal from consequential changes (Marton *et al.*, 1998). This observation implied the need for a comparative strategy to extract *causal* candidates for further study.

Next, we wanted to determine whether the Bax-induced transcriptional responses were different from those elicited by oxidative stress, and if so, whether these differences could explain how Bax expression actually generates a cellular oxidative stress.

The differences between the Bax-induced responses and those elicited by oxidative stress were determined via macroarray profiling (Chapter 6). A system was developed that mimics the

generation and effects of endogenous ROS. As H_2O_2 is an effective ROS molecule for which membranes are highly permeable, it became an obvious choice. We optimised a H_2O_2 treatment by determining the dose eliciting cell death to the same extent as induced by expression of Bax. This H_2O_2 challenge was applied in the same conditions as Bax expression and for the same duration. Transcriptional changes were compared and 23 genes were selected as candidates to have a Bax-specific transcriptional response. To elucidate whether these 23 genes represented a Bax-exclusive pathway, their transcriptional cross-response with other types of stress was determined via literature search. First, we chose to compare with data published concerning mitochondrial stress (Epstein *et al.*, 2001). For the cross-responding genes (*QCR8*, *COX12*, *PKH2*, *YMR107W*, *YPL201C*, *ADR1*, *YCK1* and *YJL142C*), the transcriptional changes due to Bax-induction may well be the consequence of mitochondrial dysfunction. Next, we compared the transcriptional effects of Bax induction with data on tunicamycin treatment published by others (Travers *et al.*, 2000). Tunicamycin inhibits N-linked protein glycosylation and induces an UPR (Unfolded Protein Response), mimicking the suspected endoplasmic reticulum-mitochondria cross talk during Bax-induced cell death. Tunicamycin treatment and Bax induction triggered comparable transcriptional responses for the genes *YGR146C* and *YLR414C*, the responses of which may be the result of ER-dysfunction. Further, only the Bax-induced transcriptional response of *YGR146C*, *YLR414C* and *YJL060W* could be independently verified by Q RT-PCR (13%). To determine the influence of these genes on Bax-induced growth arrest, growth was recorded and the logistic growth curve (Richards, 1959) was chosen for modeling purposes. This approach extended the common methods of (i) pure visual analysis (De Backer *et al.*, 2001); or (ii) extracting the exponential growth part of the curve (Gardner *et al.*, 2001; Groeneveld *et al.*, 2002; Cabral *et al.*, 2003), which for the latter is rather subjective in the case of severe growth retardation. The strain Δ*YGR146C* demonstrated sensitivity (*GAL1* Bax-expression system) and the strain knocked out for *YLR414C* demonstrated resistance (*GALL* Bax-expression system) to Bax-induced growth arrest, indicating *YGR146C* and *YLR414C* to interfere with the growth/cell cycle-disturbing function of the Bax protein. The absence of *YJL060W* did not affect Bax-induced growth arrest. Further, a suitable assay for Bax-induced cell death was searched for. Flow cytometry revealed that Bax-induced PI (Propidium Iodide) penetration only occurred 24 hours after Bax induction (data not shown) and this late cell death marker was regarded as having no discriminatory potential for screening knockout strains. Finally, the "old-fashioned" clonogenic survival assay was selected to be the best approach for the investigation of Bax-induced cell death. The knockout strains Δ*YGR146C* and Δ*YLR414C* did not demonstrate a significant change in the rate of Bax-induced cell death.

Additionally, strains knocked out for some other genes out of the 23-list demonstrated an altered growth rate upon Bax expression, although their macroarray transcriptional responses could not be validated. Sensitivity towards Bax-induced growth arrest was observed in (i) Δ*YDR504C* and Δ*YJL142C;* and in (ii) Δ*ATP1*, Δ*COX12*, and Δ*QCR9*, the latter contradictory to the view that oxidative phosphorylation is needed for Bax toxicity (Harris *et al.*, 2000). Resistance towards Bax-

induced growth arrest was observed in Δ*PKH2*, Δ*TSA1*, Δ*YHR138C*, Δ*YKL123W* and Δ*QCR8*. Further, the deficiency in *GAL*-driven Bax expression (Durrin *et al.*, 1991) may be an explanation for the resistance to Bax-induced growth arrest in Δ*HHF1* and Δ*HHF2*.

A technical repeat of the GeneFilter® macroarray profiling reduced the list of Bax-responsive (from 104 to 44) genes and Bax-specific (from 23 to 2) genes. However, both datasets demonstrated more than 60% difference, pointing towards the introduction of random and/or lot number-specific errors (Fig. 8.1A). This observation is a clear argument against the use of GeneFilter® macroarrays for gene expression profiling.

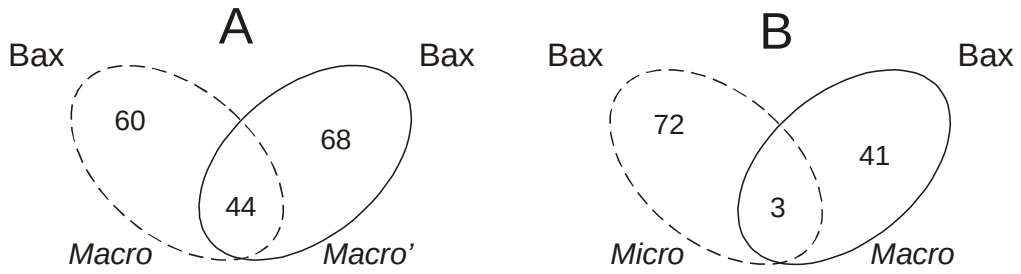

Fig. 8.1: Venn diagrams illustrating the Bax-elicited transcriptional responses as determined via different transcriptional profiling techniques. (A) Technical repeats using GeneFilter® macroarrays of a different lot number demonstrate partial overlap (40%) only, which illustrates the unreliability of this method. (B) The averaged datasets of technical repeats performed on GeneFilter® macroarrays and oligo microarrays demonstrate almost no overlap, pointing towards the utmost importance of an independent validation of the transcriptional responses.

The differences between the Bax-induced responses and those elicited by oxidative stress were also determined by microarray analysis (Chapter 7) and subsequent data normalisation via MARAN modelling. Microarray expression profiling of the early transcriptional responses to Bax expression identified 75 genes as Bax-responsive candidates. These genes only showed minor overlap with the Bax-responsive genes identified via macroarray profiling (Fig. 8.1B). The transcriptional responses of those genes to Bax expression were compared with their responses to a H_2O_2 treatment that was as lethal as Bax expression in exactly the same conditions. Nine genes were identified, the Bax-induced change in expression of which was at least 2-fold more manifest compared to the corresponding H_2O_2-induced change. In addition, one gene was identified for which the Bax-induced and H_2O_2-induced expression changes were of opposite sense. There was no overlap between the 10 microarray-defined Bax-specific genes and the 23 macroarray-defined Bax-specific genes. The transcriptional response of the 10 microarray-defined genes was independently verified by Q RT-PCR, and 4 genes (*MLS1*, *OYE3*, *ICY2* and *BTN2*) were confirmed as having Bax-specific transcriptional responses. The observed validation percentage (40%) was in accordance with previous observations (van Hal *et al.*, 2000). These 4 genes could constitute part of a mechanism by which Bax elicits oxygen stress or by which cells protect themselves against Bax-toxicity. Whatever it may be, the selection of 4 genes with a Bax-specific response reinforced our view about the difference between the Bax-induced and H_2O_2-induced cell death pathways.

We analysed the phenotypic outcome of Bax expression in yeast strains individually knocked out for each of these four genes. We observed that solely the absence of *OYE3* (Old Yellow Enzyme) increased the tolerance to Bax-induced cell death. Moreover, deletion of *OYE3 per se* did not increase generation time. Old yellow enzyme (OYE; EC 1.6.99.1), was originally isolated from brewers' bottom yeast (Warburg and Christian, 1933) as "das gelbe Ferment", and was shown to be composed of a colourless apoprotein and a redox-active flavin mononucleotide prosthetic group (FMN) that gives it its distinctive yellow colour (Theorell, 1935). The genes encoding Old yellow enzyme from *Saccharomyces carlbergensis* (Saito *et al.*, 1991) and *S. cerevisiae* have been cloned (Stott *et al.*, 1993; Niino *et al.*, 1995) and it has been established that NADPH serves as the physiological reductant for the enzyme-bound flavin (Fox and Karplus, 1994; Kohli and Massey, 1998). Old yellow enzyme reacts with several substrates, catalysing the reduction of CoQ_1 and other quinones (Massey and Schopfer, 1986; Vaz *et al.*, 1995), as well as the reduction of nitro-olefins (Meah and Massey, 2000) and the olefinic bond of α,β-unsaturated aldehydes and ketones (Matthews *et al.*, 1975; Stott *et al.*, 1993; Vaz *et al.*, 1995). As Oye3 is a NADPH dehydrogenase, we wondered whether an increased intrinsic NADPH level, due to the absence of an NADPH converting enzyme in the corresponding knockout strain, could be responsible for the decreased cell death rate of Bax-expressing cells. This was observed not to be the case. Moreover, we observed Bax-induced and H_2O_2-induced cell death were accompanied by a drop in NADPH level proportionate to the cell death rate. Our observation of NADPH consumption during Bax-induced cell death further illustrates the position of oxygen stress as a general regulator of cell death in yeast (Madeo *et al.*, 1999) and extends the observation that intracellular glutathione is shifted to the more oxidized state during Bax expression (Kampranis *et al.*, 2000). In mammalian cells, the redox state of pyridine nucleotides affects mitochondrial physiology, in that the oxidised state promotes mitochondrial Ca^{2+} efflux (Lehninger *et al.*, 1978; Vercesi and Pereira-da-Silva, 1984), mitochondrial permeability transition (Rigobello *et al.*, 1995) and apoptosis (Pias and Aw, 2002).

It has been proposed that Old yellow enzyme could keep quinones reduced in a period of redox imbalance, preventing redox cycle initiation (Joseph *et al.*, 2000; Koerkamp *et al.*, 2002). However, the predominant form of physiological quinones in yeast, CoQ_6, did not show any reactivity (Massey and Schopfer, 1986). In fact, lipid peroxidation breakdown products, which can be toxic to cells (Esterbauer *et al.*, 1991) and are known to be potent apoptotic triggers (Sandstrom *et al.*, 1994; Kruman *et al.*, 1997; Mark *et al.*, 1997; Tang *et al.*, 2002), could be the prime physiological substrates (Kohli and Massey, 1998). Indeed, many of these breakdown products are in fact α,β-unsaturated enones, and Old yellow enzyme thus could serve a defensive role against these substrates by reducing their toxicity. This has led to the suggestion that Old Yellow Enzyme may be involved in the oxidative stress response (Lee *et al.*, 1999; Gasch *et al.*, 2000; Causton *et al.*, 2001).

In the present study we demonstrated that, in contrast to the complete lack of protein and mitochondrial DNA oxidative damage following Bax expression, lipid peroxidation breakdown products accumulate in the cells during Bax-induced and H_2O_2-induced cell death in yeast. This

indicates that the oxidative damage to proteins and mitochondrial DNA (mtDNA) during Bax-induced cell death is not detectable or efficiently repaired/removed. The carbonyl assay, which is the most widely used measure of general protein oxidation (Dalle-Donne *et al.*, 2003), detects as little as 0.3-0.5 pmol carbonyl (Shacter, 2000b). The presence of protein carbonyls is reflective of more severe cases of oxidative stress (Shacter, 2000a), which are not necessarily reached by Bax protein expression. In contrast, the detection of mitochondrial DNA damage is very sensitive with a detection limit of 1 lesion/10^5 nucleotides (Santos *et al.*, 2002). The MDA (MalonDiAldehyde) test for lipid peroxidation method detects as little as 100 pmol MDA (Yagi, 1976). MDA is however neither the sole end product of lipid peroxide decomposition nor a substance that is generated exclusively through lipid peroxidation (Gutteridge, 1981; Janero, 1990). Indeed, oxidative damage to DNA and proteins can also induce MDA formation (Gutteridge and Quinlan, 1983; Cheeseman *et al.*, 1988). However, we did not detect these molecules to be oxidatively damaged, excluding this possibility.

Surprisingly, we could not observe any lipid peroxidation in Δ*OYE3* during Bax-induced cell death (Fig. 8.2). These findings demonstrate Old Yellow Enzyme Oye3 to be, directly or indirectly, implicated in Bax-induced lipid peroxidation, suggesting Oye3 to interfere with the mechanism by which Bax induces oxidative stress. Nevertheless, lipid peroxidation is unlikely to be the only determinant of Bax-induced cell death, as the absence of *OYE3* delayed but did not fully suppressed Bax-induced cell death. Accordingly, lipid peroxidation is absent in *Schizosaccharomyces pombe* (Gille *et al.*, 1993) in spite of Bax cytotoxicity (Jurgensmeier *et al.*, 1997).

Interestingly, the *OYE3* knockout strain was shown to be sensitive to H_2O_2, as reflected in a more severe NADPH decline and a higher accumulation of lipid peroxidation breakdown products following H_2O_2 treatment. This result is in agreement with the observation that *OYE3* is part of the Yap1 regulon (DeRisi *et al.*, 1997; Lee *et al.*, 1999; Gasch *et al.*, 2000; Cohen *et al.*, 2002).

Thus, the differentiality in transcriptional response after Bax protein expression (downregulation) and H_2O_2 treatment (upregulation) was reflected by the different phenotypical outcome of the *OYE3* knockout strain following these triggers. Based on the observation that the rate of Bax-induced cell death decreases and the rate of H_2O_2-triggered cell death increases upon deletion of *OYE3*, it could be argued that the absence of Oye3 somehow hampered the generation of oxygen stress, and provided a protective mechanism against Bax-induced cell death.

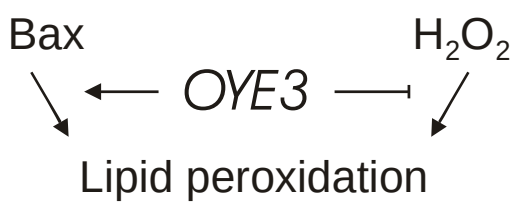

Fig. 8.2: The Bax-toxicity interfering role of Old Yellow Enzyme Oye3. Both Bax expression and H_2O_2 treatment induce lipid peroxidation. Peroxidised lipids could affect membrane physiology or may interfere with an unknown signaling mechanism. The absence of Oye3 sensitises yeast cells to H_2O_2-induced cell death but attenuates Bax-induced cell death. Oye3 is thought to protect yeast cells to H_2O_2-induced cell death but to stimulate Bax-induced cell death.

In an attempt to explain our results, we presumed Oye3 to have another cellular function besides being a NADPH dehydrogenase for antioxidant defence. Although the physiological oxidant of the yeast Old yellow enzyme is still elusive, the plant Old yellow enzyme homologue, 12-oxo-phytodienoate reductase 3 (OPR3), is known to be involved in the biosynthesis of jasmonate (Schaller *et al.*, 2000), a signalling compound which influences multiple cellular functions (Weiler, 1997). Like OPR3, *OYE3* is an active 12-oxo-phytodienoate reductase able to reduce the naturally occurring (9S,13S)-isomer. As oxylipins are the precursors of jasmonic acid in plants, it has been proposed that yeast Old yellow enzyme could be involved in oxylipin metabolism by reducing the α,β-double bonds of these cyclic oxo-fatty acids. Oxylipins have already been found in *Dipodascopsis uninucleata* (Venter *et al.*, 1997; Kock *et al.*, 1998), *Mucor genevensis* (Pohl *et al.*, 1998) and *Saccharomycopsis malanga* (Sebolai *et al.*, 2001) but are not described to be present in *S. cerevisiae*.

Based on the finding that (i) several α,β-unsaturated carbonyl compounds can act as electron acceptors of the reaction catalysed by Old Yellow Enzyme (Stott *et al.*, 1993); (ii) different steroids are able to bind to Old Yellow Enzyme (Vaz *et al.*, 1995); and (iii) Oye3 is highly homologous to the *Candida albicans* estrogen-binding protein (Niino *et al.*, 1995) and a gene product involved in bile acid metabolism by *Eubacterium* sp. (Stott *et al.*, 1993), it may be possible for Oye3 to be involved in sterol metabolism. Especially since many sterol metabolites are in fact α,β-unsaturated compounds. We decided to explore this possibility experimentally. Upon sterol analysis of the wild type and *OYE3* knockout strain, we could observe clearly elevated ergosterol levels in the absence of Oye3. To the best of our knowledge, this is the first evidence demonstrating the involvement of Old Yellow Enzyme Oye3 in ergosterol metabolism via experimental verification. Although altered sterol compositions can have an effect on cytochrome c oxidase enzyme activity (Thompson and Parks, 1974), Δ*OYE3* did not show a defect in respiration. Recently, increased cholesterol levels were found in mice with a shorter life span (Ishigami *et al.*, 2004). However, although Δ*OYE3* was observed to have higher ergosterol levels, its replicative life span was unaffected.

The fungal sterol, ergosterol, is similar to the animal sterol, cholesterol. Cholesterol is known to decrease the fluidity of lipid bilayers *in vitro* (Boggs and Hsia, 1972) at concentrations up to 25 mol%, by affecting the internal viscosity and molecular motion of the lipids within the membrane (Papahadjopoulos *et al.*, 1973). This “condensing effect” is thought to occur via van der Waal’s interactions between the rigid hydrophobic ring structure of cholesterol and the phospholipid acyl chains in the membrane bilayer (Darke et al., 1972; Gutteridge, 1978). Furthermore, *in vitro* bilayer stability was also demonstrated to be affected by cholesterol via the increase of the perimeter energy required for the critical pore formation (Taupin *et al.*, 1975; Abidor *et al.*, 1979). The role of cholesterol can thus be considered as a stabilising or dampening mechanism, inhibiting structural changes *in vivo*. Indeed, the increase of mitochondrial cholesterol has been shown to increase the mitochondrial membrane microviscosity (Lluis *et al.*, 2003). Similarly, ergosterol has also been demonstrated to have a “condensing effect” in lipid monolayers (Demel *et al.*, 1972; Ghosh and

Tinoco, 1972) and to stabilise lipid bilayers at concentrations up to 50 mol% *in vitro* (Wiseman *et al.*, 1990). Finally, yeast sterols are also known to stabilise biological membranes *in vivo* (Astin and Haslam, 1977).

S. cerevisiae differs from most other eukaryotes in that the mitochondrial ergosterol is concentrated in the inner membrane (25 mol%) rather than in the outer membrane (1 mol%) (Zinser *et al.*, 1993; Tuller and Daum, 1995), resulting in a higher lipid fluidity in the outer membrane compared to the inner one (Sperka-Gottlieb *et al.*, 1988). The increased ergosterol content in Δ*OYE3* could thus interfere with Bax docking at the mitochondrial outer membrane or with its stable insertion via the stabilisation of this membrane, thereby preventing ROS generation and lipid peroxidation (Fig. 8.3). Keeping in mind vacuolar integrity becomes affected during Bax-induced cell death (Dimitrova *et al.*, 2004), the increased ergosterol level in Δ*OYE3* may also protect these organelles against rupture.

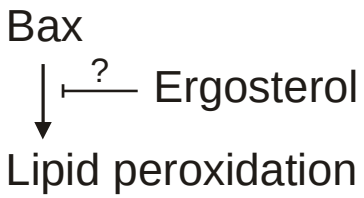

Fig. 8.3: Ergosterol may interfere with Bax cytotoxicity by interfering with lipid peroxidation. Ergosterol may prevent the pore-forming capacity of Bax proteins at the mitochondria or the labilisation of intracellular organelles in general.

Ergosterol is also known to play a role in the phospholipid transfer rate *in vitro* (Szolderits *et al.*, 1989), the activity of membrane-bound enzymes (Cobon and Haslam, 1973; Aithal *et al.*, 1976; Arami *et al.*, 1997), the formation of raft microdomains -potentially involved in signal transduction- (Bagnat *et al.*, 2000), and the regulation of sphingolipid metabolism (Swain *et al.*, 2002). The metabolites of the latter have an important role in determining cell fate in yeast (Dickson *et al.*, 1990; Fishbein *et al.*, 1993; Nagiec *et al.*, 1997) and mammalian cells (Spiegel *et al.*, 1998; Mandala, 2001). Thus, there may be many more possibilities by which the increased ergosterol levels in Δ*OYE3* influence Bax toxicity.

Coordinate ergosterol and oxidative stress responses were identified via macro- or microarray analysis (Higgins *et al.*, 2003; Bruckmann *et al.*, 2004) and mutants in the ergosterol biosynthesis pathway were observed to be highly sensitive to oxidative stress-generating compounds (Higgins *et al.*, 2003) (Schmidt *et al.*, 1999; Thorpe *et al.*, 2004). Ergosterol is able to inhibit lipid peroxidation *in vitro* (Wiseman *et al.*, 1990), which is contradictory to the observation of the Δ*OYE3* H_2O_2-sensitivity in spite of increased ergosterol levels. However, as *in vitro* lipid peroxidation was studied using the iron/ascorbate system, which is a hydroxyl radical generator (Schneider *et al.*, 1988), the different nature of the particular ROS molecule may be responsible for the apparent paradox.

Bax-induced cell death has been shown to depend on vesicular transport (Madeo *et al.*, 1997; Levine *et al.*, 2001), oxidative phosphorylation (Harris *et al.*, 2000) and mitochondrial lipid peroxidation (Priault *et al.*, 2002). As sterols may be implicated in (Munn *et al.*, 1999) or quantitatively influenced by the perturbations used to study these cellular processes (Gal'tsova *et al.*, 1971; Koh *et al.*, 1977), ergosterol could well be the common denominator influencing Bax-induced toxicity.

Given the broad-spectrum involvement of ergosterol, much is left to be explored about its role in Bax-induced cell death and more detailed physicochemical studies should shed light on this interesting problem.

For instance, comparing the transcriptional profile of wild type with Δ*OYE3* after Bax expression can identify the targets associated with Bax toxicity. The relationship between ergosterol levels and Bax toxicity can be determined via ergosterol feeding, as yeast cells take up sterols supplied in the growth medium proportionally (Lewis *et al.*, 1987). An *OYE3* overexpression in yeast and/or mammalian cells (no *OYE3* homologue present) can give additional information concerning its influence on Bax toxicity. It would be interesting to determine the extent of mitochondrial cyt-c release or Yca1 activation in Δ*OYE3* compared to WT upon Bax expression. Further, increased ergosterol incorporation in mitochondrial membranes could be investigated and the impact on membrane fluidity and/or pore formation determined.

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Chapter 9

Summary and Conclusion

Samenvatting met Besluit

In the present work, we investigated the transcriptional responses of *Saccharomyces cerevisiae* to the murine pro-apoptotic protein Bax via macroarray and microarray profiling. Both a kinetic concept and a comparative strategy were used in an attempt to unravel Bax toxicity at the molecular level.

To create a Bax expression system controlled by the *GAL1* promoter, a cDNA encoding the full-length mouse Bax-α protein was subcloned into the integrative plasmid pSCTyGAL1-L, creating pSCTyGAL1mBAX-L. The galactose-inducible nature of Bax expression was demonstrated by Western blotting. The functionality of the integrative Bax expression system was confirmed via a growth assay, demonstrating an inhibition of colony formation, and via a cell death assay, revealing a time-dependent increase in the percentage of dead cells.

Global gene expression during early and middle stages of Bax-induced cytotoxicity was determined using the yeast Genefilter® macroarray. The major drawback of this approach was illustrated by the observation that about 50% of the hybridisation signals were within background range. In total, 208 Bax-responsive genes were determined and half of them demonstrated an early response, pointing towards an active metabolic resetting of the cell upon Bax expression. In an attempt to identify the biochemical processes involved in Bax-induced cell death, the 208 Bax-responsive genes were grouped into functional categories. Due to the diversity of the affected cellular functions, this kinetic approach did not generate a clear insight into the biochemical mechanisms of Bax cytotoxicity. However, within the pool of genes that are regulated as the consequence of the cell death process, some others may encode proteins that are causal factors for Bax-induced cell death to occur.

We next used a comparative strategy in an attempt to extract the causal candidates for further study. Bax expression in *S. cerevisiae* is accompanied with the accumulation of Reactive Oxygen Species (ROS) (Madeo *et al.*, 1999) and therefore, we argued that the cellular response to induced Bax expression may contain a Bax-specific part –resulting in enhanced ROS generation- and a general pathway of oxygen stress-induced cell death. A comparative analysis of the Bax-induced and oxygen stress-induced transcriptional responses may thus identify the mechanism by which Bax proteins elicit an intracellular oxidative stress condition. A system was developed that mimics the generation and effects of endogenous ROS. As H_2O_2 is an effective ROS molecule for which membranes are highly permeable, it became an obvious choice. A H_2O_2 dose was optimised to elicit cell death to the same extent as induced by a 1-hour Bax expression, when both are used in the same conditions and for the same duration.

The transcriptional responses after Bax expression and H_2O_2 treatment were determined via a macroarray. The Bax-responsive genes that demonstrated a Bax-associated transcriptional response at least two-fold more manifest or with a different tendency compared to the H_2O_2-associated transcriptional response, were selected as Bax-specific candidate genes. The validation frequency of this approach was rather low (13%), and only 3 genes were finally verified to have a Bax-specific transcriptional response (*YLR414C*, *YGR146C* and *YJL060W*). These genes encode proteins of an unknown function not interfering with mitochondrial respiration. To analyse the effect of these genes on the rate of Bax-induced growth arrest and cell death, two additional Bax expression systems were

created. The switch towards centromeric episomal expression systems streamlined the phenotypical validation process, avoiding Southern applications for the control of integration events. Both a *GAL1*- and a *GALL*-driven Bax expression system were created, the latter promoter being a deletion variant of the former. The use of 2 Bax expression systems of different strength was chosen as a strategy to broaden the window in which to evaluate phenotypic effects. Bax protein levels were observed to be in accordance with promoter strength, as was the rate of Bax-induced growth arrest and cell death. Knocking out *YJL060W* had no effect on the rate of Bax-induced growth arrest (in both Bax expression systems). The knockout of *YLR414C* demonstrated a certain resistance (*GALL* system) and the knockout of *YGR146C* demonstrated some sensitivity (*GAL1* system) towards Bax-induced growth arrest.

In conclusion, the Bax versus H_2O_2 comparative macroarray approach identified the involvement of YLR414C and YGR146C, encoding proteins of unkown function, in the growth/cell cycle-disturbing activity of the Bax protein.

Another Bax versus H_2O_2 comparative macroarray experiment was conducted as a technical repeat for increasing the validation percentage. *However, the datasets of both repeats only demonstrated low overlap, which illustrated the unreliability of this method.* A third Bax versus H_2O_2 comparative experiment was conducted, as a technical repeat now using microarrays for transcriptional profiling. *The microarray approach identified a completely different subset of genes having a Bax-specific transcriptional response.* The validation frequency was higher (40%) and finally 4 genes (*MLS1*, *BTN2*, *OYE3* and *ICY2*) were selected to have a Bax-specific transcriptional response.

In conclusion, in our hands GeneFilter® macroarray transcriptional profiling was evaluated as unreliable because of (i) the partial overlap (40%) between datasets of technical repeats; (ii) the very low validation frequencies (13%); and (iii) the minor overlap (7%) between technically repeated datasets of GeneFilter® macroarray and oligo microarray, the latter of which has a higher validation frequency (40%).

Only the *OYE3* knockout demonstrated a reduction in Bax-induced growth arrest and cell death (*GALL* expression system), although the rate of H_2O_2-induced cell death was increased. The different phenotypical outcome of Δ*OYE3* upon oxidative stress compared to the Bax trigger was a nice reflection of the differential transcriptional response of this gene towards both stressors (Bax: 2.70-fold down, H_2O_2: 1.50-fold up). The absence of *OYE3* affected the progressive decline in NADPH, which is associated with Bax-induced and H_2O_2-induced cell death, to an extent in correlation with the cell death rate induced by these triggers in Δ*OYE3*. Bax-induced cell death was not observed to be associated with oxidative protein or mitochondrial DNA damage. However, the extent of lipid peroxidation was somehow indicative for the rate of Bax-induced or H_2O_2-induced cell death. The H_2O_2-sensitivity of Δ*OYE3* was in accordance with the more prominent lipid peroxidation during H_2O_2-induced cell death, while no lipid peroxidation was observed during Bax-induced cell death. Further, ergosterol levels were increased in the *OYE3* knockout strain and

ergosterol can increase bilayer stability both *in vitro* and *in vivo* (Astin and Haslam, 1977; Wiseman *et al.*, 1990).
In conclusion, the Bax versus H_2O_2 comparative microarray approach identified the involvement of OYE3 in the growth/cell cycle-disturbing and cytotoxic activity of the Bax protein.

As a general conclusion, our results indicate OYE3 to influence the molecular mechanism by which Bax proteins generate an oxidative stress condition and the increased ergosterol content in ΔOYE3 may interfere with Bax docking and/or its stable insertion in intracellular membranes.

In dit werk werden de transcriptionele veranderingen in *Saccharomyces cerevisiae* bepaald, tengevolge van de expressie van het pro-apoptotisch Bax eiwit van de muis. Hierbij werd gebruik gemaakt van de macro- en microarray technologie. Zowel een kinetisch concept als een vergelijkende subtractieve aanpak werden aangewend om de toxiciteit van Bax in gist tot op moleculair niveau te ontrafelen.

Een cDNA, coderend voor het volledige Bax-α proteïne, werd gekloneerd in de integratieve vector pSCTyGAL1-L, resulterend in het plasmide pSCTyGAL1mBAX-L, waardoor het open leesraam van Bax onder controle van de *GAL1* promotor werd geplaatst. Via Western blotting kon de galactose gereguleerde expressie van het Bax proteïne aangetoond worden. De functionaliteit van het Bax expressiesysteem werd enerzijds bevestigd via een groeitest, die een volledige inhibitie van kolonievorming aantoonde, en anderzijds via een celdoodtest waaruit bleek dat het percentage aan dode cellen exponentieel toenam in functie van de Bax-inductieperiode.

De globale transcriptionele veranderingen tijdens de vroege en middenstadia van de door Bax geïnduceerde cytotoxiciteit werden bepaald, gebruik makend van de GeneFilter® macroarray. Hierbij werd duidelijk dat ±50% van de hybridisatiesignalen zich in de achtergrond bevonden, wat onmiddellijk het grootste minpunt van macroarray-analyses illustreert. Tijdens het volledige Bax-geïnduceerd celdoodproces werd vastgesteld dat de transcriptionele status van 208 genen tijdens één of meerdere stadia wijzigde. De helft hiervan vertoonde een vroege respons, wat een actieve metabolische herprogrammering als gevolg van Bax expressie suggereert. In een poging om de biochemische processen, betrokken in Bax-geïnduceerde celdood, te identificeren, werden de 208 genen gegroepeerd in functionele categorieën. Door de grote diversiteit aan betrokken functionele groepen leverde de kinetische studie geen helder inzicht op in de biochemie van de Bax toxiciteit. Uiteraard werd de mogelijkheid niet uitgesloten dat een gedeelte van deze 208 genen transcriptionele veranderingen ondergingen die aan de grondslag lagen van het Bax-geïnduceerd celdoodproces. Om deze oorzakelijke regulaties van bijkomstige effecten te kunnen onderscheiden werd in eerste instantie geopteerd voor een vergelijkende macroarray strategie.

Aangezien de expressie van het Bax proteïne aanleiding geeft tot het ontstaan van reactieve zuurstof moleculen (ROS) (Madeo *et al.*, 1999), werd de hypothese gesteld dat het cellulaire antwoord volgend op Bax expressie zowel een Bax-specifiek gedeelte (resulterend in een verhoogde ROS productie) als een algemeen gedeelte (leidend tot ROS-geïnduceerde celdood) kon bevatten. Een vergelijkende studie van de Bax-geïnduceerde en ROS-geïnduceerde transcriptionele respons werd verondersteld het moleculair mechanisme, waarbij Bax proteïnen aanleiding geven tot het ontstaan van ROS, te kunnen verhelderen. Om de intracellulaire accumulatie van ROS zo goed mogelijk te benaderen, werd gekozen voor H_2O_2, dat erom bekend staat sterk membraanpermeabel te zijn. Vervolgens werd de H_2O_2 dosis geoptimaliseerd om binnen hetzelfde tijdsbestek (1 uur) en onder dezelfde condities een zelfde finale toxiciteit te bekomen als na Bax expressie.

De transcriptionele veranderingen volgend op Bax expressie of H_2O_2 behandeling werden bepaald via de GeneFilter® macroarray. We vestigden speciaal onze aandacht op die Bax-responsieve genen

waarvan de Bax-geïnduceerde transcriptionele verandering ten minste tweemaal sterker was in vergelijking met de H_2O_2-geïnduceerde wijziging of een duidelijk andere tendens vertoonde. De genen die op deze manier konden aangeduid worden, werden als Bax-specifiek beschouwd. De validatiefrequentie binnen deze groep van genen was echter laag (13%) en slechts 3 genen werden uiteindelijk weerhouden omwille van hun Bax-specfieke transcriptionele verandering (*YLR414C, YGR146C* en *YJL060W*). Deze genen coderen voor proteïnen met een onbekende functie, die niet interfereren met de mitochondriale respiratie. Om het effect van deze genen op Bax-geïnduceerde groeivertraging en Bax-geïnduceerde celdood efficiënt na te gaan, werden 2 bijkomende centromeer episomale Bax expressiesystemen ontwikkeld, zodat Southern applicaties ter controle van genomische integratie konden vermeden worden. Er werd gekozen om Bax expressie zowel onder controle van de *GAL1* promotor als onder controle van een deletievariant (*GALL* promotor) te plaatsen, waardoor het analysevenster zo groot mogelijk kon gehouden worden. De graad van promotorsterkte was in overeenstemming met het Bax expressieniveau en de daaropvolgende groeivertraging en celdood. Het verwijderen van *YJL060W* had geen invloed op de Bax-geïnduceerde groeivertraging, en dit zowel binnen het *GAL1*- als *GALL*-expressiesysteem. Echter, bij afwezigheid van *YLR414C* of *YGR146C* kon respectievelijk een zekere resistentie (*GALL*-systeem) of sensitiviteit (*GAL1*-systeem) voor Bax-geïnduceerde groeivertraging vastgesteld worden.

Samenvattend kunnen we stellen dat, via een vergelijkende Bax versus H_2O_2 macroarray studie de betrokkenheid van YLR414C en YGR146C, coderend voor proteïnen met ongekende functie, binnen de groei/celcyclus-verstorende activiteit van Bax werd vastgesteld.

Er werd een bijkomende Bax versus H_2O_2 macroarray studie uitgevoerd als een technische herhaling van het eerste macroarray experiment, met als doel de lage validatiegraad te verhogen. *De datasets van beide herhalingen vertoonden een lage overlappingsgraad, wat de onbetrouwbaarheid van de methodologie illustreerde.* Er werd een derde vergelijkende Bax versus H_2O_2 studie uitgevoerd die eveneens moest dienen als een technische herhaling van het macroarray experiment, nu echter gebruik makend van microarrays. *Er werd een volledig andere lijst van genen met een Bax-specifieke transcriptionele respons bekomen.* De validatiefrequentie was ditmaal hoger (40%) en 4 genen werden weerhouden (*MLS1*, *BTN2*, *OYE3* en *ICY2*).

Samenvattend kunnen we concluderen dat de transcriptionale profilering aan de hand van een GeneFilter® macroarrasy onbetrouwbaar blijkt te zijn en dit omwille van (i) de gedeeltelijke overlap (40%) tussen de datasets van technische herhalingen; (ii) de zeer lage validatiegraad (13%); and (iii) de minimale overlap (7%) tussen de datasets van technische herhalingen die gebruik maken van een GeneFilter® macroarrasy en oligo microarrays, waarbij deze laatste een beduidend hogere validatiegraad bezit (40%).

Het bleek echter dat enkel de afwezigheid van *OYE3* een invloed had op de Bax-geïnduceerde groeivertraging, waarbij een zekere resistentie binnen het *GALL*-systeem werd vastgesteld. De differentiële transcriptionele verandering van *OYE3* als gevolg van Bax expressie (2.70-voudige

daling) en H_2O_2-behandeling (1.50-voudige stijging) werd bovendien gereflecteerd in de mate van gevoeligheid van Δ*OYE3* voor Bax-geïnduceerde celdood (resistentie) en H_2O_2-geïnduceerde celdood (sensitiviteit). De afwezigheid van *OYE3* had ook een invloed op de daling in NADPH niveau, die optreedt tijdens Bax-geïnduceerde en H_2O_2-geïnduceerde celdood, zodat een correlatie kon vastgesteld worden tussen de mate van NADPH daling en de celdoodgevoeligheid. Verder werd nagegaan in welke mate de Bax-geïnduceerde ROS accumulatie oxidatieve beschadigingen teweeg kon brengen ter hoogte van mitochondriaal DNA, lipiden en proteïnen. We stelden vast dat de gebruikte technieken niet toelieten noch oxidatief beschadigd mitochondriaal DNA, noch carbonyl-gemodificeerde eiwitten te detecteren. Bax-geïnduceerde en H_2O_2-geinduceerde celdood gingen echter wel gepaard met lipide peroxidatie. De gevoeligheid van Δ*OYE3* voor H_2O_2 was in overstemming met de meer uitgesproken lipide peroxidatie tijdens H_2O_2-geïnduceerde celdood, en dit terwijl geen lipide peroxidatie kon aangetoond worden tijdens Bax-geïnduceerde celdood in Δ*OYE3*. Bovendien bleek in afwezigheid van *OYE3* een duidelijk toegenomen gehalte aan ergosterol, een molecule die zowel *in vitro* als *in vivo* de stabiliteit van membranen verhoogt (Astin and Haslam, 1977; Wiseman *et al.*, 1990).
Samenvattend kunnen we stellen dat, via een vergelijkende Bax versus H_2O_2 microarray studie de betrokkenheid van OYE3 binnen de groei/celcyclus-verstorende en cytotoxische activiteit van Bax werd vastgesteld.

Onze resultaten tonen aan dat OYE3 een invloed heeft op het mechanisme waarbij Bax proteïnen een intracellulaire oxidatieve stress genereren, waarbij het toegenomen ergosterol gehalte in ΔOYE3 kan interfereren met de aanhechting of de stabiele integratie van Bax proteïnen ter hoogte intracellulaire membranen.

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Addendum I

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Bax-induced cell death in *Pichia pastoris*

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Abstract

Murine Bax was expressed in the methylotrophic yeast, *Pichia pastoris*, using the alcohol oxidase 1 (*AOX1*) or alcohol oxidase 2 (*AOX2*) promoter and the *AOX1* terminator. Upon induction in methanol medium, transformants containing *BAX* cDNA under control of the strong *AOX1* promoter showed complete growth inhibition and extensive cell death. Except for chromatin condensation, morphological changes typical of apoptosis in mammalian cells could not be observed, indicating that the cell death machinery in *P. pastoris* is marked different from the endogenous cell death program of higher eukaryotes. Staining of Bax-induced cells with propidium iodide indicated that cell death was not correlated with necrosis. Electron microscopic examination revealed no striking differences in cell morphology, but showed few cells with an enlarged vacuole containing spherical bodies, which suggests autophagic cell death.

Introduction

Cell death in budding yeast is a natural process that occurs during growth of yeast cell populations; it can considerably be stimulated by several external and/or endogenous factors. A clear understanding of these factors is important during industrial fermentation to maintain culture viabilities at high levels or when eradication of undesirable yeast strains is required. Major advances in our understanding of cell death are related to the effects of certain physical stress parameters on yeast cell physiology. Yeast will die if confronted with excessive heat, extreme cold, high-voltages, ionizing radiation, and high hydrostatic and osmotic pressures. Death results when the protection responses are insufficient to counteract the cellular damage caused by severe physical stress. Besides physical parameters, there are numerous external chemicals and internal biochemical reactions which occur outside and inside the yeast cells and which may result in their death. External chemical factors lethal to yeast include the presence of toxic compounds in the culture medium, abnormal pH or nutrient starvation. With regard to endogenous factors, accumulation of toxic metabolites may occur intracellularly. In addition, numerous biological events take place in yeast cells which may lead to 'self-inflicted' death. Autophagic death occurs, for example, when vacuolar hydrolytic proteases cause dissolution of the protoplasm; autolysis, on the other hand, is caused by carbohydrases that destruct the cell envelope. Finally, also genetic factors are important. Many mutations in the genes of the baker's yeast *Saccharomyces cerevisiae* proved to be lethal. Moreover, Lydall & Weinert (1996) described how yeast cells may commit suicide when their DNA is damaged, presumably to avoid the risk of producing genetically altered progeny.

Recent studies indicate that expression of mammalian Bax triggers cell death in *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe* involving

morphological changes similar to apoptosis (Jürgensmeier *et al.* 1997, Manon *et al.* 1997, Ligr *et al.* 1998). Bax is a 21 kDa pro-apoptotic member of the Bcl-2 protein family and is widely expressed in mammalian tissues. The mechanism by which Bax promotes apoptosis is only partially elucidated, but dimerization and targeting to the mitochondrial membrane appear to be essential to exert cytotoxicity in both yeast and mammalian cells (Zha *et al.* 1996). Electron microscopic analysis of yeast cells dying in response to Bax expression demonstrated chromatin condensation, dissolution of the nuclear envelope and massive cytosolic vacuolization (Jürgensmeier *et al.* 1997, Ligr *et al.* 1998); this suggests that the basic steps of the evolutionary conserved metazoan pathway leading to programmed cell death are present in unicellular eukaryotes. The finding was confirmed by Madeo *et al.* (1997) who studied a *S. cerevisiae* mutant in cell division cycle gene *CDC48* with typical markers of early and late apoptosis. Here, we describe Bax-induced cell death in the methylotrophic yeast *Pichia pastoris*. We found that cell death could not simply be attributed to necrosis or apoptosis. Nevertheless, we believe that a basic programmed cell death machinery exists in *P. pastoris* and becomes activated by expression of mammalian Bax.

Materials and methods

Strains and culture conditions

Escherichia coli MC1061 (F^- *ara*D139 Δ(*ara leu*)7697 Δ*lac*X74 *gal*U *gal*K *hsd*R2(r_k^-, m_k^+) *mcr*B1 *rps*L str^R) was used to construct and to amplify plasmid DNA. The *Pichia pastoris* host strain used in all experiments was GS115 (*his4*) from Invitrogen (San Diego, CA). Recombinant *P. pastoris* strain GSML20 (M. Logghe, unpublished) contained an integrated cDNA expression cassette encoding secreted human interleukin-6. Yeast strains were grown under continuous shaking at 28–30 °C either in YPD [2% (w/v) dextrose, 2% (w/v) peptone (Difco Laboratories, Detroit, MI), 1% (w/v) yeast extract (Difco Laboratories)] or in minimal dextrose medium containing 1% (w/v) dextrose and 1.34% (w/v) yeast nitrogen base (Difco Laboratories). To induce the *P. pastoris* *AOX1* or *AOX2* promoter, 0.5% (v/v) methanol was added instead of dextrose. Methanol induction was maintained by adding fresh methanol to the culture medium every 12 h. *E. coli* strains were grown in Luria-Bertani (LB) or Terrific Broth (TB) at 37 °C supplemented with 100 μg ampicillin/ml for plasmid selection. Zeocine was purchased from Invitrogen and used as cytotoxic drug in both bacteria and yeast for the selection and amplification of plasmid DNA containing the bleomycin resistance gene. Low salt LB medium [1% (w/v) tryptone, 0.5% (w/v) NaCl, 0.5% yeast extract], adjusted to pH 7.5 with 5 M NaOH and supplemented with 25 μg ml^{-1} zeocine, was used for selection in *E. coli*. *P. pastoris* strains containing the zeocine resistance marker were grown in YPD supplemented with zeocine at 100 μg ml^{-1}.

Construction of Bax expression plasmids and transformation to P. pastoris

Mouse *BAX* cDNA was excised from pUC19B (R. Reekmans, unpublished) by digestion with *Xba*I and *Hin*dIII and subcloned into *Eco*RI (blunted)-opened pHIL-D2 (Invitrogen), obtaining the expression plasmid pHILBAX. The *AOX2* promoter was generated by *Pfu* DNA polymerase (Stratagene, Heidelberg, FRG) PCR from *P. pastoris* GS115 genomic DNA by using the *AOX2* primers 5′-GACGAGATCTTTTTTTCAGACCATATGACCGG-3′ and 5′-GCGGAATTCTTTTCTC-AGTTGATTTGTTTGT-3′. The PCR-product was *Eco*RI-*Bgl*II digested and cloned in the similarly opened plasmid pPICZB (Invitrogen), resulting in pAOX2ZB. Subsequent insertion of the *AOX2* promoter, as a *Bgl*II-*Not*I fragment from pAOX2ZB, in *Not*I-partial *Bgl*II treated pPIC9 (Invitrogen) resulted in pAOX2, thereby replacing the *AOX1* promoter with that of *AOX2*. Plasmid pAOX2 was linearized with *Xho*I, end-filled with T4 DNA polymerase and dNTPs and subsequently digested with *Eco*RI. The pAOX2 vector fragment was religated with a *Hin*dIII (blunted)-*Eco*RI fragment from pUC19B containing *BAX* cDNA so that pAOX2BAX was obtained. Retransformation of recombinant *P. pastoris* GS115, containing one or more copies of pHILBAX, with an additional Bax expression cassette was possible after construction of pPICZBBAX. This plasmid was created as follows: pUC19B was linearized with *Hin*dIII, end-filled with Klenow polymerase and dNTPs, and subsequently digested with *Eco*RI. This fragment was ligated into *Not*I (blunted)-*Eco*RI opened pPICZB.

Spheroplast transformations were carried out with a *Pichia* expression kit (Invitrogen) according to the manufacturer's instructions. Expression plasmid pPICZBBAX containing the *Streptoalloteichus*

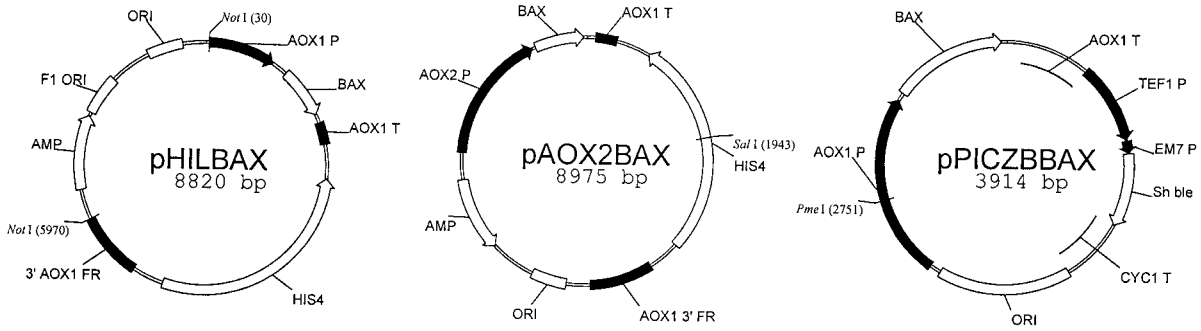

Fig. 1. Schematic representation of the constructed *P. pastoris* expression vectors for the production of murine Bax. AOX1 P, *AOX1* promoter; AOX2 P, *AOX2* promoter; AOX1 T, *AOX1* transcription termination signal; 3′ AOX1 FR, *AOX1* 3′ untranslated region; AMP, ampicillin resistance gene; ORI, ColE1 origin of replication; Sh ble, *Streptoalloteichus hindustanus* bleomycin gene.

hindustanus bleomycin gene as dominant selection marker was transformed to *P. pastoris* GS115 by electroporation according to the recommendations of Invitrogen.

Immunoblot assays

SDS/PAGE (15% acrylamide gels) and Western blotting of cell lysates were performed according to standard procedures. After transfer to nitrocellulose membranes (Schleicher & Schuell, Dassel, FRG), Bax was detected by using an affinity-purified anti-Bax rabbit polyclonal antiserum (BaxΔ21; Santa Cruz Biotechnology, Santa Cruz, CA) followed by an alkaline phosphatase-conjugated anti-rabbit immunoglobulin G from goat (Sigma).

Characterization of Bax-induced cell death

Necrotic cell death was investigated by labeling cells with propidium iodide (PI). Briefly, 10^7 cells were harvested by centrifugation and resuspended in 1 ml sterile water. After addition of PI (1.5 μl from a 3 mM stock solution; Molecular Probes, Eugene, OR), the mixture was incubated for 1 min at room temperature. Next, cells were centrifuged and resuspended in 1 ml of water to remove unbound PI. A 150 μl aliquot was added to the wells of a microtiter plate and excited immediately in a CytoFluor multi-well plate reader (PerSeptive Biosystems, Cambridge, MA) at 530 nm. Fluorescence was measured at 620 nm. In the case of chromatin condensation analysis, cells were fixed in acetic acid/methanol (1:9, v/v) prior to PI staining of DNA. To determine DNA degradation, genomic DNA was isolated with a Nucleon MiY yeast DNA extraction kit (Amersham Pharmacia Biotech, Rainham, UK) according to the manufacturer's instructions. A CHEF yeast genomic DNA plug kit (Bio-Rad, Richmond, CA) was used to prepare chromosome-sized DNA. Pulsed-field gel electrophoresis was carried out on a Bio-Rad CHEF-DRIII apparatus in 0.8% Seakem gold agarose (FMC, Rockland, ME) at 3 V cm^{-1} in 1 $\times$ TAE-buffer and chilled to 14 °C. Running time was 40 h with an included angle of 106° and with a switch time of 750 s. Chromosomes of *Hansenula wingei* (Bio-Rad) were used as size markers. For nuclear staining, cells were washed with PBS, incubated with 1 μg ml^{-1} diaminophenylindole (DAPI) in PBS for 10 min, and then rinsed three times with PBS. An in situ cell death detection kit (Boehringer, Mannheim, Germany) was used according to a previously described protocol for TUNEL detection of fragmented nuclear DNA in yeast (Madeo *et al.* 1996). Externalization of phosphatidylserine (PS) was examined by reaction with FITC-coupled annexin V (Annexin-V-FLUOS staining kit; Boehringer) according to Madeo *et al.* (1996).

Electron microscopy

Cells were fixed for 24 h at 4 °C in fixation reagent [2% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1% (w/v) cacodylate buffer, containing 1 M sorbitol and 0.05% (w/v) $CaCl_2$, and adjusted to pH 7.4]. The fixative was replaced by cacodylate buffer and the cells were pelleted in a microcentrifuge tube. Post-fixation (1 h) was carried out in OsO_4 [1% (w/v) in cacodylate buffer]. During dehydration the cells were stained 'en bloc' with uranyl acetate [2% (w/v) in 50% ethanol] and in saturated lead acetate in ethanol-acetone (1:1, v/v), both for 1 h. The pellets were embedded in Araldite (Araldite-DDSA-MNA-BDMA = 1:0.85:0.15:0.04). Thin and ultrathin sections were made with a Reichert-Jung Ultracut E.

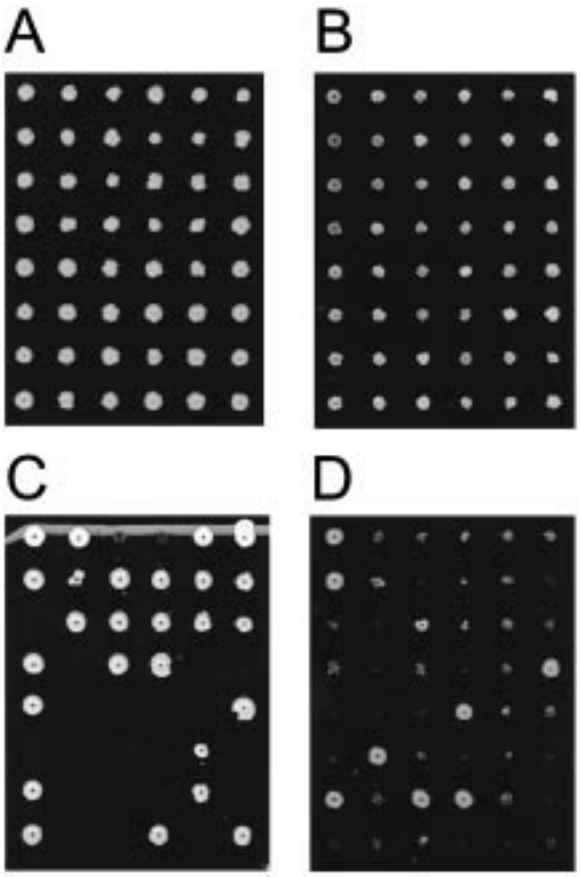

Fig. 2. Growth of *P. pastoris* pHILBAX transformants on minimal medium, containing 1% dextrose (A), 1% mannitol (B) or 0.5% methanol (C). Cells were grown in a 96-well microtiter plate, filled with minimal dextrose medium, and then gridded to semi-solid medium. Only one half of the plates is shown. (D) growth of *P. pastoris* on methanol after transformation with pAOX2BAX.

The sections were post-stained in a Reichert-Jung Ultrostainer, programmed for 30 min uranyl acetate (40 °C) and 10 min lead citrate (20 °C) staining. The micrographs were made on a JEOL JEM 1010 transmission electron microscope.

Results

A cDNA encoding the full-length mouse Bax protein was placed under transcriptional control of the methanol-inducible *AOX1* or *AOX2* promoter and the *AOX1* terminator sequences from *P. pastoris*, resulting in expression plasmids pHILBAX and pAOX2BAX, respectively (Figure 1). According to Cregg *et al.* (1989), the promoter of the *AOX1* gene is responsible for the vast majority of alcohol oxidase activity in the cell (typically ∼5% of total soluble protein after methanol induction) and is tightly regulated at the transcriptional level; *AOX2* is transcribed at a much lower level than *AOX1*, but is regulated similarly (Ohi *et al.* 1994). Plasmids pHILBAX and pAOX2BAX were integrated into the *P. pastoris* GS115 (*his4*) genome by transformation of spheroplasts with 10 μg *Not*I-digested (pHILBAX) or *Sal*I-linearized (pAOX2BAX) plasmid DNA; transformants were selected on histidine-free minimal medium. Ninety-six randomly chosen transformants of both constructs were grown in the wells of a microtiter plate filled with minimal dextrose medium and, after 24 h incubation at 30 °C, gridded to different solid media containing 1% dextrose (non-inducing condition), 1% mannitol (weak induction; Sears *et al.* 1998) or 0.5% methanol (strong induction) as sole carbon source. All transformants showed growth on glucose (Figure 2A) and to a lesser extent on mannitol as well (Figure 2B). Several candidates transformed with pHILBAX, however, failed to grow on methanol (Figure 2C). Southern blot analysis of these candidates revealed one copy of the transformed DNA integrated into the endogenous *AOX1* locus (data not shown). In a few instances, a gene replacement event had occurred due to a double crossover event between the *AOX1* promoter and the 3′ *AOX1* regions of the vector and the genome. This resulted in complete removal of the *AOX1* coding region, yielding a His^+Mut^S (Methanol utilization Slow) phenotype. A significant percentage of His^+ transformants did not contain the expression vector. This appeared to be the result of gene conversion events between the *HIS4* gene on the vector and the *P. pastoris his4* locus, such that the wild-type *HIS4* gene recombined into the genome without any additional vector sequences. In general, these events account for 10–50% of His^+ transformant colonies (Higgins & Cregg 1998), explaining why many pHILBAX transformants survived methanol induction (Figure 2C). Compared to His^+Bax^+ cells, His^+Bax^- transformants demonstrated equal growth impairment on mannitol-containing medium. Therefore, it is concluded that the decreased growth rate of pHILBAX transformants on mannitol was the result of less efficient carbon source assimilation and not the consequence of weak Bax expression. Most pAOX2BAX transformants showed poor growth on methanol (Figure 2D), indicating that the Bax expression level driven by the *AOX2* promoter was too low to inhibit growth completely. One positive transformant

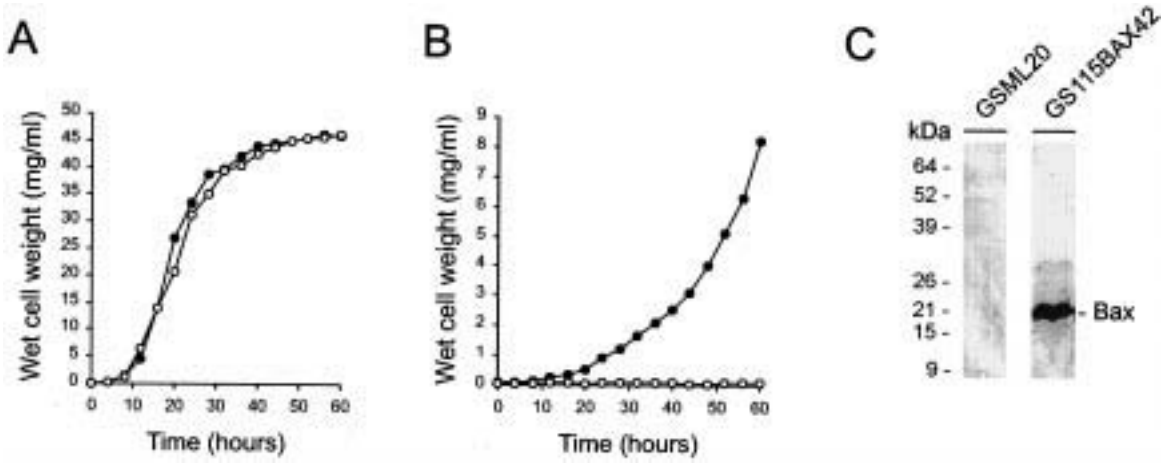

Fig. 3. Growth kinetics of GSML20 (●) and GS115BAX42 (○) in minimal dextrose (A) or methanol (B) medium. Intracellular accumulation of Bax was observed within 24 h after methanol induction of GS115BAX42 cells (C).

of pHILBAX with a $His^+Bax^+Mut^S$ phenotype was selected for further use and named GS115BAX42.

P. pastoris strain GSML20, secreting the non-toxic human interleukin-6, as well as the Bax-producing strain GS115BAX42 were grown in liquid media under induced and non-induced conditions to determine growth kinetics. Since GSML20 has a Mut^S phenotype and since heterologous interleukin-6 expression is regulated by the *AOX1* promoter, we preferred this strain as a negative control to study differences in growth, rather than untransformed GS115 or GS115 transformed with the parental plasmid pHIL-D2. GSML20 and GS115BAX42 were inoculated in minimal dextrose medium and incubated for several hours at 30 °C until the wet cell weight was approximately 65 mg l^{-1}. Subsequently, cells were switched to fresh medium containing either dextrose or methanol. Growth of both strains in dextrose was comparable (Figure 3A), but expression of Bax in methanol-containing broth inhibited growth of GS115BAX42 completely (Figure 3B), thereby confirming the data mentioned above. Western blot analysis showed accumulation of Bax protein of the correct length in GS115BAX42 cells shortly after induction, but not in untransformed control cells nor in GSML20 (Figure 3C). Cell viability was tested at several time points by plating known cell numbers on a minimal dextrose solid medium. Viability of GS115BAX42 declined rapidly as shown in Figure 4. After 72 h incubation in methanol-containing broth, about 99% of the culture was no longer viable, whereas approximately 0.05% appeared to be death-resistant. Introduction of an additional Bax expression cassette by retransforming GS115BAX42 with 10 μg *Pme*I-linearized pPICZBBAX did not influence the course of cell death, suggesting that the level of Bax protein generated from a single copy was sufficient to execute the cell death process. Taken together, we demonstrate that growth inhibition under inducing conditions was caused by cell death and not simply by growth arrest, an observation which is in accordance with previous results described for *S. cerevisiae* and *S. pombe*.

Furthermore, we characterized cell death by labeling cells with PI. The amount of PI-positive cells gradually increased during fermentation (Figure 5), indicating necrotic cell death. However, considering the fast decline of GS115BAX42 viability early after induction (Figure 4), one can imagine that necrosis is the natural outcome of nutrient starvation, decrease in pH and accumulation of toxic compounds in the culture medium, but not the result of Bax expression. Because cells were not preadapted to methanol, the level of necrotic cell death immediately after inoculation was higher in methanol compared to dextrose cultures. In contrast, the increase in PI-positive cells was more pronounced in non-induced conditions, probably due to the more efficient growth and faster depletion of nutrients in dextrose.

In order to identify morphological changes similar to apoptosis in mammalian cells, typical indicators of apoptosis, such as exposure of PS on the outer leaflet of the cytoplasmic membrane, chromatin condensation, DNA breakage and abnormal cell morphology were studied. Externalization of PS to the outer phase of the cytoplasmic membrane, a phenomenon which

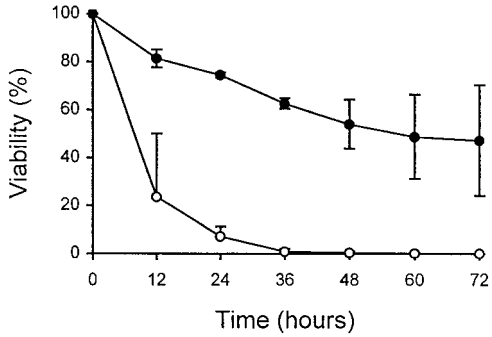

Fig. 4. Viability of GSML20 (●) and GS115BAX42 (○) cells after induction in methanol. Cells were grown in minimal dextrose medium and then switched to fresh minimal medium containing methanol. Every 12 h, samples were taken and equal cell amounts were spread on dextrose semi-solid medium. The appearing colonies represented the viable fraction of the total pool. The viability was considered 100% when the number of appearing colonies equals the amount of plated cells.

is considered an early event during apoptosis, was verified 8 h and 16 h after induction by removing the cell wall with Zymolyase and incubating the resulting spheroplasts with FITC-labeled annexin V. In contrast to *S. cerevisiae*, where this approach led to the detection of green halos surrounding the cells (Ligr *et al.* 1998), overexpression of Bax in *P. pastoris* did not lead to a significant loss of membrane asymmetry and exposure of PS on the cell exterior. Chromatin condensation was followed by PI staining of acetic acid/methanol treated cells. At 24 h after induction of *BAX* transcription, many GS115BAX42 cells showed clear signs of chromatin condensation (Figure 6). However, nuclear fragmentation or changes in the physical intactness of DNA could not be detected. Analysis of genomic DNA preparations at different times after induction did not point to smearing as a result of DNA degradation nor to DNA laddering. Electrophoretic separation of GS115BAX42 chromosomes by pulsed-field gel electrophoresis showed intact bands, excluding the possibility of specific DNA cleavage as previously shown in *S. pombe* cells expressing the closely related death-promoting protein Bak (Ink *et al.* 1997). Finally, the TUNEL assay was used to visualize DNA fragmentation in individual cells. Two days after induction, GS115BAX42 cells were fixed with formaldehyde, digested with Zymolyase and incubated with FITC-labeled dUTP and terminal deoxynucleotidyl transferase. Bax-expressing cells as well as negative control cells showed unstained or only slightly green nuclei, indicating that DNA cleavage was weak or non-existing.

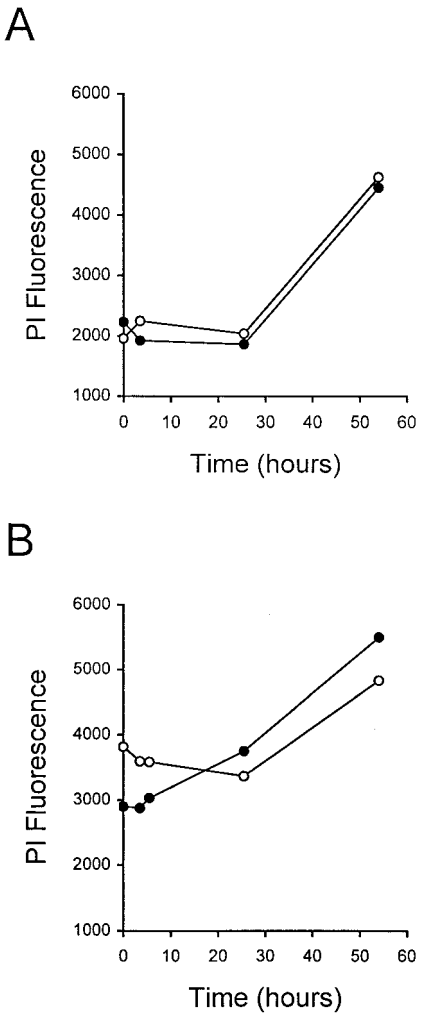

Fig. 5. Fluorescence of GSML20 (●) and GS115BAX42 (○) cells after labeling with PI. Cells were grown in minimal dextrose medium and then switched to either fresh minimal dextrose medium (A) or minimal medium containing methanol (B). From that moment, samples were taken at several time points and labeled with PI.

Electron micrographs of representative GS115BAX42 cells were taken 24 h after methanol induction. In comparison with GSML20 control cells, no striking differences in cell morphology could be demonstrated (Figure 7A-C). The cells maintained an intact contour and contained an unfragmented nucleus. The plasma membrane showed many large invaginations. This is not due to Bax expression, but most likely the result of hyperosmotic stress during preparation of samples. However, some cells appeared to contain enlarged vacuoles in which several spherical bodies (approximately 100 nm diameter) were present (Figure 7D). In these

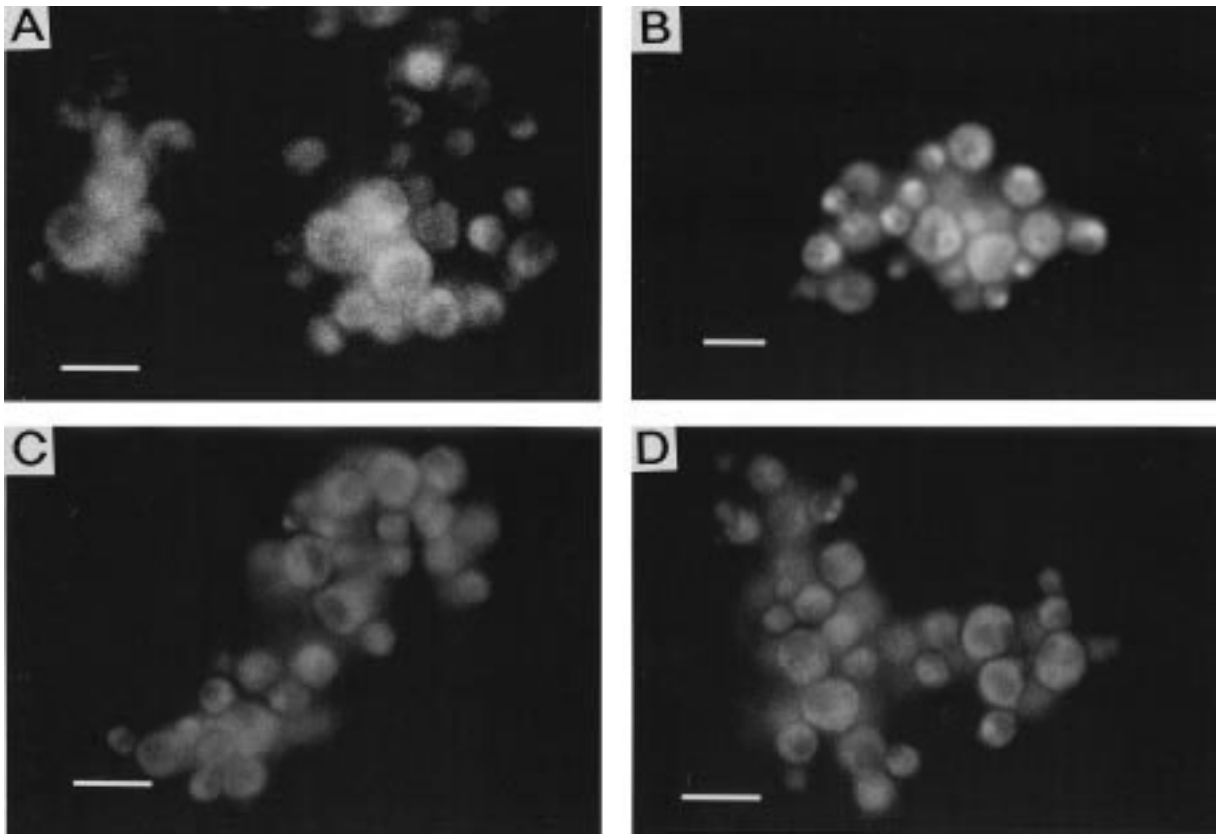

Fig. 6. Chromatin condensation in *P. pastoris* cells expressing Bax as shown by PI staining. GS115BAX42 and GSML20 control cells were grown in minimal dextrose or methanol medium for 24 h. The cells were then fixed in acetic acid/methanol (1:9, v/v) and labeled with PI. (A) GS115BAX42 in dextrose medium. (B) GS115BAX42 in methanol medium. (C) GSML20 in dextrose medium. (D) GSML20 in methanol medium. Bars represent 5 μm.

cells, most cytosolic organelles were lost, suggesting that the cellular components were sequestered to the vacuole for degradation.

Discussion

Recently, a number of groups have shown that the cell-killing activity of Bax appears to function in several biological systems ranging from eukaryotic unicellular organisms to mammalian cells (Zha *et al.* 1996, Manon *et al.* 1997, Jürgensmeier *et al.* 1997, Ligr *et al.* 1998). This includes the methylotrophic yeast *P. pastoris*, as described in the present study. Bax-induced cell death in *P. pastoris* could not simply be contributed to necrosis. On the contrary, there is accumulated evidence that the cytotoxic effects of Bax in yeast are relevant to their mechanism of pro-apoptotic action in mammalian cells. This is consistent with our observation that Bax-induced cell death in *P. pastoris* leads to chromatin condensation, a common hallmark of cells programmed to die. Two models that could explain this finding have been proposed (Fraser & James 1998). One is that yeast species contain an endogenous cell death program that is in part homologous to the metazoan cell death machinery. Even though yeast does not contain obvious homologues of known metazoan cell death regulators, such as caspases or Bcl-2 member proteins, both metazoan and the yeast death machinery could presumably have descended from a hypothetical ancestral death program. An alternative interpretation is that Bax is not activating an endogenous 'death program', but cell death is caused solely by the toxicity of the Bax protein itself, mediated by a hypothetical pore-forming function. Indeed, overexpression of Bax-like proteins (or their enforced dimerization) killed mammalian cells even in the presence of a caspase inhibitor, provoking DNA condensation and membrane alternations without caspase activation and DNA degradation (Xiang *et al.* 1996, McCarthy

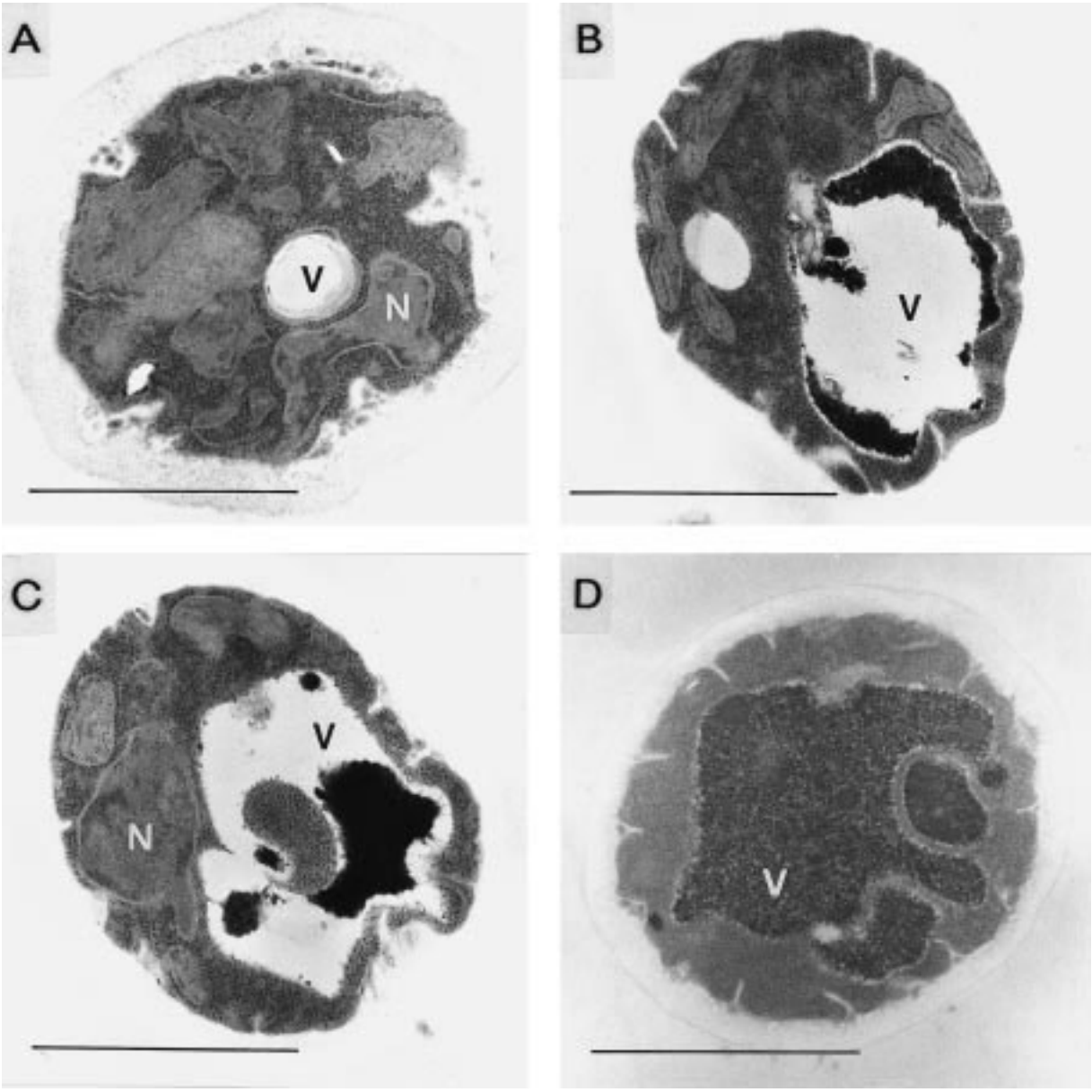

Fig. 7. Electron microscopy analysis of Bax-mediated cell death in *P. pastoris* 24 h after methanol induction. (A) GSML20 control cell. (B-D) GS115BAX42 cells expressing murine Bax. A vacuolar autophagosome-like structure with cytosolic content (ribosomes) is clearly visible in panel C. A representative cell with increased vacuolization and presence of small spherical bodies in the vacuole is shown in panel D. Bars represent 1 μm. N, Nucleus; V, Vacuole.

et al. 1997, Gross *et al.* 1998). Moreover, expression of pro-apoptotic effector proteins such as granzyme B (Pham *et al.* 1998) or caspase-3 (Sun *et al.* 1997) in *P. pastoris* did not activate pathways leading to cell death. Here, we demonstrate that certain morphological changes, typical of apoptosis in mammalian cells, including exposure of PS, DNA breakage and nuclear fragmentation, were absent in *P. pastoris*. These data indicate that the cell death process in *P. pastoris* (and presumably in other yeast species as well) induced after intracellular Bax accumulation is marked different from the caspase network in mammalian cells. Interestingly, Zha *et al.* (1996) claimed that Bax-induced cell death in *S. cerevisiae* resembles autophagy, with dissolution of the internal organelles and vacuolization. In this regard, Hammond *et al.* (1998) isolated

and characterized a human apoptosis-specific protein that is expressed in the cytoplasm of cultured mammalian cells following induction of apoptosis and that shows striking homology with the yeast *APG5* gene necessary for autophagy. This may be consistent with our observation that some Bax-expressing cells contained enlarged vacuoles with a few autophagic body-like structures inside. These data indicate a possible relationship between apoptosis and autophagy, and suggest evolutionary conservation in mammalian apoptosis of a degradative process present in yeast. Taken together, our observations and that of other groups suggest that the type of Bax-induced cell death in yeast is rather controversial and thus requires further investigation. However, at least some aspects of Bax protein function may be operative in yeast, leading to some kind of programmed cell death.

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(54) Title: CELL DEATH RELATED DRUG TARGETS IN YEAST AND FUNGI

(57) Abstract: The invention describes the use of nucleic acids and polypeptides which are involved in a pathway eventually leading to programmed cell death of yeast or fungi for the preparation of a medicament for treating diseases associated with yeast or fungi or for the treatment of proliferative disorders or for preventing apoptosis in certain diseases. Methods are provided to identify compounds which selectively modulate the expression or functionality of said polypeptides in the same or a parallel pathway. Also provided are compounds as well as pharmaceutical compositions, medicaments and vaccines. The invention also comprises new nucleic acid sequences, probes and primers derived thereof, expression vectors and host cells transformed with said vectors, polypeptides and antibodies raised against said polypeptides.

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(54) Title: BAX-RESPONSIVE GENES FOR DRUG TARGET IDENTIFICATION IN YEAST AND FUNGI

(57) Abstract: The invention describes the use of nucleic acids and polypeptides which are involved in a pathway eventually leading to programmed cell death of yeast or fungi for the preparation of a medicament for treating diseases associated with yeast or fungi or for the treatment of prolifeative disorders or for preventing apoptosis in certain diseases. Methods are provided to identify compounds which selectively modulate the expression or functionality of said polypeptides in the same or a parallel pathway. Also provided are compounds as well as pharmaceutical compositions, medicaments and vaccines. The invention also comprises new nucleic acid sequences, probes and primers derived thereof, expression vectors and host cells transformed with said vectors, polypeptides and antibodies raised against said polypeptides.