Marta Pinheiro Torres Marques

Oxidative stress in *Saccharomyces cerevisiae*: the key role of Isc1p sphingomyelinase in cell resistance, and the molecular mechanisms associated to the recovery from hydrogen peroxide stress

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Instituto de Ciências Biomédicas de Abel Salazar
Universidade do Porto

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Dissertação de Candidatura ao grau de Doutor em Ciências Biomédicas, especialidade de Bioquímica, submetida ao Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto.

Orientador - Professor Doutor Pedro Moradas-Ferreira (Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Portugal).

Universidade do Porto

2004

Aos meus Pais, à Be

Ao Paulo e à Matilde

De acordo com o nº 2 do Artigo 8º do Decreto-Lei nº 388/70, parte dos resultados apresentados nesta dissertação encontram-se em preparação para publicação, como a seguir se discrimina:

Marta Marques, Dominik Mojzita, Maria A. Amorim, Teresa Almeida, Stefan Hohmann, Pedro Moradas-Ferreira and Vítor Costa. The Pep4 vacuolar protease is required for the turnover of oxidised proteins in *Saccharomyces cerevisiae*.

No cumprimento do Decreto-Lei acima mencionado, foi decidido apresentar esta dissertação na forma de artigos. Declara-se ainda serem da nossa responsabilidade o planeamento e execução das experiências, assim como a interpretação, discussão e redacção dos resultados.

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ABSTRACT

Hydrogen peroxide is a weak oxidant, but is able to induce oxidative damages to lipids, carbohydrates, proteins and nucleic acids, probably due to its conversion into the highly reactive hydroxyl radicals by the Fenton reaction (Halliwell and Gutteridge, 1999). These oxidative modifications affect the structural and metabolic integrity of the cell, and disturb the internal redox homeostasis. To recover after oxidative stress, cells must repair and degrade irreversibly damaged molecules, and re-establish the redox balance. This study aimed to analyse the molecular mechanisms underlying the recovery of *Saccharomyces cerevisiae* cells from H_2O_2 stress.

Changes in gene expression at the genome-wide level were analysed during exposure to high concentration of H_2O_2 and during recovery after H_2O_2 stress. The major changes are related to the expression of genes encoding DNA and lipid repair enzymes, chaperones and proteins involved in cell rescue and defence, protein turnover, redox control, transcription, ionic homeostasis, cell wall and cytoskeleton organization. In addition, the glucose-6-phosphate dehydrogenase (Zwf1p) activity and *ZWF1* gene expression increased during cellular recovery, supporting the role of NADPH production, required for the activity of the antioxidant defences.

The upregulation of genes associated with protein turnover was correlated with a decrease in the level of oxidised proteins, as assessed by the carbonyl content. The turnover of carbonylated proteins occurred at a lower level rate in mutants deficient in the Pep4p vacuolar protease, but was not affected in mutants deficient in the deubiquitinating enzyme Doa4p, which contain low ubiquitin levels. Besides, the disruption of *PEP4* increased basal protein carbonylation. These results indicate that Pep4p, in contrast with the ubiquitin-26S proteosome pathway, plays a critical role in the turnover of the proteins oxidised by H_2O_2 or by ROS generated as by-products of normal aerobic metabolism. Consistently, genes related to aminoacid catabolism were also up-regulated.

The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Tdh) is a main protein target oxidatively inactivated during exposure to H_2O_2, and the decrease of Tdh activity has been attributed to S-thiolation and carbonylation (Grant *et al.*, 1999; Cabiscol *et al.*, 2000; Costa *et al.*, 2002). This study also provides new evidence that the degradation of oxidised protein and enzyme synthesis *de novo* (turnover), contributes to restore Tdh activity during cellular recovery. Moreover, the turnover of both Tdh2p and Tdh3p contribute to restore Tdh activity.

Sphingolipids have emerged as bioactive molecules in mammalian cells. Specifically ceramide has been shown to have roles in differentiation, senescence, cell cycle arrest and apoptosis, leading to the proposal of ceramide as a stress-responsive lipid. In particular, the

accumulation of ceramides by activation of sphingolyelinases has been observed in response to a variety of stimuli, namely DNA damage and oxidants. In *Saccharomyces cerevisiae*, the inositolphosphosphingolipid-phospholipase C (Isc1p) also displays neutral sphingomyelinase activity. These features led us to analyse the role of Isc1p in oxidative stress resistance.

In this study, we show that Isc1p is essential to constitutive, but not induced H_2O_2 resistance. Indeed, *isc1Δ* mutant cells are more sensitive to H_2O_2, but still able to induce an adaptive response. Our results provide evidences that the induction of major antioxidant defences and heat shock proteins as part of the adaptive response is not dependent on ceramide generated by Isc1p. In addition, the higher sensitivity of *isc1Δ* mutant cells is not associated with a limited antioxidant capacity or reduced energy charge. However, the constitutive protein carbonyl, as well as the H_2O_2-induced protein carbonylation and lipid peroxidation content increased. These results suggest that ceramide generated by Isc1p may regulate cellular functions or structures important for oxidative stress resistance. The analyses of the changes in the transcriptome of *isc1Δ* mutant cells revealed that the major changes associated with *ISC1* disruption are related with the secretory pathway, and plasma membrane and cell wall proteins. Most of these proteins are involved in iron transport, multidrug or long chain sphingoid base resistance, cell wall biogenesis or cell signalling. Although the higher sensitivity of *isc1Δ* mutant cells to H_2O_2, the disruption of *ISC1* gene did not result in a general stress sensitivity. Interestingly, these cells are more resistant to heat-shock, ethanol and acetic acid stress, what may be related with the constitutive up-regulation of genes known to be involved in the cellular response to these stressful conditions. The overall results suggest that Isc1p may be involved in cell signalling in response to H_2O_2.

RESUMO

O peróxido de hidrogénio é um oxidante fraco, mas capaz de induzir danos oxidativos nos lípidos, carbohidratos, proteínas e ácidos nucleicos, provavelmente devido à sua conversão através da reacção de Fenton, em radicais hidroxilo, altamente reactivos (Halliwell and Gutteridge, 1999). Estas modificações oxidativas afectam a integridade estrutural e metabólica da célula, e alteram a homeostase redox interna. Para recuperar de um stress oxidativo, as células têm de reparar ou degradar as moléculas irreversivelmente danificadas, e restabelecer o balanço redox. Este estudo teve como objectivo, analisar os mecanismos moleculares associados à recuperação de células de *Saccharomyces cerevisiae* de stress por H_2O_2.

As alterações do transcriptoma foram analisadas durante a exposição a altas concentrações de H_2O_2, e durante a recuperação após o stress. As principais alterações estão relaccionadas com a expressão de genes que codificam enzimas envolvidas na reparação de DNA e de lípidos, chaperones e proteínas envolvidas na defesa e recuperação das células, turnover de proteínas, controlo das condições redox, transcrição, homeostase iónica, parede celular e organização do citoesqueleto. Além disso, a actividade da glucose-6-fosfato desidrogenase (Zwf1p) e a expressão do gene *ZWF1* aumentaram durante a recuperação celular, evidenciando o papel da produção de NADPH, necessário para a actividade das defesas antioxidantes.

O aumento da expressão de genes associados ao turnover de proteínas esteve correlacionado com uma diminuição dos níveis de proteínas oxidadas, determinados por análise do conteúdo em carbonilos. O turnover de proteínas carboniladas ocorreu a uma taxa mais baixa em mutantes deficientes na protease vacuolar Pep4p, mas não foi afectado em mutantes deficientes na enzima desubiquitinante Doa4p, que contêm níveis baixos de ubiquitina. Além disso, a disrupção do gene *PEP4* aumentou os níveis basais de proteínas carboniladas. Estes resultados indicam que a protease vacuolar Pep4p, em contraste com a via da ubiquitina-26S proteasoma, desempenha um papel crítico no turnover de proteínas oxidadas pelo H_2O_2 ou pelas espécies reactivas de oxigénio geradas como produto no metabolismo aeróbico normal. De acordo com estas evidências, verificou-se ainda um aumento da expressão de genes relaccionados com o catabolismo de aminoácidos.

A enzima glicolítica gliceraldeído-3-fosfato desidrogenase (Tdh) é a principal proteína oxidativamente inactivada durante a exposição ao H_2O_2, e a diminuição da actividade tem sido atribuída à ocorrência de S-tiolação e carbonilação (Grant *et al.*, 1999; Cabiscol *et al.*, 2000; Costa *et al.*, 2002). Este estudo fornece ainda novas evidências de que a degradação da proteína oxidada e a síntese *de novo* da enzima, contribuem para o restabelecimento da

actividade da Tdh durante a recuperação celular. Além disso, quer o turnover da Tdh3p, quer o da Tdh2p, contribuem para o restabelecimento da actividade.

Os esfingolípidos têm sido referidos como moléculas bioactivas em células de mamíferos. Especificamente a ceramida, tem sido referenciada por desempenhar funções na diferenciação, senescência, paragem do ciclo celular e apoptose, e por isso considerada um lípido de resposta ao stress. A acumulação de ceramida por activação de esfingomielinases tem sido observada em resposta a vários estímulos, nomeadamente danos no DNA e presença de oxidantes. Em *Saccharomyces cerevisiae*, a inositolfosfoesfingolípido fosfolipase c (Isc1p) também possui actividade de esfingomielinase neutra. Estes factos levaram-nos a analisar o papel da Isc1p na resistência ao stress oxidativo.

Neste estudo, mostrou-se que a Isc1p é essencial para a resistência constitutiva ao H_2O_2, mas não para a resistência induzida. Na verdade, as células deficientes em Isc1p são mais sensíveis ao H_2O_2, mas capazes de induzir uma resposta adaptativa. Os resultados fornecem evidências de que a indução das principais defesas antioxidantes e proteínas de choque térmico, como parte da resposta adaptativa, não depende da ceramida gerada por Isc1p. Além disso, a maior sensibilidade do mutante *isc1Δ* não está associada a uma capacidade antioxidante limitada ou a uma carga energética reduzida. No entanto, os níveis constitutivos de carbonilos proteicos, assim como os níveis de carbonilação proteica e peroxidação lipídica induzidos pelo H_2O_2, estão aumentados neste mutante. Estes resultados sugerem que a ceramida gerada pela Isc1p poderá regular funções celulares ou estruturas importantes para a resistência ao stress oxidativo. A análise do transcriptoma de células do mutante *isc1Δ* revelou que as principais alterações associadas à disrupção do gene *ISC1* estão relaccionadas com a via de secrecção e proteínas de membrana e de parede. A maior parte destas proteínas está envolvida no transporte de ferro, resistência a drogas ou bases esfingoides de cadeia longa, biogénese da parede ou sinalização celular. Apesar da maior sensibilidade do mutante *isc1Δ* ao H_2O_2, a disrupção do gene *ISC1* não resultou numa sensibilidade geral ao stress. Curiosamente, estas células são mais resistentes a choque térmico e a stress por etanol ou ácido acético, o que poderá estar relaccionado com uma expressão constitutiva mais elevada no mutante *isc1Δ*, de genes que se sabem estar envolvidos na resposta celular a estas condições de stress. De uma forma geral, os resultados sugerem que a Isc1p pode estar envolvida na sinalização celular em resposta ao H_2O_2.

RESUMÉ

Le peroxyde d'hydrogène est un oxydant faible, mais peut entraîner des dommages aux lipides, hydrates de carbone, protéines et acides nucléiques, probablement en raison de sa conversion dans les radicaux fortement réactifs d'hydroxyle par la réaction de Fenton (Halliwell et Gutteridge, 1999). Ces modifications oxydantes affectent l'intégrité structurale et métabolique de la cellule, et dérangent l'homéostasie redox interne. Pour récupérer après le stress oxydant, les cellules doivent réparer et dégrader les molécules irréversiblement endommagées, et rétablire l'équilibre redox. Cette étude vise à analyser les mécanismes moléculaires du rétablissement des cellules de *Saccharomyces cerevisiae* après un stress oxydant de H_2O_2.

Des changements de l'expression des gènes au niveau du génome totale ont été analysés pendant l'exposition à concentration élevée de H_2O_2 et pendant le rétablissement après le stress oxydant de H_2O_2.

Les changements principaux sont liés à l'expression des gènes codant des enzymes de réparation d'ADN et des lipides, chaperones et protéines impliqués dans la délivrance et la défense des cellules, “turnover” des protéines, contrôle redox, transcription, homéostasie ionique et la organisation du mûr et du cytosquelette des cellules. En plus, l'activité de la déshydrogénase de glucose-6-phosphate (Zwf1p) et l'expression du gène *ZWF1* ont augmenté pendant le rétablissement cellulaire, ce qui support le rôle de la production de NADPH, requis pour l'activité de défenses antioxydantes.

L'augmentation du niveau d' expression des gènes liés au “turnover” des protéines a été corrélé avec une diminution au niveau des protéines oxydées, évalué par le contenu de carbonylique. Le “turnover”de carbonylation des protéines s'est produit à un taux plus bas dans les mutants déficients en protéase vacuolar Pep4p, mais n'a pas été affecté dans le cas des mutants déficientes en l'enzyme de deubiquitination Doa4p, ce qui contiennent les niveaux bas d'ubiquitin. En plus, la rupture de *PEP4* a augmenté la carbonylation basal des protéines. Ces résultats indiquent que Pep4p, contrairement à la voie proteosome d'ubiquitin-26S, joue un rôle critique dans le “turnover” des protéines oxydées par H_2O_2 ou par ROS generée comme sous-produits du métabolisme aérobie normal. En effect, les gènes liés au catabolisme d'aminoacides étaient également augmentées.

L' enzyme glytolytique glyceraldehyde-3-phosphate déshydrogénase (Tdh) est une protéine cible principal, inactivée par l'oxidation pendant l'exposition à H_2O_2. La diminution de l'activité de Tdh a été attribuée à S-thiolation et à carbonylation (Grant *et autres*, 1999 ; Cabiscol *et autres*, 2000 ; Costa *et autres*, 2002). Cette étude fournit également la nouvelle évidence que la dégradation des protéines oxydées et de la synthése de novo l'enzymes (turnover), contribuent à la restauration de l'activité de Tdh pendant le rétablissement

cellulaire. D'ailleurs, le “turnover” de Tdh2p et de Tdh3p contribuent aussi à restauration de l'activité de Tdh.

Les sphingolipids ont émergé en tant que molécules bioactives en cellules mammifères. Spécifiquement le céramide a été montré capable de jouer des rôles dans la différentiation, sénescence, arrestation du cicle cellulaire et apoptose, ce que améne à la proposition du céramide comme lipide sensible au stress. En particulier, on a observé l'accumulation des céramides par l'activation des sphingolyelinases en réponse à une variété de stimulus, à savoir dommages d'ADN et oxydants. Chez la levure *Saccharomyces cerevisiae*, l'inositolphosphosphingolipid-phospholipase C (Isc1p) montre aussi une activité neutre de sphingomyelinase, ce que nous a amenés à analyser le rôle d'Isc1p dans l'effort á la résistance au stress oxydant.

Dans cette étude, nous prouvons que l'isc1p est essentiel à résistance constitutif au H_2O_2, mais pas à la resistence induite par ce stress. Les mutants d' *isc1*Δ sont plus sensibles à H_2O_2, mais encore capables d'induire une réponse adaptative. Nos résultats indiquent que l'induction des principales défenses antioxydantes et des protéines de choc térmique, en tant qu'élément de la réponse adaptative, ne dépendent pas du céramide induit par Isc1p. En plus, la sensibilité plus élevée des mutants *isc1*Δ n'est pas associée à une capacité antioxydante limitée ou à une diminuition de charge d'énergie. Ces résultats suggèrent que le céramide induit par Isc1p puisse réguler des fonctions ou des structures cellulaires importantes pour la résistance au stress oxydant. Les analyses des changements du transcriptome des mutants d' *isc1*Δ ont indiqué que les changements principaux liés à la rupture *ISC1* sont reliés avec la voie de secretion, et protéines de la paroi et de la membrane plasmatique des cellules. La plupart de ces protéines sont impliquées dans le transport de fer, la résistance de base aux drogues et à longue chaîne de sphingoid, biogénèse de la paroi des cellules ou la signalisation cellulaire. Bien que la sensibilité des cellules mutants d' *isc1*Δ à H_2O_2 est plus élevée, l'interruption du géne *ISC1* n´a pas eu comme résultat une sensibilité générale au stress. De façon intéressante, ces cellules sont plus résistantes au choque térmique, stress induit par l'éthanol et l'acide acétique, ce qui peut être relié avec l'augmentation de léxpression constitutif des gènes impliqués dans la réponse cellulaire à ces conditions. Les résultats globaux suggèrent que l'Isc1p puisse être impliqué dans la signalisation cellulaire en réponse au H_2O_2.

1D	one-dimensional
2D	two-dimensional
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AP	apurinic/apyrimidinic
ATP	adenosine triphosphate
AXP	adenine nucleotide
cAMP	cyclic AMP
cDNA	complementar DNA
CHAPS	3-[(3-chloramidopropyl)dimethyllammonio]1-propanesulfonate
CRD	cysteine rich domain
CTP	cytidine triphosphate
DHS	dihydrosphingosine
DNA	deoxyribonucleic acid
DNP	dinitrophenyl
DNPH	2,4-dinitrophenylnydrazine
dsDNA	double strand DNA
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
ER	endoplasmic reticulum
ESR	environmental stress response
Gpx	glutathione peroxidase
GSH	reduced glutathione
GSSG	oxidised glutathione
GTP	guanosine triphosphate
HOG	high osmolarity growth
HPLC	high performance liquid chromatography
HSP	heat shock protein
IEF	isoelectric foccusing
IgG	immunoglobulin G
IPC	inositol phosphoceramide
IPG	immobilised pH gradient
IPS	inositol phosphosphingolipid
LCB	long chain base
LOOH	lipid hydroperoxides
MDA	malondialdehyde

MEC	DNA damage checkpoint
MetSO	methionine sulfoxide
MIPC	mannosyl-inositol phosphoceramide
$M(IP)_2C$	mannosyl diphosphoryl-inositol ceramide
MIPS	Munich information center for protein sequences
MMR	mismatch repair
mRNA	messenger RNA
MT	metallothionein
mtRNA	mitochondrial RNA
NAD^+	oxidised nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	oxidised nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NER	nucleotide excission repair
NES	nuclear export sequence
NLS	nuclear localization signal
O.D.	optical density
ON	overnight
PCR	polymerase chain reaction
PDRE	pleiotropic drug resistance
PHGPx	phospholipid hydroperoxide glutathione peroxidase
PHS	phytosphingosine
PKA	protein kinase A
PKC	protein kinase C
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
ROS	reactive oxygen species
S1P	sphingosine-1-phosphate
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrilamide gel electrophoresis
SOD	superoxide dismutase
SSC	sodium chloride / sodium citrate
STRE	stress response element
TBARS	thiobarbituric acid reactive species

t-BOOH	tert-butyl-hydroperoxide
TBA	thiobarbituric acid
TGO	tris glucose oxidase
TOR	target of rapamyccin
YPD	yeast extract peptone glucose
YRE	Yap1p response element
WT	wild type
Yap1	yeast activator protein 1

CHAPTER 1

General introduction

1.1 - Yeast as a model to study stress responses

Yeasts are generally regarded as a model eukaryotic cell to disclose fundamental mechanisms common to higher eukaryotes. Indeed, from both the genetic and physiological point of view, yeast is a favourite organism for molecular cell biologists. Furthermore, yeasts have been utilized in traditional biotechnologies, such as backing, brewing distiller's fermentations, and wine making, and in modern biotechnologies, namely in recombinant protein production. In nature and during industrial applications, yeast cells are exposed to numerous environmental stress conditions, such as supraoptimal temperatures, oxidation, hyperosmolarity, ionic stress, organic acids, alcohols and nutrient limitations and starvation (Attfield, 1997). However, while this might pose problems for the process in many instances, stress effects could sometimes be used for practical purposes, particularly if their effects on the physiology of yeast are well understood.

Unicellular organisms require specific internal conditions for optimal growth and function and sudden changes in the external environment can perturb the internal milieu, disrupting the metabolic balance. Thus, cells must be able to protect and maintain the critical features of the internal homeostasis when facing variable external conditions. The adaptation of *Saccharomyces cerevisiae* cells to stress conditions, and the defences and mechanisms of regulation of the stress response have been a major research topic in recent years (Hohmann and Mager, 2002). Both specific and general stress responses exhibit a complex array of events that involve cell sensing, signal transduction, transcriptional and post - translational control, protein targeting to organelles, accumulation of protectants, and repair of damaged molecules.

Understanding osmotolerance or cryotolerance of baker's yeast, ethanol tolerance of wine yeast, or protection of foodstuffs from spoilage has impact on biotechnological applications. Stress responses are well conserved from yeast to plant and mammalian cells. Yeasts have remarkable similarities to human cells at the macromolecular and organelle level, and a number of yeast proteins have been shown to be functionally interchangeable with the highly homologous human proteins. Indeed, a number of heat shock proteins and antioxidant defences, as well as redox sensitive transcription factors, are well conserved. Stress responses are related to aging, apoptosis, disease states (neurodegenerative and inflammatory diseases, cancer and atherosclerosis) and immunological responses (Halliwell and Gutteridge, 1999; Gutteridge, 1993), and therefore have implications on medical issues.

Saccharomyces cerevisiae, in particular, has become a very powerful model to elucidate the complexity of these biologically important responses, as it offers a relative simplicity of a single celled eukaryotic organism that enables to combine and integrate

genetic, biochemical, physical-chemical, cell biological and genome-wide experimental approaches. Indeed, yeast cells can be easily manipulated, genetically, through defined cell mutants, and physiologically, through alteration of the growth and environmental conditions. Besides, the *S. cerevisiae* yeast genome sequence is complete and the availability of DNA and protein microarrays allows the study of changes in the entire transcriptome, and large-scale analysis of protein-protein and protein-ligand interactions or enzymatic activities.

These features turn *S. cerevisiae* an ideal system to study oxidative stress responses, a topic that gained a particular interest in recent years, mainly due to the role of ROS in diseases, cell aging and cell death. Studies using yeast cells have contributed to elucidate the role of ROS in diseases related to oxidative stress, namely in Amyotrophic lateral sclerosis (Rosen *et al.*, 1993) and Friedreich's ataxia (Patel and Isaya, 2001).

1.2 - The general stress response in yeast. The environmental stress response (ESR)

Details on the mechanisms that *S. cerevisiae* uses to adapt to changes in environment conditions have emerged over the years. An interesting feature is the fact that yeast cells gain cross protection against different kind of stresses. Indeed, cells exposed to a mild dose of one stress agent become resistant to lethal doses of other stress agents (Mitchel and Morrison, 1982; Blomberg *et al.*, 1988; Wieser *et al.*, 1991; Flattery-O'Brien *et al.*, 1993, Lewis *et al.*, 1995). Several observations sparked the idea that yeast cells use a general mechanism of cellular protection that is triggered when cells are challenged with different stress agents, such as heat shock, ethanol, metal ions, high osmolarity and oxidants. The common events have been identified as the general stress response. Genes considered as part of the general stress response are listed in Table 1 (Mager and De Kruiftt, 1995; Ruis and Schuller, 1995; Boy-Marcotte *et al.*, 1998; Kurtz *et al.*, 1986; Werner-Washburne *et al.*, 1989; Susek and Lindquist, 1990).

The recent increase in popularity of whole-genome studies is expanding and allowing our definition and understanding of yeast stress responses. Characterization of genomic transcript abundance and global protein synthesis levels allowed to explore these mechanisms of the cellular responses and provided insights into the defence mechanisms against environmental changes. Many of the observed cellular responses are specifically triggered to counteract features that are unique to each environment. However, in addition to these specific responses, global studies have identified the players in a common response, providing data into the complex regulation of this cellular programme. Characterization of the genomic expression re-programming in yeast in response to different environmental conditions revealed that a substantial fraction of each of the responses is not specific to the

stimulus but instead, represents a common response to all of the conditions tested. In a study using DNA microarrays, approximately 900 genes whose expression was typically altered in *S. cerevisiae* in response to a variety of stressful environmental transitions were identified (Gasch *et al.*, 2000; Causton *et al.* 2001). Despite similarities to the previously defined "general" stress response (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996), the coordinate fluctuations in the expression of these genes were referred to as the environmental stress response. Although this program is commonly initiated in response to diverse conditions, the precise levels and timing of the gene expression changes appear to be specific to the feature of each new environment, hinting at the sensitivity with which the program is regulated (Gasch *et al.*, 2000).

Table 1. Genes considered as part of the general stress response

Gene	***Gene Product***	***Function***
CTT1	cytoplasmic catalase T	Decomposition of hydrogen peroxide
DDR2	heat shock protein	DNA damage repair
HSP12	heat-shock protein	Molecular chaperone
HSP104	heat-shock protein	Molecular chaperone
GLC3	1,4-glucan-6-(1,4-glucano)-transferase	Formation of 1,6-glucosidic linkages of glycogen
CYC7	iso-2-cytochrome c	Electron carrier activity
SOD2	mitochondrial superoxide dismutase	Dismutation of superoxide radicals
UBI4	polyubiquitin	Tagging proteins for degradation by the 26S proteasome
TPS1	trehalose-6-phosphate phosphatase complex 56 kDa synthase subunit	Trehalose biosynthesis
TPS2	trehalose-6-phosphate phosphatase	Trehalose biosynthesis
GPD1	glycerol-3-phosphate dehydrogenase	Glycerol biosynthesis

1.2.1 - Genes repressed in the ESR

The expression of 600 genes is reduced as part of the ESR, and many of these genes can be directly related to protein synthesis (Gasch *et al.*, 2000). The reduced expression of these genes correlates with the transient decrease in overall translation initiation, coupled with a transient cell cycle and growth arrest regulated by the transcriptional factor Rap1p (McAlister and Finkelstein, 1980; Fuge *et al.*, 1994; Blomberg 1995; Ashe *et al.*, 2000; Kuhn *et al.*, 2001; Teige *et al.*, 2001; Moehle and Hinnebusch, 1991; Li *et al.*, 1999). The combined effects of the decrease in transcription and protein synthesis may help to preserve mass and energy while the cell adapts to its new conditions. In additon to genes involved in protein synthesis, other functional processes are downregulated, including those related to cell wall

biosynthesis, cytoskeletal and chaperonin functions, protein glycosylation and secretion, amino acid and pyruvate metabolism, nucleotide biosynthesis, DNA replication and nonsense mediated mRNA decay (Gasch *et al.*, 2000).

1.2.2 - Genes induced in the ESR

Of the more than 300 genes induced in the ESR, the known functions have been related to carbohydrate metabolism, metabolite transport, fatty acid metabolism, maintenance of the redox potential, detoxification of reactive oxygen species, autophagy, protein folding and degradation, cell wall modification, DNA-damage repair, secretion, vacuolar and mitochondrial functions, intracellular signalling, and others (Gasch *et al.*, 2000). The functions of these genes hint to the cellular processes that may be affected in response to diverse environmental changes, and suggest mechanisms that the cell uses to protect itself when facing cellular stress. The induced expression of these genes may lead the cell to acquire critical features for the internal homeostasis, such as glucose stores, ATP levels, osmolarity, the cellular redox balance and the integrity of cellular structures, such as proteins and DNA.

Glucose is the preferred carbon source in yeast and upon a stress the cell induces the expression of genes associated with glucose metabolism, including genes encoding glucose transporters (*HXT5, YGL104C*) and glucose kinases (*HXK1*, *GLK1*). In response to stress conditions, the fate of glucose is then divided between trehalose synthesis, glycogen storage, ATP synthesis through glycolysis and NADPH regeneration by the pentose phosphate pathway.

The disaccharide trehalose, comprised of alpha-(1,1) linked glucose molecules, has long been implicated in stress responses, namely to hyperosmolarity, heat-shock and oxidative stress (reviewed in Singer and Lindquist, 1998a; Francois and Parrou, 2001). A number of evidences indicated that trehalose may stabilize protein structures and prevents the unfolding and aggregation of proteins during heat-shock (De Virgilio *et al.*, 1994; Singer and Lindquist, 1998b), while it appears to protect cellular structures from oxidative damage triggered by H_2O_2 (Benaroudj *et al.*, 2001). As part of the ESR, it is observed the concomitant induction of genes affecting the synthesis (*TPS1*, *TPS2* and *TSL1*) and degradation (*NTH1*, *ATH1*) of trehalose. Although these enzymes are co-induced, trehalose levels increase after the neutral trehalase Nth1p is phosphorylated and inactivated (Zahringer *et al.*, 1998).

Glycogen is also known to be critical to cell survival under starvation and likely also plays an important role in response to a wide variety of stressful environments (Francois and Parrou, 2001). Similar to the case of trehalose, genes encoding enzymes that promote

glycogen synthesis (*UGP1, GLG1, GSY2*) and degradation (*GPH1, PGM2, GLC3, GDB1*) are co-induced in the ESR, and are post-translationally regulated (Hwang *et al.*, 1989; Parrou *et al.*, 1997). Both the glycogen synthase (Gsy2p) and glycogen phosphorylase (Gph1p) are inactivated by phosphorylation, while Gsy2p is activated by high levels of glucose-6-phosphate.

Genes affecting the synthesis of fructose-2,6-biphosphate, a key regulator of glycolytic flux are induced, namely the genes encoding the kinase (*PFK26*) and the phosphatase (*FBP26*). The activity of these enzymes is regulated post-translationally by phosphorylation and by allosteric regulation of Pfk26p and Pbp26p by ATP and fructose-6-phosphate, respectively (Kretschmer *et al.*, 1987; Manhart and Holzer, 1988; Hofmann *et al.*, 1989; Thevelein and Hohmann, 1995). The concomitant induction of these synthetic and catabolic enzymes may allow the cell to modulate glycolysis without altering the expression of the entire pathway genes.

In cells growing with glucose as carbon source, the genes involved in respiration are repressed, but they can be derepressed by a reduced ATP:ADP ratio in the cell, even in the presence of the sugar (Hardie *et al.*, 1998; Hardie, 1999). As some stress defence mechanisms consume ATP, there is an induced expression of respiration components. As part of the ESR, the gene encoding the rate-limiting step of the tricarboxylic acid cycle (*CIT1*) as well as an alternate isoform of cytochrome c (*CYC7*), two factors that affect the synthesis and assembly of the oxidative phosphorylation components, cytochrome c oxidase (*COX15*) and ubiquinone (*COQ5*), are induced. This induction may be associated with ATP synthesis or alternatively it can play a role in the defence against oxidative stress, as ubiquinone can act as an antioxidant against lipid peroxides (see 1.3.2.2) (Schultz and Clarke, 1999; Soballe and Poole, 1999).

During oxidative stress, glutathione and other thiol specific antioxidants, such as the thioredoxin and glutaredoxin systems, are involved in maintaining the reducing environment while protecting cellular structures from oxidative damage (see 1.3.2.) (Kuge and Jones, 1994; Grant *et al.*, 1996a; Grant *et al.*, 1996b; Luikenhuis *et al.*, 1998). The importance of the redox balance is highlighted by the induction of the genes listed in Table 2.

It is well known that genes encoding chaperones are induced in response to heat denatured proteins, and a subset of chaperone genes is induced by a variety of stressful conditions (Kurtz *et al.*, 1986; Werner-Washburne *et al.*, 1989): the small heat shock proteins (*HSP12, HSP26*, and *HSP48*), members of the HSP70 family of chaperones (*SSA4, SSE2, HSP78*) and *HSP104*. However, denaturated proteins that cannot be properly refolded are targeted for degradation mainly by the ubiquitin pathway. Thus, it is not surprising that genes as *UBA1* (ubiquitin-activating enzyme e1) and *UBC4* (ubiquitin-conjugating enzyme e2), *HUL4* (ubiquitin ligase), *UBC5* (ubiquitin-conjugating enzyme), *UBC8* (ubiquitin-conjugating

enzyme / ubiquitin-protein ligase), *UBI4* (polyubiquitin), and *UBP15* (a putative deubiquitinating enzyme), are involved in the ESR (Figure 1).

Table 2. Antioxidant defences induced by the ESR

Gene	***Gene Product***	***Function***
TRX2	cytoplasmic thioredoxin	Reduction of hydrogen peroxide and alkyl hydroperoxides
mTPx	mitochondrial thioredoxin peroxidase	Reduction of hydrogen peroxide
cTPxII	cytoplasmic thioredoxin peroxidase II	Reduction of hydrogen peroxide and alkyl hydroperoxides
ECM38	gamma-glutamyl-transferase homolog	Involved in glutathione synthesis
GPX3	glutathione peroxidase	Reduction of hydrogen peroxide and alkyl hydroperoxides Hydroperoxide receptor that transduce a redox signal to Yap1p
GPX1	glutathione peroxidase	Reduction of hydrogen peroxide and alkyl hydroperoxides
GRX2	glutaredoxin	Reduction of protein disulphides
ZWF1	glucose-6-phosphate dehydrogenase	Reduction of $NADP^+$ to NADPH
GND2	6-phosphogluconate dehydrogenase	Reduction of $NADP^+$ to NADPH
SOD1	cytoplasmic superoxide dismutase	Dismutation of superoxide radicals
CTT1	cytoplasmic catalase T	Decomposition of hydrogen peroxide
MCR1	NADH-cytochrome b5 reductase	Transfer electrons from external NADH to cytochrome c
CCP1	cytochrome c peroxidase	Reduction of hydrogen peroxide

In addition to the ubiquitin pathway, some proteins can be degraded in the vacuole. Indeed, autophagy is a process by which engulfed materials such as proteins, small molecules and organelles are degraded in the vacuole, so that their components can be recycled in the cell. Thus, in addition to autophagy genes (*APG1, APG7 and AUT1*), genes encoding vacuolar proteins are also induced in the ESR. These include genes encoding the vacuolar calcium pump Pmc1p, a protein implicated in vacuolar targeting (*VAB2*), vacuolar proteases (*PRC1, YPS6, LAP4, PEP4 and PRB1*) as well as the protease inhibitor Pai3p and Pbi2p, which are thought to inhibit the Pep4p and Prb1p proteases, respectively. The products of these genes may aid in the degradation of cargo delivered to the vacuole by autophagy as well as other protein that arrive through endocytosis or the secretory pathway (van den Hazel *et al.*, 1996).

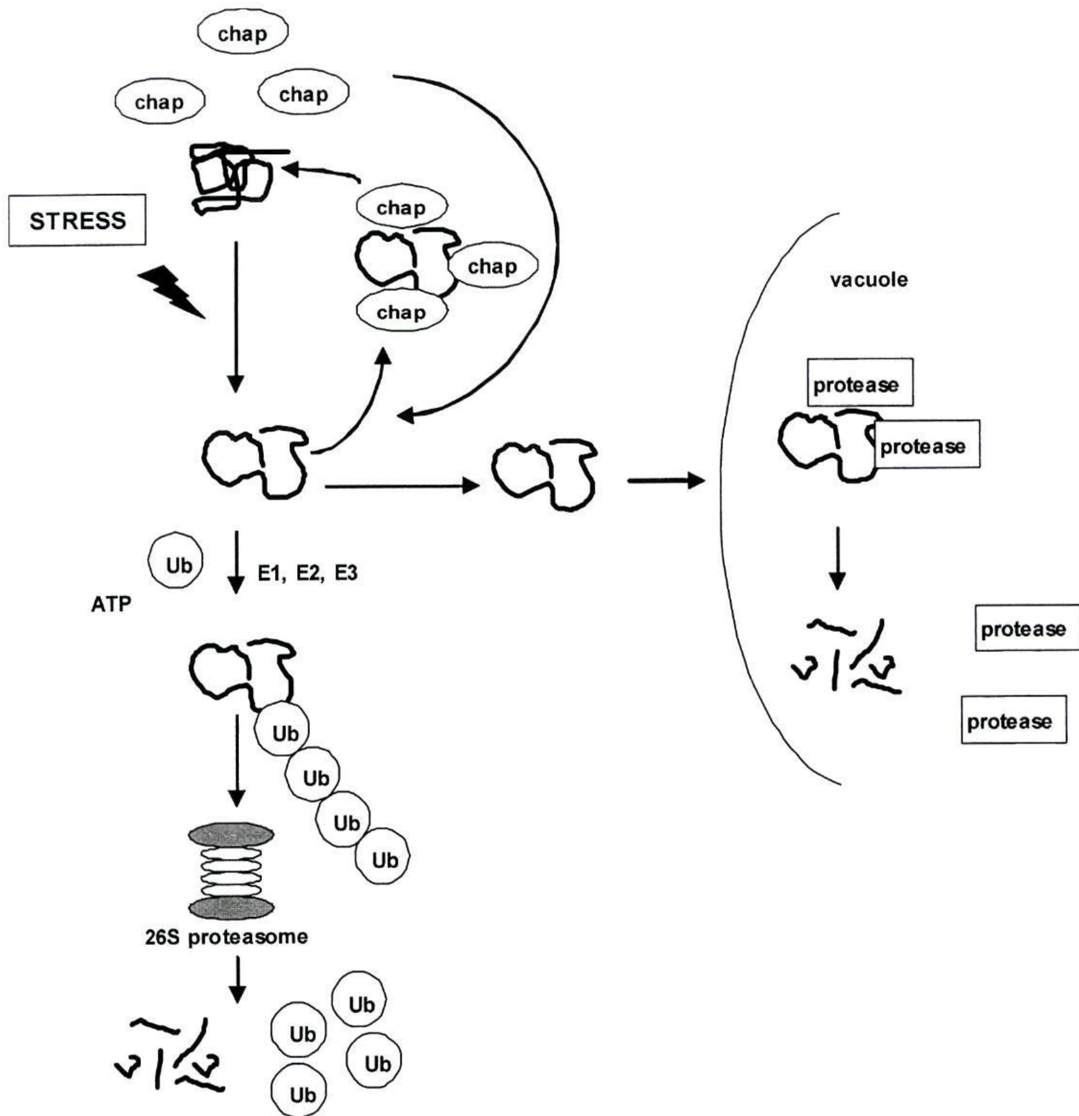


Figure 1.
Role of molecular chaperones, ubiquitin-26S proteasome and vacuolar proteases under stress conditions. Under stress conditions, damaged proteins can be stabilized by molecular chaperones, which facilitate their renaturation and / or degradation. Denatured proteins that cannot be properly refolded are targeted for degradation by the ubiquitin-26S proteosome or can be delivered for vacuolar degradation, by specific proteases. Ub, ubiquitin; chap, molecular chaperones; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase.

1.2.3 – Regulation of the ESR

Although initiation of the ESR is a common response to stressful conditions, regulation of ESR gene expression is condition-specific and is controlled at multiple levels, including transcription initiation, silencing through chromatin remodelling, and regulated mRNA turnover (reviewed in Gash and Werner-Washburne, 2002). As an example, a group of the

transcription factors that control ESR gene expression are active only under specific conditions (Table 3): the transcription factors Hsf1p, Hot1p and Yap1p independently affect the expression of subsets of ESR genes in response to heat shock, osmotic shock, or oxidative stress, respectively, but are not involved in regulating the expression of these ESR genes under other conditions (Treger *et al.*, 1998; Rep *et al.*, 1999, 2000; Gash *et al.*, 2000; Amoros and Estruch, 2001).

Table 3. Factors and pathways implicated in regulating genes in the ESR

Factor / Pathway	*Conditions* [a]
Transcription factors	
Msn2p/Msn4p	Diverse conditions
Yap1p	Oxidative stress
Hot1p	Osmotic shock
Hsf1	Heat shock
Protein Kinase Pathways	
MEC pathway	DNA - damaging agents
Ste11p/Ssk1p-dependenr pathways	Osmotic stress
HOG1 pathway	Osmotic stress
PKC pathway	Heat shock, secretion defects Cell wall damage
PKA pathway	Nutrition repletion, others?
TOR pathway	Nutrition repletion, others?

[a] Conditions under which each factor is known to be active

Promoter analysis revealed the presence of two novel and conserved motifs (GCGATGAGCTG and GAAAAA/(T)TTTTC) in the upstream elements of ESR genes, one of which (the second refered above) was similar to a motif identified in the promoter of RNA processing genes (Hughes *et al.*, 2000). In addition, a number of condition-specific signalling pathways have been implicated in mediating the coordinated expression of the induced and repressed ESR genes (see Table 3). Pathways that suppress the ESR, including the TOR and Protein Kinase A pathways, have also been implicated.

1.2.3.1 – The cAMP - PKA pathway and the stress response

In *Saccharomyces cerevisiae*, the Ras/PKA (cAMP-protein kinase A) signalling pathway is involved in the regulation of a number of cellular functions, including nutrient sensing, regulation of cell proliferation and carbon storage. The activation of PKA causes transient changes, including in trehalose and glycogen metabolism, glycolysis and

gluconeogenesis, which are dependent on components controlled by PKA-mediated phosphorylation (Thevelein, 1994). This pathway also participates in the regulation of the general stress response (reviewed in Thevelein, 1994; Hohmann and Mager, 2002).

Yeast PKA is a heteromeric protein consisting of three catalytic (encoded by *TPK1*, *TPK2* and *TPK3* genes), and one regulatory subunit (encoded by *BCY1*). The regulatory subunit Bcy1p is released upon binding of cAMP. Thus, the regulation depends on the availability of cAMP, by affecting PKA activity. The concentration of cAMP is controlled by cAMP phosphodiesterase activities of Pde1p and Pde2p, which converte cAMP in AMP, and by adenylate cyclase (Cyr1p) that generates cAMP (Werner-Washburne *et al.*, 1991; Zaremberg and Moreno, 1996). Under optimal growth conditions the PKA activity is high. When cells are stressed, cAMP levels decrease and thus, it is observed a decrease in PKA activity that allows the activation of the stress adaptive mechanisms. Hyperactivity of Ras/PKA pathway causes sensitivity to several stress conditions and starvation (Varela *et al.*, 1995; Siderius *et al.*, 1997; Gorner *et al.*, 1998). Reduced PKA activity, in contrast, results in preadaptation to stress (Hirata *et al.*, 1995). Indeed, it was revealed that Ras/PKA pathway have a general inhibitory effect on Msn2/4p, Skn7p and Yap1p transcription factors (Marchler *et al.*, 1993, Martinez-Pastor *et al.*, 1996, Boy-Marcotte *et al.*, 1998; Fernandes *et al.*, 1997; Charizanis *et al.*, 1999a). This negative control is exerted, at least in part, by a PKA-directed phosphorylation of Msn2/4p, resulting in the inhibition of its stress-induced nuclear redistribution (Gorner *et al.*, 1998) (see 1.2.3.2). Otherwise, upon activation, Yap1p becomes phosphorylated by an unidentified kinase, and the role of this phosphorylation awaits the identification of the cognate kinase (Delaunay *et al.*, 2000). Data showing that the Ras-cAMP-PKA pathway has a negative effect on Yap1p -dependent transcription, led to suggest that Yap1p is a downstream factor of the Ras/PKA pathway (Fernandes *et al.*, 1997, Charizanis *et al.*, 1999b). This effect might result partly from the reduction of Yap1p protein levels and from the inhibition of Yap1p function at the level of promoter occupancy (Fernandes *et al.*, 1997). In addition, Yap1p also upregulates the *RPI1* gene, which codes for a protein that represses the Ras/PKA pathway (Dumond *et al.*, 2000).

Interestingly, genes encoding both positive effectors and negative regulators of protein kinase A (PKA) signalling were co-ordinately induced in the ESR. The expression of the genes encoding two of the three catalytic subunits of PKA (*TPK1, TPK2*) is induced concomitantly with the cAMP-dependent inhibitory subunit, *BCY1*, the phosphodiesterase *PDE1*, and the gene encoding the Yak1p kinase that is proposed to counteract PKA signalling (Garrett and Broach, 1989; Hartley *et al.*, 1994). The cell may concomitantly increase protein levels of positive and negative regulators of PKA signalling to allow sensitive post-translational control through this pathway, and to provide a mechanism of feed-back

regulation to aid in the eventual suppression of the ESR after the cell has adapted to the new conditions.

1.2.3.2 – The transcription factors Msn2p and Msn4p

Msn2p and Msn4p, are zinc finger transcription factors (Estruch and Carlson, 1993; Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996) harbouring a DNA-binding domain almost identical and specific to a sequence element that is common in the promoter of several genes, the STRE sequence - Stress Response Element – CCCCT (C_4T) (Kobayashi and McEntee, 1990; Marchler *et al.*, 1993). These two related transactivators become active under most stress conditions, including nutritional changes (Martinez-Pastor *et al.*, 1996; Boy-Marcotte *et al.*, 1998), and induce the transcription of a large set of genes (Figure 2) (Boy-Marcotte, 1998; Moskovitz *et al.*, 1998; Gasch *et al.*, 2000; Causton *et al.*, 2001). Msn2p and Msn4p transcription factors have been implicated in regulating many of the induced ESR genes (Gash *et al.*, 2000; Causton *et al.*, 2001), although the role of these factors also varies under different conditions (Treger *et al.*, 1998; Rep *et al.*, 1999, 2000; Garreau *et al.*, 2000; Gash *et al.*, 2000; Amoros and Estruch, 2001). Strains lacking *MSN2* and *MSN4* are sensitive to various stress agents and fail to accumulate stress-regulated messages following heat and osmotic stress, as well as nutrient starvation and DNA damage (Martinez-Pastor, 1996; Schmitt and McEntee, 1996).

Although Msn2p and Msn4p can both activate the induction of gene expression through the STRE promoter element, they appear to be differentially regulated and may play distinct roles in the regulation of specific genes induced in the ESR. Deletion of *MSN2* results in decreased induction of STRE-regulated stress genes in response to stress, whereas *MSN4* deletion often has no effect on gene expression (Martinez-Pastor, 1996; Schmitt and McEntee, 1996; Treger *et al.*, 1998; Amoros and Estruch, 2001). However, in the absence of Msn2p, Msn4p clearly contributes to transcriptional induction, as double deletion results in significantly weaker gene induction in comparison to that of *MSN2* single deletion (Treger *et al.*, 1998; Garreau *et al.*, 2000). Under certain conditions, STRE-regulated stress genes are normally induced despite of *MSN2* and *MSN4* deletion, hinting that the regulation of the stress response was more complex than suggested by the initial model (Schuller *et al.*, 1994).

Gorner *et al.* (1998) used epitop-tagged versions of these factors to demonstrate that in non-stress conditions, Msn2p and Msn4p are in the cytosol, and in response to stress, these factors are rapidly translocated to the nucleus, where they activate target genes. The accumulation in the nucleus occurs within minutes of exposure to heat shock,

hyperosmolarity, salt stress, ethanol exposure, and glucose starvation. When the cells are returned to their standard growth conditions, Msn2p and Msn4p are relocalized to the cytosol within minutes (Figure 2).

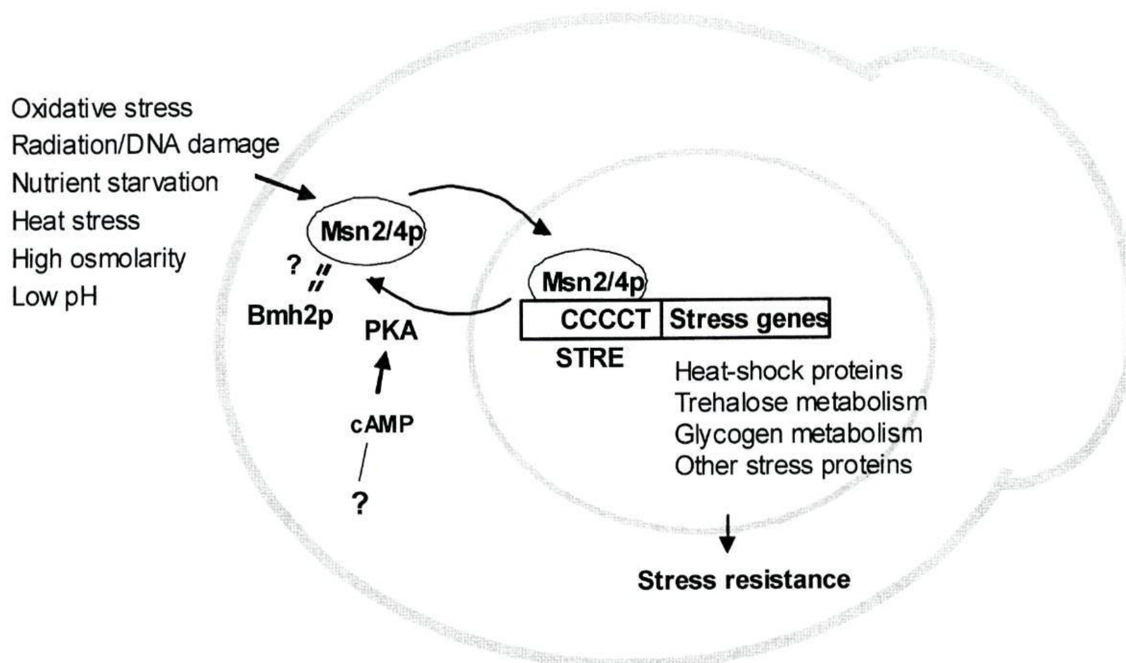


Figure 2.

Regulation of Msn2/4p activity by PKA. Msn2p and Msn4p, both are Cys_2His_2 zinc finger proteins, required for the transcription of several genes coding for proteins with stress-protective functions, by binding to the stress response element (STRE) present in the promoter of these genes. Under optimal growth conditions, PKA activity is high and both factors accumulate in the cytoplasm. Bmh2p may serve to anchor Msn2p and Msn4p in the cytosol. Stress-induced nuclear localization was found to be correlated to decreased cAMP levels and protein kinase (PKA) activity, leading to the induction of stress-responsive genes.

As referred in 1.2.3.1, PKA signalling is known to negatively affect the induction of STRE regulated genes and is involved in the regulation of Msn2p and Msn4p localization (Marchler *et al.*, 1993; Boy-Marcotte *et al.*, 1998; Gorner *et al.*, 1998). In mutants with constitutive low PKA activity (*bcy1tpk1^{w}tpk2tpk3*), Msn2p is localized predominantly in the nucleus, increasing stress-inducible gene expression and stress resistance (Schuller *et al.*, 1994; Ruis, 1997). In contrast, mutants with high PKA activity (*e.g. bcy1Δ*) repress the expression of stress genes by preventing nuclear accumulation of Msn2p, and are stress sensitive (Thevelein, 1994; De Winde *et al.*, 1997). In agreement with the evidence that the mutation of serines in the central domain of Msn2p led to a constitutively nuclear localization

of the factor, Gorner *et al.* (1998) proposed that PKA might directly phosphorylate Msn2p and Msn4p to trigger cytoplasmic relocalization of the proteins. Later, Garreau *et al.* (2000) observed that under standard growth conditions both Msn2p and Msn4p exist in a phosphorylated state, but become hyperphosphorylated in response to multiple stresses. This hyper-phosphorylated state is reversed by addition of cAMP that triggers relocalization of the factors to the cytosol. It now appears that the inhibitory effect of PKA signalling leads to the dephosphorylation of Msn2p and Msn4p, and their relocalization to the cytosol. In the absence of cellular stress, Msn2p and Msn4p interact with the constitutively cytosolic protein Bmh2p (Beck and Hall, 1999), homologous to mammalian 14-3-3 proteins that mediate the localization of signalling proteins. Bmh2p and its paralog Bmh1p have been implicated in RAS-dependent signalling in yeast (Gelperin *et al.*, 1995; Roth *et al.*, 1999). That mammalian 14-3-3 proteins bind phosphoserine residues in proteins (Muslin *et al.*, 1996; Bertram *et al.*, 1998; Muslin and Xing, 2000), hint that Bmh2p may bind to Msn2p and Msn4p in a manner dependent on the precise phosphorylation state of these proteins and serve to anchor these factors in the cytosol under standard growth conditions.

Recently, it has been shown that phosphorylation of the nuclear localization signals (NLS) of Msn2p (between residues 576 and 704), specifically controls the cytoplasmic relocalization of the protein in response to glucose repletion. Indeed, a PKI-NES-Msn2 (576-704)-GFP3 fusion protein, that contains both NLS domains of Msn2p and the heterologous protein kinase A inhibitor nuclear export signal (Wen *et al.*, 1994), is cytoplasmic under optimal growth conditions, but nuclear upon glucose starvation stress. In contrast, the relocalization of Msn2p in response to other stress conditions that normally induce nuclear accumulation of full-length Msn2p, is independent of NLS domains and may be primarily controlled by alterations in nuclear export, as the PKI-NES-Msn2 (576-704)-GFP3 fusion protein did not localize in the nucleus after any of those stresses (Gorner *et al.*, 2002).

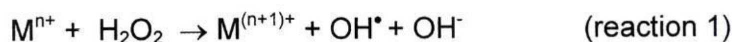
1.3 – Oxidative stress

1.3.1 - Generation of reactive oxygen species

Yeast, as aerobic cells, have to face the toxic side effect of molecular oxygen. Oxidative stress is a generic term for the stress associated with cells or organisms sensing, responding to and protecting themselves from the generation of reactive oxygen species (ROS). The ROS, which are highly unstable molecules, represent different oxidation states of dioxygen (O_2), and include the singlet oxygen ($^1O_2$), the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH^\bullet$), are formed during normal

cellular metabolism (Halliwell and Gutteridge, 1999). Superoxide anion is generated via the one electron reduction of dioxygen during mitochondrial respiration, and several oxidases are known to produce H_2O_2 via their normal reaction mechanisms.

The production of ROS can be induced under several stress conditions. ROS can originate from the presence of pro-oxidants, such as menadione and paraquat in the medium, since they can efficiently cross biological membranes and generate highly reactive species, such as superoxide radicals. An increase in the oxygen pressure (hyperoxia or re-oxygenation of hypoxic cells) can also generate ROS. The hydroxyl radical is also efficiently generated upon exposure to ionizing radiation, which causes homolytic fission of O-H bonds in water. In addition, H_2O_2 easily crosses membranes and serves as substrate to generate the hydroxyl radical via several mechanisms: by the metal (Fe^{2+}, Cu^{+}) catalysed Fenton reaction (reaction 1), or by a slow spontaneous decomposition of H_2O_2 to yield $OH^{\bullet}$. Furthermore, the Haber-Weiss reaction (reaction 2) can be coupled to the Fenton reaction for the generation of the hydroxyl radical (Halliwell and Gutteridge, 1999).



$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^{\bullet} + OH^{-} \qquad \text{(reaction 1)}$$

$$M^{(n+1)+} + O_2^{\bullet-} \rightarrow M^{n+} + O_2 \qquad \text{(reaction 2)}$$

Several features of ROS contribute to their toxicity, including site of generation, rates of chemical reactivity, abundance and diffusion. The hydroxyl radical is the most reactive ROS. This high reactivity limits its diffusion in biological systems, causing $OH^{\bullet}$ to generate cellular damage as soon as it is formed. $OH^{\bullet}$ can abstract hydrogen from a molecule leading to a new radical species. It can also react by addition on molecules containing aromatic rings, or to metals, forming metal-centered radicals. Lastly, $OH^{\bullet}$ can transfer its unpaired electron to another molecule and thus create new radical species, which can then diffuse from their site of generation, producing oxidative damages in other target sites.

1.3.2 – Antioxidant defences

ROS generated endogenously as by-products of mitochondrial respiration and enzymatic activities or exogenously by environmental factors, can damage cellular structures such as proteins, lipids and DNA, and prevent proper enzymatic activity by perturbing the internal redox potential (Halliwell and Gutteridge, 1999). As a result, the cell has evolved a

number of defences to prevent and repair the effects of oxidative stress, as well as mechanisms to maintain its internal redox balance.

Under physiological conditions, the cell defence mechanisms are able to avoid molecular damages, as they are almost certainly adequate to maintain ROS at basal unharmful levels. These defences operate at different levels: primary defences (Table 4) neutralise ROS through electronic transfer, while secondary defences repair or remove the products of oxidative damages to cellular components (Table 5). Thus, cells possess both enzymatic and non-enzymatic defence systems to protect their cellular constituents and to keep an adequate redox balance in the different cellular sub-compartments (Jamieson, 1998; Moradas-Ferreira and Costa, 2000).

1.3.2.1 – Primary antioxidant defences

All aerobic organisms contain superoxide dismutase (SOD) activity, which disproportionates $O_2^{\cdot-}$ into H_2O_2 and O_2 (Fridovich, 1995). This dismutation is catalized by a transition-metal center in SOD, that can be iron, copper, or manganese, and does not require the supply of an external source of reducing power, such as NADPH. *S. cerevisiae* like other eukaryotes, contains two intracellular SODs: a cytosolic Cu,Zn-superoxide dismutase, Sod1p (Gralla and Kosman, 1992; Culotta, 2000; Bermingham-McDonogh *et al.*, 1988), that also localizes in peroxisomes, vacuoles, nucleus (Chang *et al.*, 1988; Keller *et al.*, 1991; Geller and Winge, 1982), and in the intermembrane space of mitochondria (Sturtz *et al.*, 2001), and a Mn-superoxide dismutase, Sod2p, localized strictly in the mitochondrial matrix (Autor, 1982; van Loon *et al.*, 1986).

The primary source of $O_2^{\cdot-}$, is the electron transport chain, located in the inner membrane of mitochondria. Thus, the main physiological role of Sod2p appears to be the protection of mitochondrial targets from $O_2^{\cdot-}$ generated during respiration, as *sod2*Δ mutants are unable to grow on a non-fermentable carbon source, and are highly sensitive to 100% O_2 and environmental oxidants (*e.g.* paraquat) (van Loon *et al.*, 1986). In these *sod2*Δ mutants, oxygen sensitivity can be suppresses by rho^{o} mutations in mitochondrial DNA that block respiration (Guidot *et al.*, 1993). In addition, Sod2p also protects mitochondria from $O_2^{\cdot-}$ generated by other stress conditions such as heat-shock or ethanol (Pereira *et al.*, 2001). Indeed, ethanol exposure leads to an increase of the MnSOD activity and *sod2*Δ mutant cells display high ethanol sensitivity (Costa *et al.*, 1993, 1997).

Table 4. Yeast primary antioxidant defences

Gene	**Product**
SOD1	cytoplasmic superoxide dismutase
SOD2	mitochondrial superoxide dismutase
CTA1	catalase A, peroxisomal and mitochondrial
CTT1	cytoplasmic catalase T
CCP1	cytochrome c peroxidase, mitochondrial
GSH1 [a]	glutathione
GPX1-GPX3	glutathione peroxidases
CUP1	metallothionein
CRS5	metallothionein
TRX2	cytoplasmic thioredoxin
TRX3	mitochondrial thioredoxin
cTPXI /TSA1	cytoplasmic thioredoxin peroxidase I
cTPXII / TSA2	cytoplasmic thioredoxin peroxidase II
mTPX	mitochondrial thioredoxin peroxidase
cTPXIII / AHP1	cytoplasmic thioredoxin peroxidase III
nTPX / DOT5	nuclear thioredoxin peroxidase
COQ3 [b]	ubiquinone
SPE2 [c]	polyamines

[a]*GSH1* encodes gamma-glutamyl-cysteine synthetase, which catalyses the first and rate-limiting step in glutathione biosynthesis; [b]*COQ3* encodes 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase, which catalyzes different o-methylation steps in ubiquinone (Coenzime Q) biosynthesis; [c]*SPE2* encodes S-adenosylmethionine decarboxylase, which is involved in polyamine biosynthesis.

Sod2p is encoded by a nuclear gene and the polypeptide acquires manganese after import into the mitochondrial matrix. A number of candidates for manganese trafficking has been identified: Smf1p and Smf2p are members of the Nramp family of metal transporters and appear to function in manganese homeostasis. Unlike Smf1p, Smf2p shows no evidence of plasma membrane localization but appears to reside in intracellular vesicles (Portnoy *et al.*, 2000). Luk and Culotta (2001) showed that Smf2p is critical for activation of Sod2p with manganese and that intracellular Smf2p containing vesicles play a pivotal role in regulating manganese availability to mitochondrial Sod2p, as well as to other cellular targets.

The insertion of the copper cofactor *in vivo* into Sod1p requires an accessory protein, the so-called copper chaperone for superoxide dismutase or Ccs1p (Culotta *et al.*, 1997; Wong *et al.*, 2000). Indeed, Ccs1p is specific for Sod1p, and although *ccs1*Δ cells have normal levels of Sod1p, these mutants fail to incorporate copper into Sod1p, and therefore lack superoxide dismutase activity (Culotta *et al.*, 1997). Ccs1p co-localizes with Sod1p

within the cytosol (Culotta *et al.*, 1997; Rothstein *et al.*, 1999), and interacting with Sod1p, directly inserts copper into the active site of the enzyme (Casareno *et al.*, 1998; Rae *et al.*, 1999; Schmidt *et al.*, 2000; Rae *et al.*, 2001; Lamb *et al.*, 2000). Copper loading to Sod1p by Ccs1p may represent a novel method of controlling enzyme activity at the post-translational level.

Cu,Zn-superoxide dismutase is essential for protection against $O_2^{\cdot-}$ generated in the cytoplasm. Yeast strains lacking Sod1p show several defects including reduced rates of growth, auxotrophies for lysine and methionine, higher rates of spontaneous mutations and are sensitive to $O_2^{\cdot-}$ generating agents (*e.g.* paraquat) (Gralla and Valentine, 1991; Chang and Kosman, 1990; Longo *et al.*, 1996; Gralla, 1997; Liu *et al.*, 1992; Bilinski *et al.*, 1985). The *sod1Δ* mutants are also sensitive to oxidants and hyperoxia, but unlike *sod2Δ* cells, the oxygen sensitivity is not overcome by rho^{o} mutations blocking respiration (Guidot *et al.*, 1995). Yeast cells lacking Sod1p also exhibit mitochondrial defects, including poor growth on non-fermentable carbon sources (Longo *et al.*, 1996), a deficiency in mitochondrial aconitase (Strain *et al.*, 1998), a rapid death in stationary phase (Longo *et al.*, 1996), and increased carbonylation damage to mitochondrial proteins (Sturtz *et al.*, 2001). All these data point to a role of Sod1p in protecting against $O_2^{\cdot-}$ anions produced in the mitochondria. This hypothesis is further supported by the localization of Sod1p and its copper chaperone Ccs1p in the mitochondrial intermembrane space (Sturtz *et al.*, 2001). As ubisemiquinone anion of the respiratory chain can release $O_2^{\cdot-}$, not only to the mitochondrial matrix but also in the inter-membrane space (Turrens, 1980; Turrens *et al.*, 1985; Zhang *et al.*, 1998; Trumpower, 1990), Sod1p may in fact have a role in protection against these $O_2^{\cdot-}$ in the inter-membrane space, or alternatively, may preclude exit of mitochondrial $O_2^{\cdot-}$ and as such, protect extra-mitochondrial cell components from oxidative damage. Indeed, Longo *et al.* (1996) showed that inactivation of coenzyme Q partially alleviates the aminoacid auxotrophies of *sod1Δ* cells, suggesting that Sod1p does play a role in protection from superoxide anion produced during mitochondrial respiration.

Superoxide radicals specifically oxidize one iron of (4Fe-4S) clusters in enzymes, such as the mitochondrial aconitase (citric acid cycle), homoaconitase (lysine biosynthesis) and isopropyl malate isomerase (leucine biosynthesis), causing its release from the cluster and inactivating the enzyme (Halliwell and Gutteridge, 1999; Fridovich, 1995; Liochev and Fridovich, 1994). This process leads to both enzyme inactivation and further oxidative damage of other cellular components, as "free" iron can promote the formation of hydroxyl radical, via the Fenton reaction (Liochev and Fridovich, 1994). Thus, elevated levels of "free" iron are detected in yeast cells exposed to superoxide generating drugs, as well as in *sodΔ* mutants, deficient in superoxide dismutase (either the cytosolic Sod1p or the mitochondrial Sod2p, or both) (Srinivasan *et al.*, 2000; De Freitas *et al.*, 2000). Thus, Sod1p protects the

(4Fe-4S) enzymes from inactivation by preventing the accumulation of excess intracellular iron. However, a new mechanism suggests that the phenotype of *sod1Δ* mutants is due to both loss of essential (4Fe-4S) clusters and increased demand of iron, needed for their reconstitution, despite the increased intracellular "free" iron (De Freitas *et al.*, 2000). The released iron might not be in a form that can be used for the biogenesis of the clusters, and may be sequestered in the vacuole (reported to be the site of iron storage). Indeed, the increase of iron mediated oxidation within the vacuole leads to vacuole fragmentation in *sod1Δ* mutants (Corson *et al.*, 1999). According to this, the poor respiratory growth of *sod1Δ* mutants is improved by excess of exogenous iron and worsened by deletion of the high affinity iron transport *FET3* gene (De Freitas *et al.*, 2000).

In agreement with Sod1p functions, three of the genetic suppressors of *sod1Δ* methionine auxotrophy are involved in mitochondrial function and act by decreasing respiration (Strain *et al.*, 1998). These are Jac1p and Ssq1p, two mitochondrial chaperones and Nsf1p, a protein involved in (4Fe-4S) cluster assembly. The search for high copy suppressors of the *sod1Δ* methionine auxotrophy identified transketolase (*TKL1*) (Slekar *et al.*, 1996), an enzyme of the non-reductive part of the pentose phosphate pathway that controls the production of NADPH by glucose-6-phosphate dehydrogenase (Zwf1p). A mutant lacking Zwf1p also exhibits methionine auxotrophy and is oxidative stress sensitive (Nogae and Johnston, 1990; Thomas *et al.*, 1991), which supports the idea that low NADPH is causing the *sod1Δ* methionine auxotrophy by blocking sulphate assimilation at the level of PAPS (3'-phosphoadenosine-5'-phosphosulphate) and sulphite reductases. The *sod1Δ* lysine auxotrophy is not explained by low NADPH levels, but instead could be caused by a direct attack of a (4Fe-4S) cluster of an enzyme of the lysine biosynthetic pathway (Culotta, 2000).

Aerobic defects of *sod1Δ* mutants can be suppressed by genetic manipulations that increase copper or manganese levels. Deletion of *BSD2* gene, which is involved in copper and cadmium homeostasis, causes an increase in the intracellular copper levels (Liu and Culotta, 1994). Mutants in *PMR1* gene, which encode a Golgi localized P-type ATPase (Pmr1p/Bsd1p) that functions in the delivery of manganese and calcium ions into the Golgi (Lapinskas *et al.*, 1995; Mandal *et al.*, 2000), accumulate manganese in the cytoplasm. *ATX1* encodes a small copper binding protein that delivers copper to the copper transporter Ccc2p for the secretory pathway (Lin *et al.*, 1997; Lin and Culotta, 1995), and *ATX2* is another manganese homeostasis factor that localizes to intracellular vesicles (Lin and Culotta, 1996). A mutation in the *PMR1* or *BSD2* genes, leading to an increase of intracellular manganese and copper, respectively, rescue the lysine and methionine auxotrophies of the *sod1Δsod2Δ* double mutants, and reverse the aerobic defects of *sod1Δ* (Liu *et al.*, 1992). Overexpression

of *ATX1* or *ATX2* genes (that increase intracellular ion concentrations), also rescue the metabolic deficiencies of *sod1Δsod2Δ* mutants. Both manganese and copper ions are scavengers of $O_2^{\cdot-}$ *in vitro* (Liao *et al.*, 1994; Halliwell and Gutteridge, 1984) and a similar activity *in vivo* may be the mechanism by which all these mutations suppress the defects of *sod1Δsod2Δ* mutants.

In addition to $O_2^{\cdot-}$ scavenging, Sod1p also exerts a role in copper ion buffering. Copper represents a paradox to living organisms, because trace amounts of this ion are essential to promote growth, yet high concentrations can drastically impair cell growth and function due to its ability to participate in the Fenton or Haber-Weiss chemistry (Fridovich, 1978; Fridovich, 1983; Bostek, 1989). Sod1p functions as a metallothionein, by direct binding and sequester the metal, or by binding biological metal ligands (Culotta *et al.*, 1995).

Metallothioneins (MT) are a class of small cysteine-rich metal-binding proteins that function in metal detoxification and oxidative stress protection. The cysteine residues are usually present in Cys-X-Cys or Cys-X-X-Cys sequence motifs. One function of MT proteins is to buffer the intracellular concentration of metal ions such as copper and zinc, by chelating and sequestering metal ions, and thus minimizing metal cytotoxicity (Kagi, 1991; Hamer, 1986; Karin, 1985; Petering and Fowler, 1986). *S. cerevisiae* contains two metallothionein encoding genes, *CUP1* and *CRS5*, that are transcriptionally activated by copper ions (Thiele, 1995; Culotta *et al.*, 1994; Furst *et al.*, 1988). Both basal and induced levels of *CUP1* and *CRS5* expression are dependent on Ace1p/Cup2p (Buchman *et al.*, 1989; Thiele, 1988; Welch *et al.*, 1989; Szczypka and Thiele, 1989; Culotta *et al.*, 1994; Furst *et al.*, 1988). Cup1p also guards against the oxidative damage associated to $O_2^{\cdot-}$ (Tamai *et al.*, 1993). Indeed, MTs have been suggested to protect against oxidants, by scavenging superoxide and hydroxyl radicals (Culotta *et al.*, 1994; Tamai *et al.*, 1993). In agreement, overexpression of *CUP1* suppresses defects associated with loss of *SOD1* (Tamai *et al.*, 1993). According to the role in scavenging, *CUP1* gene expression is induced by the $O_2^{\cdot-}$ generator, menadione, through a Hsf1p (heat shock factor 1) – dependent mechanism (Liu and Thiele, 1996).

The degradation/removal of H_2O_2 is catalyzed by different enzymes, including catalases and peroxidases. Catalases are homotetrameric iron containing enzymes, with a haem reactive group. Catalases degrade H_2O_2 into O_2 and H_2O. *S. cerevisiae* has two forms of it: a cytosolic catalase, Ctt1p, and a peroxisomal / mitochondrial catalase, Cta1p (Cohen *et al.*, 1988; Hartig and Ruis, 1986; Petrova *et al.*, 2004). The main physiological role of Cta1p appears to be the remotion of H_2O_2 produced by fatty acid β-oxidation (peroxisomes) and by mitochondrial respiration. *CTT1* gene expression is induced by oxidative and osmotic stress, and by starvation (Ruis and Hamilton, 1992). Both the *ctt1Δ* and *cta1Δ* mutants show resistance to H_2O_2 similar to that of wild type cells (Grant *et al.*, 1998).

In *S. cerevisiae*, cytochrome c peroxidase (Ccp1p) uses H_2O_2 as an electron acceptor to oxidize cytochrome c in the mitochondrial intermembrane space (Godon *et al.*, 1998). Consequently, electrons to be transferred to oxygen are consumed by Ccp1p and, as result, water is produced from hydrogen peroxide. Thus, Ccp1p might be used to detoxify hydrogen peroxide produced in mitochondria by Sod2p. Indeed, Ccp1p expression increases under respiratory culture conditions and by treatment with H_2O_2 (Kwon *et al.*, 2003).

Unlike catalase, Ccp1p or SOD, the peroxidases use active-site thiols to reduce peroxides. The catalytic activity of these enzymes is dependent on NADPH and two distinct electron flow systems: the glutathione and the thioredoxin pathways. In addition to their scavenging functions, these pathways are essential for the maintenance of a reduced cytoplasmic thiol redox balance, and are essential as electron donors for enzymes with a reducing step in their catalytic cycle, such as ribonucleotide reductase. The electron source for both glutathione and thioredoxin pathways is NADPH obtained from the oxidation of glucose by the pentose phosphate pathway (see 1.3.2.2).

Glutathione (γ-glutamyl-cysteinyl-glycine, GSH) is the most abundant thiol antioxidant molecule in yeast and has a low redox potential. GSH is synthesized by the action of gamma-glutamylcysteine synthase (*GSH1*), that catalyses the rate-limiting step, and glutathione synthase (*GSH2*) (Grant and Dawes, 1996; Ohtake and Yabuuchi, 1991). GSH acts as a radical scavenger, reacting with oxidants and resulting in oxidised glutathione. The sulphydryl group of the cysteine residue is responsible for its redox properties. Oxidized glutathione (GSSG) is reduced back to GSH by the NADPH-dependent glutathione reductase (*GLR1*) (see 1.3.2.2). Thus, a high GSH:GSSH ratio functions as a redox buffer, playing a crucial role in keeping a reducing cytoplasmic thiol redox balance and in absorbing oxidizing equivalents. The *gsh1*Δ mutants display a petite (respiration deficient cells) phenotype, being unable to grow on non-fermentable carbon sources such as glycerol, and can only grow when GSH is supplemented. The role of glutathione can be extended to mitochondria protection from oxidants produced during respiration (Wu and Moye-Rowley, 1994; Grant *et al.*, 1996b; Stephen and Jamieson, 1996; Lisowsky, 1993). The *gsh1*Δ mutants, but not *gsh2*Δ mutants, are hypersensitive to H_2O_2, plumbagin and menadione (superoxide anion generators) (Grant *et al.*, 1996b; Grant *et al.*, 1997; Izawa *et al.*, 1995), and the transcription of *GSH1* is induced by H_2O_2 (Stephen *et al.*, 1995; Wu and Moye-Rowley, 1994). GSH is also involved in detoxification, as it can also be conjugated to toxic electrophiles and the conjugate transported to the vacuole through specific membrane efflux pumps. For example, GSH provides protection from cadmium toxicity, in association with the vacuolar membrane ATP-binding cassette transporter, Ycf1p (Li *et al.*, 1997). In addition to its protective role against oxidative damage, glutathione performs a specific function in the

maturation of cytosolic Fe/S proteins. In *gsh1*Δmutants, the maturation of extra mitochondrial Fe/S proteins is substantially decreased (Sipos *et al.*, 2002).

The redox balance is also dependent on thioredoxin. Thioredoxin is a small sulphydryl rich protein, with two redox active cysteines within the conserved active site motif, Trp-Cys-Gly-Pro-Cys. This protein is able to reduce the dissulfide bonds of other proteins. Oxidised thioredoxin is then reduced back to the active dithiol form by the flavoenzyme NADPH dependent thioredoxin reductase (see 1.3.2.2). *S. cerevisiae* contains two apparently redundant isoforms of cytosolic thioredoxins, Trx1p and Trx2p (Muller, 1991) and a mitochondrial thioredoxin, Trx3p (Pedrajas *et al.*, 1999). *TRX2* is strongly induced by H_2O_2 (Kuge and Jones, 1994; Morgan *et al.*, 1997) and deletion of *TRX2*, but not *TRX1* gene, renders cells hypersensitive to H_2O_2 (Muller 1996). Deletion of both *TRX1* and *TRX2* genes results in a marked increase of oxidized glutathione, and in peroxide hypersensitivity (Muller, 1996).

Peroxiredoxins (Prx), also known as thioredoxin peroxidases, reduce H_2O_2 and alkyl hydroperoxides to H_2O or the corresponding alcohols with the use of hydrogens provided by thioredoxin, thioredoxin reductase and NADPH (Chae *et al.*, 1994a; Rhee *et al.*, 1999). All Prxs have one conserved domain containing one (1-Cys) or two (2-Cys) cysteine residues. *S. cerevisiae* possesses four 2-Cys Prx and one 1-Cys Prx, which are not redundant, as they differ in subcellular localization and peroxide specificities.

S. cerevisiae contains three 2-Cys cytosolic thioredoxin peroxidases, cTPxI/Tsa1p (Thiol Specific Antioxidant) (Chae *et al.*, 1994a,b), cTPxII (Park *et al.*, 2000) and cTPxIII (Jeong *et al.*, 1999). Expression of cTPxI, cTPxII and cTPxIII, is induced by H_2O_2 (Lee *et al.*, 1999a; Park *et al.*, 2000). cTPxI is more specific towards H_2O_2, whereas cTPxIII is more specific towards organic peroxides (Chae *et al.*, 1993; Lee *et al.*, 1999b; Park *et al.*, 2000; Jeong *et al.*, 1999). Based on its capacity to confer resistance to t-BOOH (tert-butyl-hydroperoxide), cTPxIII was named as Ahp1p, for alkyl hydroperoxidase (Lee *et al.*, 1999b). The proposed catalytic model for Tsa1p involves direct reduction of H_2O_2 by Cys47, which is oxidized to a sulphenic acid. Oxidized Cys47 can then react with Cys170 of another Tsa1p molecule to form a disulphide anti-parallel homodimer, which is then reduced by the thioredoxin system (Chae *et al.*, 1994a,b; Netto *et al.*, 1996). Similar to Tsa1p, cTPxIII forms a disulphide linked homodimer upon oxidation (Lee *et al.*, 1999b).

In addition, *S. cerevisiae* contains a nuclear 2-Cys Prx, nTPx, and a mitochondrial 1-Cys Prx, mTPx (also known as Prx1p) (Park *et al.*, 2000; Pedrajas *et al.*, 2000). In contrast to nTPx (Dot5p) (Izawa *et al.*, 2003), mTPx appears to have a more specific role in combating peroxides produced during respiration (Pedrajas *et al.*, 2000). mTPx expression is induced by H_2O_2 and diamide (Park *et al.*, 2000). mTPx peroxidase activity is assisted by the

mitochondrial thioredoxin system, and is therefore the first 1-Cys peroxiredoxin shown to receive its electrons from thioredoxin.

Other thiol dependent antioxidants are glutathione peroxidases. In *S. cerevisiae*, three Gpx were identified, Gpx1, Gpx2 and Gpx3 (Inoue *et al.*, 1999). A careful alignment of these proteins with GPxs from higher eukaryotes showed higher sequence identities with phospholipids hydroperoxide glutathione peroxidases (PHGPx) than with GPx, suggesting that all three *S. cerevisiae* GPx are PHGPx (Avery and Avery, 2001). GPx1-3 are able *in vitro* to reduce a phospholipid hydroperoxide (Avery and Avery, 2001), in addition to H_2O_2 and t-BOOH (Inoue *et al.*, 1999). Gpx3p is the most important of the three Gpx isoforms, as *gpx3*Δ but not *gpx1*Δ and *gpx2*Δ mutants, are hypersensitive to H_2O_2 and t-BOOH. Although *gpx2*Δ mutants are not hypersensitive to H_2O_2, *GPX2* is induced by H_2O_2 (Inoue *et al.*, 1999; Avery and Avery, 2001).

Aminoacid derived polyamines have also been implicated in protecting yeast against oxidant stress. Polyamines bind copper, thus preventing the Fenton reaction effects, and protecting lipids from oxidation. *SPE2* encodes S-adenosylmethionine decarboxylase, which is involved in polyamines biosynthesis, and a *spe2*Δ null mutant was found to be hypersensitive to oxygen (Balasundaram *et al.*, 1991, 1993).

1.3.2.2 – Secondary antioxidant defences

Secondary antioxidant defences (Table 5) repair or remove oxidative damaged molecules, including DNA, lipids or proteins.

It is known that $O_2^{\cdot-}$ and H_2O_2 do not damage DNA directly, but only through the Haber Weiss and Fenton-catalysed $OH^{\bullet}$ production (Imlay and Linn, 1998). In addition, any other free radicals that are formed from lipid and protein peroxidation can induce DNA damage. Oxidation of nucleic acids leads to bases and sugar damages, single strand breaks, apurinic/apyrimidinic (AP) sites, DNA-protein cross-links (Halliwell and Aruoma, 1993), and increases the frequency of intrachromossomal recombination (Frankenberg *et al.*, 1993; Brennan *et al.*, 1994). Amongst DNA oxidative damages, base modification is an important class of lesions due to its lethal and mutagenic effect. Indeed, oxidative DNA damage is involved in pathological processes such as cancer, neurodegenerative diseases and ageing (Feig *et al.*, 1994; Ames and Shigenaga, 1993; Wiseman and Halliwell, 1996). Organisms have developed robust DNA repair mechanisms and enzymatic systems to replace oxidised bases. DNA damages caused by ROS are repaired by the base excision repair (BER) and nucleotide excision repair (NER) pathways. While both BER and NER are DNA repair

pathways, owing to remove and replace DNA damages, recombination (REC) and translation synthesis (TLS) are known as bypass / tolerance mechanisms, since neither may actually remove DNA lesions (Cox, 2002; Hoeijmakers, 2001). The BER pathway is initiated by the action of glycosylases that excise specific damaged bases from DNA (Nielsen and Krokan, 2001). The resulting AP site is subsequently cleaved either by a separate AP endonuclease or by an AP lyase activity that is associated with the DNA glycosylase. This generates a single strand break with a 3'-OH end that can be used as a substrate by a DNA polymerase. Finally, the successive action of a DNA polymerase and a DNA ligase complete the damage repair.

In *S. cerevisiae*, three DNA-N-glycosylases/AP lyases are involved in the repair of oxidatively damaged DNA bases: Ntg1p, Ntg2p and Ogg1p (Girard and Boiteux, 1997). Ntg2p is exclusively nuclear, whereas Ntg1p is both nuclear and mitochondrial (Alseth *et al.*, 1999; You *et al.*, 1999). Furthermore, Ntg1p is DNA damage-inducible, whereas Ntg2p is constitutively expressed (Eide *et al.*, 1996; Augeri *et al.*, 1997; Alseth *et al.*, 1999). Recently, it has been reported that an *ntg1Δntg2Δ* mutant is not sensitive to H_2O_2, and it does not exhibit a mutator phenotype (Swanson *et al.*, 1999; Gellon *et al.*, 2001). However, a *ntg1Δntg2Δrad1Δ* mutant shows an increased rate of spontaneous and oxidative stress induced mutagenesis, which is correlated to a hypersensitivity to H_2O_2 and $O_2^{\cdot-}$ generating drugs (Gellon *et al.*, 2001). Since Rad1p mediates a nuclear nucleotide excision repair (Swanson *et al.*, 1999; Gellon *et al.*, 2001), these results show that oxidatively damaged bases are not only removed by BER enzymes such as Ntg1p and Ntg2p, but also by the NER pathway. Accordingly, a recent study showed that under conditions of H_2O_2 exposure, the BER-defective and NER-defective strains contain significant levels of unrepaired oxidative DNA damage (Salmon *et al.*, 2004).

In *S. cerevisiae*, *APN1* and *APN2* encode AP endonucleases that remove AP sites generated by glycosylases or during the oxidative attack by $OH^{\bullet}$ on the deoxyribose moiety. Apn1p possessess a 3' phosphatase and a 3' phosphodiesterase activity that can remove the 3' terminal groups formed in DNA (Seeberg *et al.*, 1995; Wallace, 1997). Apn2p, besides these activities, also has a 3'→5' exonuclease activity specific for double stranded DNA (Wallace, 1997). Furthermore, Apn2p has a 3' phosphodiesterase activity in the removal of 3' blocked ends formed in DNA upon treatment with H_2O_2 and other oxidative DNA damaging agents. It has also been shown that the *apn1Δapn2Δ* double mutation confers a large increase in H_2O_2 sensitivity although neither of the individual mutations causes an increase over that in the wild type strain (Unk *et al.*, 2001).

Recent studies strongly suggest that the mutagenic action of endogenous oxidative DNA damage is also counteracted by the mismatch repair pathway (MMR), which corrects

misaligned frameshift intermediates. The most extensively characterized MMR system is the methyl-directed MutHLS system of the bacterium *E. coli* (Modrich and Lahue, 1996), which includes MutS, MutL and MutH proteins. *S. cerevisiae* has six MutS homologs (Msh1p to Msh6p) and four MutL homologs (Pms1p and Mlh1p to Mlh3p). Msh2p functions with Pms1p and Pms2p in a complex that interacts with Msh6p to repair single-base and insertion-deletion mispairs. Consequently, these classes of base substitutions are greatly enhanced in yeast strains deficient in Msh2p and Msh6p (Earley and Crouse, 1998; Ni *et al.*, 1999), and yeast cells containing mutations in *MSH2* gene are deficient in the repair of thymine glycols resulting from oxidative DNA damage (Leadon and Avruiskaya, 1998). Taken together the results show that in *S. cerevisiae* BER, NER and MMR pathways are involved in the repair of oxidative DNA damage.

Table 5. Yeast secondary antioxidant defences

Gene	**Product**
OGG1	mitochondrial 8-oxoguanine glycosylase / lyase
NTG1 = OGG2	mitochondrial and nuclear 8-oxoguanine glycosylase / lyase
NTG2	nuclear endonuclease three-like glycosylase
APN1	mitochondrial and nuclear AP endonuclease
APN2	nuclear AP endonuclease
MSH2, MSH6	mutS homologs
MGM101	mitochondrial nucleoid protein
MHR1	involved in mitochondrial homologous recombination
PIF1	DNA helicase
ATM1	ATP binding cassette transporter
OCA1	tyrosine phosphatase
MSRA	methionine sulphoxide reductase
SRX1	sulphiredoxin
PDI	protein disulphide isomerase
GSH1	glutathione
TRX2	thioredoxin
GLR1	glutathione reductase
TRR1	cytoplasmic thioredoxin reductase
TRR2	mitochondrial thioredoxin reductase
ZWF1	glucose-6-phosphate dehydrogenase
GRX1-GRX5	glutaredoxins
UBI4	polyubiquitin

[a]GSH1 encodes gamma-glutamyl-cysteine synthetase, which catalyses the first and rate-limiting step in glutathione biosynthesis

Mitochondria are important cellular targets for spontaneous and induced DNA damage (Sawyer and van Houten, 1999). The susceptibility of mtDNA to oxidative damage has been

associated to the proximity of mtDNA to ROS generated as by products of normal respiration and the lack of a compact nucleosome structure to protect mtDNA (Clayton *et al.*, 1974). Damaged mitochondrial DNA, if not repaired, leads to disruption of the electron transport chain and to the production of ROS. This vicious cycle of ROS production and mtDNA damage ultimately leads to energy depletion and apoptosis. Several components of the BER pathway have been localised to mitochondria in yeast. This includes the major AP endonuclease Apn1p (Vongsamphanh *et al.*, 2001), and the mitochondrial forms of DNA glycosylases, Ogg1p (Singh *et al.*, 2001) and Ntg1p (Alseth *et al.*, 1999; You *et al.*, 1999), that initiate the repair of oxidatively damaged purines and pyrimidines, respectively. In addition, yeast mitochondria contain the repairing enzyme Mgm101p, and *mgm101*Δ mutants show higher levels of H_2O_2 induced mtDNA damage (Meeusen *et al.*, 1999). Recombination also appears to be a pathway involved in mtDNA damage resistance. Yeast mitochondria contain two proteins implicated in mtDNA recombination, Pif1p, a DNA helicase (Lahaye *et al.,* 1991) and Mhr1p, a protein of unknown function, both of which appear to be also involved in mtDNA repair or damage tolerance (Foury and Lahaye, 1987; Ling *et al.*, 2000). *mhr1-1*Δ mutant cells show extensive vegetative petite induction by UV radiation at 30°C, and the number of mtDNA lesions increase 2-fold when submitted to a temperature shift from 30 to 37°C. Malonic acid, an analog of succinic acid and inhibitor of succinate dehydrogenase (a tricarboxylic acid cycle enzyme), decreases the flow of electrons in the electron transport chain, and the oxidative stress in mitochondria (Senbongi *et al.*, 1999). Using malonic acid, both the petite induction and the temperature-induced increase in the amount of mtDNA damage were partially supressed in *mhr1-1*Δ cells at 37°C. Thus, active *MHR1* may keep the extent of spontaneous oxidative damage in mtDNA within a tolerable level (Ling *et al.*, 2000). These observations indicate that BER, recombination and MMR pathways exist in mitochondria to help in the maintenance of the mitochondrial genome.

Other factors are important for the protection of mtDNA. Glutathione has a key role in the mitochondrial function, since yeast strains deleted for *GSH1* loose the mtDNA and are unable to grow on non-fermentable carbon sources (Kistler *et al.*, 1986). In addition, *gsh1*Δ mutants show increased H_2O_2 and superoxide anion sensitivity (Grant *et al.*, 1996b; Izawa *et al.*, 1995; Stephen and Jamieson, 1996). However, the role of glutathione in the mitochondrial function is independent of that in oxidative stress resistance. Indeed, grande *gsh1*Δ mutants are stable but generate petites at an elevated frequency, and still induce an adaptive stress response to H_2O_2 (Lee *et al.*, 2001). Atm1p, an ATP binding cassette transporter is also important for mtDNA integrity. Loss of Atm1p increases free iron levels and leads to the accumulation of dsDNA breaks and generation of petite mutants (Senbongi *et al.*, 1999). In addition, *atm1*Δ mutants are hypersensitive to H_2O_2 (Kispal *et al.*, 1997).

Damage to lipids involves oxidation of polyunsaturated fatty acids (PUFA) by an autocatalytic process leading to the production of fatty acid hydroperoxides which undergo fragmentation, generating a variety of highly reactive products, such as epoxides, aldehydes and alkanes. Lipid peroxidation affects membrane integrity (by generating shorter fatty acid chains and increasing membrane fluidity), and leads to inactivation of membrane proteins and cell lysis.

S. cerevisiae synthesise only monounsaturated fatty acids (Paltauf *et al.*, 1992), which are very resistant to lipid peroxidation (Halliwell and Gutteridge, 1999). However, PUFA can easily be introduced since yeast cells take up exogenously fatty acids and rapidly incorporate them into glycerolipids (Kohlwein and Paltauf, 1984). *S. cerevisiae* cells grown aerobically in the presence of PUFA will preferentially internalize and incorporate them into membranes, rather than expend energy synthesizing fatty acids *de novo*. In yeast, high concentrations of H_2O_2 increase malondialdehyde (MDA) formation, resulting from lipid peroxidation, and it is likely that MDA content is dependent on the amount of PUFA present in membrane phospholipids (Steels *et al.*, 1994; Howlett and Avery, 1997).

In *S. cerevisiae*, one of the products of the fragmentation of lipid hydroperoxides (LOOH), 4-hydroxynonenal, inhibits growth at G_0/G_1 phase and the resumption of cell growth requires an increased synthesis *de novo* of glutathione (Wonisch *et al.*, 1997; Turton *et al.*, 1997). Recent studies revealed that linoleic acid hydroperoxide (LoaOOH), at low doses, also leads to a G_1 cell cycle delay that is prevented upon deletion of *OCA1*, which encodes a tyrosine phosphatase. Although not essential for adaptation or immediate cell survival, *OCA1* is required for growth in the presence of linoleic acid hydroperoxide (Alic *et al.*, 2001).

The toxicity of lipid hydroperoxides has been associated to a decrease of ubiquinone and glutathione levels. Ubiquinone (QH_2) is a membrane antioxidant that scavenges free radicals and prevents lipid peroxidative damage by reducing lipid peroxyl radicals into lipid hydroperoxides (Beyer, 1992; Ernster and Forsmark-Andree, 1993; Ernster and Dallner, 1995). Thus, ubiquinone plays an important role in protecting cells from PUFA breakdown products that mediate cell killing. Indeed, QH_2-deficient yeast cells are hypersensitive to the products of PUFA autoxidation (Clarke *et al.*, 1991). In addition, vitamin E functions as donor of single hydrogen atoms leading to nonradical forms of lipid derivatives. By scavenging lipid peroxyl radicals, vitamin E protects cells against LOOH toxicity and inhibits the propagation of lipid peroxidation (Buettner, 1993; Wolff *et al.*, 1986; Do *et al.*, 1996).

Glutathione peroxidases and thioredoxin peroxidases, acting in concert with phospholipases, reduce LOOH esterified to biomembranes into LOH (see 1.3.2.1), thus preventing their fragmentation catalysed by Fe^{2+} (Ursini *et al.*, 1995; Chen *et al.*, 2000). Glutathione and thioredoxin peroxidases use GSH and thioredoxin, respectively, to reduce peroxides. Once oxidized, GSH dimerizes to the disulphide GSSG, which is reduced back to

GSH by the NADPH-dependent glutathione reductase (*GLR1*), that works in the maintenance of the GSH:GSSG ratio in cells. *GLR1* is not essential in *S. cerevisiae* (Collinson and Dawes, 1995), but *glr1*Δ mutants accumulate an excess of GSSG and are hypersensitive to oxidants in the stationary phase (Grant *et al.*, 1996a; Grant *et al.*, 1996c; Izawa *et al.*, 1998). In addition, *GLR1* expression is induced by H_2O_2 and diamide (a free thiol oxidizing agent) (Grant *et al.*, 1996a).

S. cerevisiae also contains two flavoenzyme NADPH dependent thioredoxin reductases which are able to reduce thioredoxin back to the active dithiol form. The citosolic thioredoxin reductase is encoded by *TRR1* gene (Chae *et al.*, 1994a) and the mitochondrial thioredoxin reductase is encoded by *TRR2* gene (Pedrajas *et al.*, 1999). *trr1*Δ and *trr2*Δ mutants are sensitive to H_2O_2 (Lee *et al.*, 1999a) and *TRR1* transcription is induced by H_2O_2 (Lee *et al.*, 1999b; Charizanis *et al.*, 1999a). These results suggest that cells lacking thioredoxin reductase have diminuished capacity to detoxify oxidants and / or to repair oxidative stress-induced damages (Carmel-Harel *et al.*, 2001) (see 1.3.2.1).

Glutathione reductase and thioredoxin reductase both require NADPH as reductant to reduce GSSG and thioredoxin. Thus, disruption of *ZWF1* gene, encoding glucose-6-phosphate dehydrogenase, which catalyses the first rate-limiting oxidative step of the pentose phosphate pathway, and also of other enzymes of this pathway, render the cells hypersensitive to H_2O_2 (Nogae and Johnston, 1990; Juhnke *et al.*, 1996).

The oxidative damages to proteins involves oxidation of aminoacid residues side chains to hydroxy and carbonyl derivatives, scission of the polypeptide chain and intermolecular protein cross-linking, leading to increased proteolytic susceptibility, decreased biological activity (Stadtman, 1993; Levine *et al.*, 1994) and eventually to cell death (Coan *et al.*, 1992). Aminoacids readly prone to oxidation by ROS include cysteine, histidine, triptophan, tyrosine, phenylalanine and methionine, being cysteine and methionine the most sensitive (Vogt, 1995; Coan *et al.*, 1992; Davies *et al.*, 2001).

Cysteine residues can be irreversibly oxidised to higher oxidation states (e.g. to cysteine sulphinic acid (Cys-SO_2H) and cysteic acid (–SO_3H) forms), and this reaction can be prevented by S-thiolation with a number of low molecular weight thiols such as GSH (Thomas *et al.*, 1995; Klatt and Lamas, 2000; Grant *et al.*, 1999; Demasi *et al.*, 2003). Indeed, the basal levels of S-thiolation are low, but they are increased following treatment with H_2O_2, together with a decrease of the GSH/GSSG ratio (Grant *et al.*, 1998). Dethiolation of protein mixed disulphides and reactivation of proteins containing oxidised Cys residues (cysteine-sulphenic acids, Cys-SOH) can occur via direct reduction by glutathione or catalyzed by glutaredoxins, thioredoxins or protein dissuphide isomerase. All of these antioxidant defences are induced by H_2O_2 (Lee *et al.*, 1999a).

Glutaredoxins are a class of small proteins that carry a thioltransferase activity similar to thioredoxin, thus acting in the reduction of protein disulphides. They form part of the glutaredoxin system comprising of NADPH, GSH, and glutathione reductase, which transfer electrons from NADPH to glutaredoxin via GSH (Holmgren, 1990). Depending on the number of cysteines, two classes of glutaredoxins have been identified: the dithiol glutaredoxins with the consensus Cys-Pro-Tyr-Cys (that might function to reduce protein disulphide bonds), and the monothiol glutaredoxins with the consensus Pro-X-Cys-Gly/Ala-Phe-Ser/Pro (that might be involved in the reduction of S-thiolated substrates). *S. cerevisiae* contains two dithiol glutaredoxins, Grx1p and Grx2p (Gan, 1992; Luikenhuis *et al.*, 1998) and three monothiol enzymes, Grx3p, Grx4p, and Grx5p (Rodriguez-Manzaneque *et al.*, 1999).

The *grx1*Δ mutants are hypersensitive to the superoxide anion, but not to H_2O_2, in contrast with *grx2*Δ mutants that are sensitive to H_2O_2 but not to superoxide (Luikenhuis *et al.*, 1998). The *GRX1* and *GRX2* genes are the two members of a gene family that are induced by H_2O_2, and when overexpressed confer an increased resistance to H_2O_2. The *grx5*Δ single mutant has a slow growth phenotype, is unable to grow on a respiratory carbon source and is hypersensitive to H_2O_2 (Luikenhuis *et al.*, 1998; Rodriguez-Manzaneque *et al.*, 1999). Grx5p is a mitochondrial protein involved in iron sulphur cluster biogenesis and in protein S-dethiolation during recovery after H_2O_2 stress (Shenton *et al.*, 2002; Rodriguez-Manzaneque *et al.*, 2002). The accumulation of iron as a consequence of *GRX5* gene disruption seems to promote the formation of ROS, such as the hydroxyl radical that leads to protein carbonylation and protein S-thiolation (Rodriguez-Manzaneque *et al.*, 1999).

Curiously, Collinson *et al.* (2002) showed that yeast glutaredoxins can directly reduce hydroperoxides, using reducing power provided by NADPH, GSH and glutathione reductase, and thus are active as glutathione peroxidases. The corresponding alcohols produced can be conjugated to GSH by glutathione–S–transferase and transported into the vacuole by Ycf1p.

Cysteine-sulphinic acid derivatives have been viewed as irreversible protein modifications. However, it has been recently identified a sulphiredoxin (Srx1p), that is conserved in higher eukaryotes and that reduces cysteine-sulphinic acid of the yeast peroxiredoxin Tsa1p. This protein is likely to be involved in the repair of protein containing cysteine-sulphinic acid modifications and in signalling pathways involving protein oxidation (Biteau *et al.*, 2003).

Although several proteins are S-thiolated during exposure of yeast cells to H_2O_2 (Shenton and Grant, 2003), the glyceraldehyde-3-phosphate dehydrogenase (Tdh3p) was identified as the major target (Grant *et al.*, 1999). Protein S-thiolation affects enzyme activity but Tdh3 activity is restored after removal of H_2O_2 by a mechanism that involves dethiolation mediated by the monothiol glutaredoxin Grx5p (Shenton and Grant, 2003). In agreement, the Tdh2 isoform is not S-thiolated and its activity is only partially restored during recovery from

H_2O_2 stress (Shenton *et al.*, 2002), indicating that this protein modification is tightly regulated. Thus, in a mutant that lacks glutathione, glycolytic enzyme activities are irreversibly inhibited by H_2O_2 (Shenton and Grant, 2003).

H_2O_2 also oxidises methionine to Met-sulphoxides or sulphone. Methionine oxidation to methionine sulphoxide (MetSO) is reversible and the reduction of MetSO is catalysed by methionine sulphoxide reductase (MsrAp), an enzyme present in all living organisms (Brot *et al.*, 1981; Brot and Weissbach, 2000). Indeed, yeast cells deleted for *MSR* gene present a considerable lower growth in the presence of oxidants and accumulate high amounts of both free and protein-bound methionine sulphoxide when exposed to H_2O_2 (Moskovitz *et al.*, 1997). Conversely, overproduction of MsrAp leads to resistance to ROS (Moskovitz *et al.*, 1998).

Yeast cells exposed to H_2O_2 accumulate free and protein bound *ortho*- and *meta*-tyrosine (Poljak *et al.*, 2003), resulting from the hydroxyl radical attack on phenylalanine (Davies *et al.*, 2001). According to the role of GSH in the cellular protection against free radical attack, yeast mutants deficient in glutathione metabolism are shown to accumulate significant levels of *o*- and *m*- tyrosine during normal aerobic growth conditions (Poljak *et al.*, 2003).

Among the various oxidative modifications, protein carbonylation is an early marker during protein oxidation and results from oxidative modification on Arg, Pro, Lys and His residues and by oxidative cleavage of the peptide chain at Pro, Glu or Asp residues (Stadtman, 1993; Levine *et al.*, 1994). Carbonyl groups may also be formed in proteins by Michael addition reactions of 4-HNE or by modifications with reducing sugars. It has been shown that the H_2O_2 induced protein carbonylation is specific and probably due to its conversion into hydroxyl radicals, catalysed by redox active metal ions, such as copper and iron (Halliwell and Gutteridge, 1999). Glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate mutase, mitochondrial enzymes and Cu,Zn-superoxide dismutase were identified as the major targets oxidatively inactivated (Cabiscol *et al.*, 2000; Costa *et al.*, 2002). Besides, the high sensitivity of yeast cells deficient in the oxidative stress response factors Yap1p and Skn7p (see 3.3.1) to H_2O_2, was correlated with an increased induction of protein carbonylation (Costa *et al.*, 2002).

Trehalose was shown to have a protective role towards protein carbonylation, presumably acting as a free radical scavenger. Indeed, trehalose accumulation induced by exposure to mild heat shock or proteasome inhibitors, increased H_2O_2 resistance associated with a reduced protein oxidation, and cells lacking trehalose revealed a faster protein oxidation and are more sensitive to H_2O_2 (Benaroudj *et al.*, 2001). Since oxidatively damaged proteins tend to aggregate, the protection of protein from oxidation mediated by trehalose may also be associated to the fact that trehalose reduces protein aggregation and maintains

polypeptide chains in a partially folded state, thus facilitating their refolding by cellular chaperones (Singer and Lindquist, 1998b).

Proteins irreversibly inactivated by formation of methionine sulphone and carbonyl derivatives cannot be repaired and are targeted to proteolytic pathways. In fact, the increase in protein carbonyl content is one of the major factors that have been implicated in increasing the rate of proteolysis and decreasing the half life of oxidised proteins (Dunlop, 2002). Both the multicatalytic protease (26S proteasome) and vacuolar proteases probably mediate the degradation of these irreversibly oxidised proteins. Indeed, in *S. cerevisiae* enzymes of the ubiquitin-proteasome pathways and vacuolar proteases are induced by H_2O_2 (Godon *et al.*, 1998; Lee *et al.*, 1999a; Gasch *et al.*, 2000). However, several evidences have been suggesting that protein ubiquitination is not required for the degradation of oxidised proteins. Although the *ubi4*Δ mutants have an increased sensitivity to H_2O_2 (Cheng *et al.*, 1994), no protein multiubiquitination was observed in H_2O_2 treated cells. In addition, exposure to H_2O_2 increased the activity of the 20S proteasome, which degrades oxidised proteins in an ATP- and ubiquitin-independent manner. Concomitantly, the activity of the 26S proteasome is repressed. The key role of the 20S proteasome in the degradation of oxidised proteins is further supported by the fact that yeast cells deficient in the Rpn9p subunit of the 19S regulatory complex, exhibit a higher activity of 20S proteasome and are able to degrade carbonylated proteins more efficiently than wild type cells (Inai and Nishikimi, 2002).

1.3.3 – Oxidative stress response

The levels of antioxidant defences present in the cells under physiological conditions confer a limited capacity to resist to a sudden oxidative aggression. Indeed, under specific stress conditions, the ROS levels exceed the antioxidant capacity of the cells, leading to an oxidative stress. This unbalance can result from a decrease in antioxidants, an increased production of ROS, or both. To survive, the yeast cells are able to sense the oxidative stress and to build a response at the molecular level involving the induction of both primary and secondary antioxidant defences (Jamieson, 1998; Moradas-Ferreira and Costa, 2000). The adaptation to stress conditions involves early responses and late responses that allow cells to return to non-stress conditions, in a rapid and ordered way. Early responses result in the post-translational activation of pre-existing defences, as well as the activation of signal transduction pathways that initiate late responses, namely the synthesis *de novo* of stress proteins and antioxidant defences. Both specific and general stress responses are triggered by oxidative challenges.

1.3.3.1 – Hydrogen peroxide stress response

The adaptive response to hydrogen peroxide has been extensively analysed and most of the data were obtained from the effect of a transient stress induced with sublethal doses (0.2 - 0.4 mM H_2O_2), which renders the cells tolerant to a subsequent lethal stress (Collinson and Dawes, 1992; Jamieson, 1992; Davies *et al.*, 1995; Flattery-O'Brien *et al.*, 1993). Upon H_2O_2 challenge, the adaptive responses involve changes in gene expression within minutes and subsequently, changes in protein synthesis.

The main data related with the biochemical changes observed with peroxide treatment were obtained by analysis of the alteration of the proteome using two dimensional gel electrophoresis (Godon *et al.*, 1998; Lee *et al.*, 1999a) or by DNA microarrays mRNA profiling (Gash *et al.*, 2000; Causton *et al.*, 2001). Studies using Northern Blot analysis or gene fusions β-galactosidase assays have also been performed (see Table 6 for references). More than 100 induced proteins and about 50 repressed proteins were identified on two dimensional maps in response to H_2O_2.

The H_2O_2 responsive proteins can be sorted into different functional classes. As expected, several antioxidants were induced by H_2O_2, such as members of the glutathione and thioredoxin systems (Gash *et al.*, 2000; Godon *et al.*, 1998; see Table 6). It is interesting to note that cells lacking glutathione synthesis (*gsh1*Δ) are hypersensitive to H_2O_2 but are still able to induce an adaptive response, indicating that GSH level is not a key factor for the response (Grant *et al.*, 1996; Grant *et al.*, 1997; Izawa *et al.*, 1995; Stephen and Jamieson, 1996).

Catalases were shown to be required for the acquisition of H_2O_2 resistance following pre-treatment with low doses of H_2O_2 (Izawa *et al.*, 1996). Indeed, *cta1*Δ*ctt1*Δ mutant cells have a decreased ability to adapt to H_2O_2 (Izawa *et al.*, 1996; Grant *et al.*, 1996). The increase of Ccp1p expression under respiratory culture conditions and by H_2O_2 treatment may be associated with the detoxification of mitochondrial generated and exogenous H_2O_2. In addition, cytochrome c peroxidase activity is important in a signalling pathway to activate both the transcription factors Skn7p and Yap1p, which are involved in the response to H_2O_2 (see 3.3.1) (Charizanis *et al.*, 1999b). All these putative roles may be associated with the high sensitivity of *ccp1*Δ mutants to hydrogen peroxide.

Heat shock proteins, proteases, as well as polyubiquitin (Ubi4p) and protein disulphide isomerase (Pdi1p) are also induced by H_2O_2 (Godon *et al.*, 1999; Lee *et al.*, 1999a; Gash *et al.*, 2000; Schuller *et al.*, 1994). Most of HSPs are molecular chaperones that assist unfolded proteins accumulating under stress conditions, to regain their proper folding or to be targeted for proteolytic degradation. The induction of subunits of the proteasome along with enzymes

of the ubiquitin pathway, mitochondrial and vacuolar proteases is consistent with an increased proteolytic activity during the stress response.

Table 6. Antioxidant defences induced by hydrogen peroxide

Gene	Product	Factors[c]	Sensitivity	References
		Regulation and sensitivity of the respective mutant		
Glutathione system				
GSH1[a]	glutathione	Yap1p	S	Stephen *et al*., 1995; Gash *et al*., 2000; Grant *et al*., 1996; Grant *et al*., 1997; Grant *et al*., 1998; Izawa *et al*., 1995
GSH2[b]	glutathione	Yap1p	R	Sugiyama *et a*l., 2000; Gash *et al*., 2000; Grant *et al*., 1996; Grant *et al*., 1997; Izawa *et al*., 1995
GLR1	glutathione reductase	Yap1p	S	Grant *et al*., 1996a; Gash *et al*., 2000; Godon *et al*., 1998; Lee *et al*., 1999a; Grant *et al*., 1998
GPX1	glutathione peroxidase		R	Gash *et al*., 2000; Inoue *et al*., 1999; Avery and Avery, 2001
GPX2	glutathione peroxidase	Yap1p	R	Inoue *et al*., 1999; Gash *et al*., 2000; Avery and Avery, 2001
GRX1	glutaredoxin	Msn2/4p	R	Luikenhuis *et al*., 1998; Gash *et al*., 2000; Godon *et al*., 1998; Lee *et al*., 1999a
GRX2	glutaredoxin		S	Luikenhuis *et al*., 1998; Gash *et a*l., 2000
Thioredoxin system				
TRX2	thioredoxin 2	Yap1, Skn7p	S	Kuge and Jones, 1994; Morgan *et al*., 1997; Gash *et al*., 2000; Godon *et al*., 1998; Lee *et al*., 1999a; Muller, 1996
TRR1	thioredoxin reductase	Yap1, Skn7p	S	Morgan *et al*., 1997; Gash *et al*., 2000; Godon *et al*., 1998; Lee *et al*., 1999a, Charizanis *et al*., 1999a
cTPXI	cytoplasmic thioredoxin peroxidase I	Yap1, Skn7p	S	Lee *et al*., 1999b; Charizanis *et al*., 1999a; Gash *et al*., 2000; Godon *et al*., 1998; Wong *et al*., 2002, Chae *et al*., 1993, Park *et al*., 2000
cTPXII	cytoplasmic thioredoxin peroxidase II	Yap1, Skn7p	S	Park *et al*., 2000;Gash *et al*., 2000; Godon *et al*., 1998; Lee *et al*., 1999a; Wong *et al*., 2002
cTPXIII	cytoplasmic thioredoxin peroxidase III	Yap1, Skn7p	R	Lee *et al*., 1999a,b; Gash *et al*., 2000; Godon *et al*., 1998; Chae *et al*., 1993; Park *et al*., 2000
mTPX	mitochondrial thioredoxin peroxidase		R	Park *et al*., 2000; Gash *et al*., 2000; Chae *et al*., 1993; Lee *et al*., 1999a
Other antioxidants				
CCP1	cytochrome c peroxidase	Yap1, Skn7p	S	Lee *et al*., 1999a,b; Gash *et al*., 2000; Godon *et al*., 1998; Kwon *et al*., 2003
CTA1	catalase A, mitochondrial / peroxisomal	Yap1p	R	Gash *et al*., 2000; Grant *et al*., 1998; Petrova *et al*., 2004
CTT1	catalase T, cytoplasmic	Yap1, Skn7, Msn2/4p	R	Jungmann *et al*., 1993; Marchler *et al*., 1993; Amoros *et al*., 2001; Gash *et al*., 2000; Godon *et al*., 1998; Lee *et al*., 1999a; Grant *et al*., 1998
SOD1	cytoplasmic superoxide dismutase	Yap1, Skn7p	S	Galiazzo and Labbe-Bois, 1993; Gash *et al*., 2000; Godon *et al*., 1998; Lee *et al*., 1999a; Bilinski *et al*., 1985; Bermingham McDonogh *et al*., 1988
SOD2	mitochondrial superoxide dismutase	Yap1, Skn7p		Gash *et al*., 2000; Godon *et al*., 1998; Lee *et al*., 1999a

[a] *GSH1* encodes gamma-glutamyl-cysteine synthetase, which catalyses the first and rate-limiting step in glutathione biosynthesis; [b] *GSH2* encodes glutathione synthetase, which catalyzes the ATP-dependent synthesis of glutathione from gamma-glutamylcysteine and glycine; S (sensitive); R (resistant); [c] Results assessed by Proteomics (2D gels) (Godon *et al*., 1998 and Lee *et al*., 1999a), and /or by DNA microarrays (Gash *et al*., 2000) , and / or Northern Blot and gene fusions beta-galactosidase assays (other references).

The changes in enzyme activities are tied up with changes in the metabolic flux related to the upregulation of antioxidant defences. Indeed, all the results point to a metabolic fluxes redistribution occurring in response to H_2O_2. The decrease in glycolytic activity, associated with a decrease of the activity of Tdh2p and Tdh3p, and enolase (Eno2p), is coupled with a slowdown of the tricarboxylic acid cycle (Godon *et al.*, 1998). Accordingly, there is a decreased expression of pyruvate decarboxylase (Pdc1p) and pyruvate dehydrogenase (beta subunit) (Pdb1p). These results suggest a redirection of glucose utilization, leading to decreased ATP levels (Osorio *et al.*, 2003). These changes dramatically affect carbohydrate metabolism, which appears to be diverted to the generation of NADPH (Figure 3).

As the conversion of glucose-6-phosphate to pyruvate decreases, the pool of glucose-6-phosphate is shifted to the pentose phosphate pathway, with an increase of NADPH production. Several enzymes of the pentose phosphate pathway are induced by H_2O_2, namely, glucose-6-phosphate dehydrogenase (Zwf1p), that regulates the carbon flux through this pathway by catalysing its first step (Schaaff-Gerstenschlager and Zimmermann, 1993), transketolase (Tkl2p) and transaldolase (Tal1p) (Godon *et al.*, 1998). Therefore, the increased production of NADPH makes this dinucleotide available for glutathione and thioredoxin antioxidant systems (Kuge and Jones, 1994; Izawa *et al.*, 1995; Morgan *et al.*, 1997). The role of the pentose phosphate pathway is further supported by the H_2O_2 hypersensitivity of strains mutated for any of the enzymes of the pentose phosphate pathway (*zwf1*Δ, *rpe1*Δ, *tkl1*Δ*tkl2*Δ, *tal1*Δ and *gnd1*Δ), (Juhnke *et al.*, 1996) and by the capacity of *TKL1* overexpression to suppress the oxygen sensitivity of a *sod1*Δ mutant (Slekar *et al.*, 1996). In addition, *zwf1*Δ mutants are unable to adapt to H_2O_2 (Izawa *et al.*, 1998).

An increased NADPH production is also associated with a favoured glycerol cycle, with the up-regulation of genes encoding glycerol phosphate dehydrogenase (Gpd1p), glycerol phosphate phosphatase (Gpp1p), glycerol dehydrogenase (YBR149wp) and dihydroxyacetone kinase (Dak1p). The inhibition of glycolysis at the step catalized by glyceraldehyde-3-phosphate dehydrogenase increases the pool of dihydroxyacetone phosphate available for the glycerol cycle. This cycle may function as a transhydrogenase activity to convert NADH to NADPH at the expense of one ATP (Norbeck and Blomberg, 1997).

The up-regulation of the glutamate catabolic pathway in response to H_2O_2 is also associated with the requirement of NADPH. Indeed, the expression of succinate semi-aldehyde dehydrogenase gene (*UGA5*) is induced, and the loss/overexpression of glutamate decarboxylase gene (*GAD1*) decreases / increases H_2O_2 resistance (Coleman *et al.*, 2001).

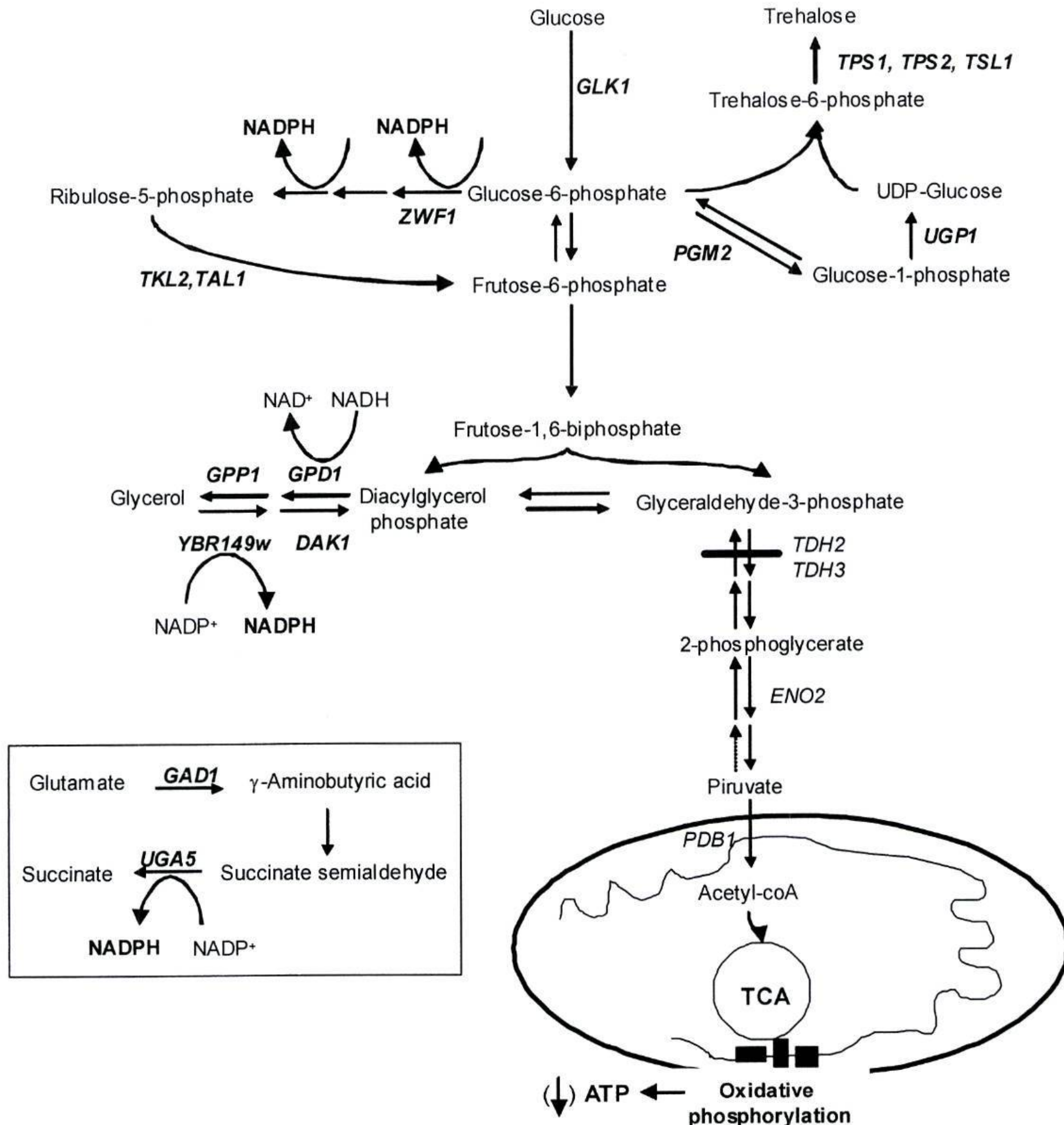


Figure 3.

Hydrogen peroxide (H_2O_2)-induced metabolic pathways. In response to H_2O_2, carbohydrate metabolism appears to be diverted to the generation of the cellular reducing power, NADPH. Enzymes of the pentose phosphate pathway, trehalose synthesis, glycerol cycle and glutamate catabolism are up-regulated (bold), whereas glycolytic activity is decreased, leading to decreased ATP levels. *GLK1*, glucokinase, *ZWF1*, glucose-6-phosphate dehydrogenase, *TKL2*, transketolase, *TAL1*, transaldolase, *PGM2*, phosphoglucomutase, *UGP1*, UDP-glucose phosphorylase, *TPS1*, trehalose-6-phosphate synthase, *TPS2*, trehalose-6-phosphate phosphatase, *TSL1*, trehalose-6-phosphate synthase / phosphatase 123 kDa regulatory subunit, *GPD1*, glycerol phosphate dehydrogenase, *GPP1*, glycerol-phosphate phosphatase, YBR149w, glycerol dehydrogenase, DAK1, dihydroxyacetone kinase, *TDH*, glyceraldehyde-3-phosphate dehydrogenase, *ENO2*, enolase, *PDB1*, pyruvate dehydrogenase (beta subunit), *GAD1*, glutamate decarboxylase, *UGA1*, succinate semialdehyde dehydrogenase.

The adaptive response to H_2O_2 involves changes in gene expression within minutes of exposure. So far regulators of this response that have been identified are the Yap1p, Skn7p

and Msn2/4p transcription factors (Table 6; see 1.2.3.2 and 1.3.3.1-3). Strains inactivated for either of these regulators are hypersensitive to H_2O_2 (Kuge and Jones, 1994; Krems *et al.*, 1995, 1996; Morgan *et al.*, 1997). Strains deleted for *YAP1* are not able to induce 32 proteins of the H_2O_2 stimulon (Lee *et al.*, 1999a) and strains deleted for *SKN7* are not able to induce 15 of the Yap1p targets in response to H_2O_2 (Morgan *et al.*, 1997; Lee *et al.*, 1999a). Hence, two subsets of the Yap1p regulon can be delineated, distinguishing the antioxidant scavenging enzymes from the metabolic pathways that regenerate the main cellular reducing power, GSH and NADPH. The Skn7p dependent subset of the Yap1p regulon specifies genes important for peroxide tolerance. The Skn7p independent gene subset comprises several activities of glutathione and pentose phosphate pathways.

In addition to Yap1p and Skn7p, Msn2p and Msn4p transcription factors were also found to regulate the H_2O_2 response. DNA microarrays identified 180 genes whose induction by H_2O_2 is affected in *msn2Δmsn4Δ* double mutants (Gash *et al.*, 2000). A total of 27 proteins were considered as dependent upon Msn2/4p for their induction (Hasan *et al.*, 2002). Seven of these proteins were previously identified as targets of Yap1p and an additional one as target of Yap1p/Skn7p (Lee *et al.*, 1999a). This pointed to a small overlap between the Msn2/4p and Yap1p/Skn7p regulons. These results suggest an independent and distinctive role of Yap1p and Msn2/4p H_2O_2 - response regulons. It was proposed that whereas Yap1p is important to prevent oxidative stress, Msn2/4p might be more important during the period of recovery from this stress. The Msn2/4p regulon only contains two antioxidant genes (Ctt1p and Grx1p), and includes several proteases and activities of the ubiquitin and proteasome degradation pathways, chaperones and heat shock proteins and metabolic enzymes (Hasan *et al.*, 2002).

1.3.3.1.1 – The Yap1p transcription factor

Yap1p is a member of a family of eight AP-1-like transcription factors and plays a major role in the activation of gene expression in response to H_2O_2 (Fernandes *et al.*, 1997). Yap1p was first identified and cloned on the basis of its ability to bind to an SV40 AP-1 recognition element (TTAGTCA) (Moye-Rowley *et al.*, 1989), and has subsequently been identified based on its capacity, when overexpressed, to confer resistance to a variety of cytotoxic compounds. Thus, the *YAP1* gene is also known by the name *PDR4* (pleiotropic drug resistance) (Hussain and Lenard, 1991; Hertle *et al.*, 1991; Schnell and Entian, 1991). However, the Yap1p basic domain differs from AP-1 factors at two of the five highly conserved residues that directly contact DNA, conferring to this factor a distinctive DNA binding specificity (Fernandes *et al.*, 1997). The Yap1p controls a large oxidative stress

regulon (Lee *et al.*, 1999a) by binding the sequence T(T/G)ACTAA termed the Yap1 response element (YRE) (Fernandes *et al.*, 1997; Kuge and Jones, 1994; Wu and Moye-Rowley, 1994) (Table 6). Deletion of *YAP1* results in hypersensitivity to oxidative stress, cadmium and cycloheximide (Schnell *et al.*, 1992; Kuge and Jones 1994). Mutants deficient in Yap1p have reduced activities of several enzymes with antioxidant activity such as superoxide dismutase, glucose-6-phosphate dehydrogenase and glutathione reductase (Schnell *et al.*, 1992). Although the response to H_2O_2 is affected in *yap1*Δ cells (Stephen *et al.*, 1995), these mutants still retained a small H_2O_2-adaptive stress response, suggesting that additional factors are involved in this response (Stephen *et al.*, 1995).

Yap1p protein levels remain constant and Yap1p DNA binding activity only slightly increases in response to H_2O_2. However, the cellular localization of Yap1p is severely affected (Kuge *et al.*, 1997). Indeed, Yap1p activity is regulated primarily by stress-induced changes in subcellular localization. Yap1p is normally cytoplasmic and translocates to the nucleus in response to oxidative stress (Kuge *et al.*, 1997). The mechanism governing this stress-dependent relocalization operates at the level of nuclear export. A cysteine rich domain at the C terminus (c-CRD), containing three Cys-Ser-Glu repeats, plays a critical role in the control of the intracellular localization of Yap1p (Kuge *et al.*, 1997; Wemmie *et al.*, 1997). A second cysteine rich domain at the N terminus (n-CRD), also containing three cysteine residues, is important for H_2O_2 resistance and for appropriate cellular relocalization of Yap1p (Kuge *et al.*, 1998, Yan *et al.*, 1998; Coleman *et al.*, 1999). Changes in the redox status of CRD prevent the interaction of Yap1p with the nuclear export factor Crm1p (also called Xpo1p) (Kuge *et al.*, 1997; Kuge *et al.*, 1998; Yan *et al.*, 1998; Coleman *et al.*, 1999; Delaunay *et al.*, 2000; Kuge *et al.*, 2001). The CRD functions not as a cytoplasmic anchor, but as a nuclear export sequence (NES) being modified by redox signals. In the presence of Ran GTP, Crm1p can bind certain NESs (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997, Neville *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997). In contrast, the import of Yap1p into the nucleus appears to be constitutive, and is mediated by Pse1p, a member of the importin β-family. This import has been found to be unaffected by the redox status of Yap1p and thus, not affected by oxidative stress (Isoyama *et al.*, 2001). Under non-stressed conditions the reduced form of Yap1p interact with Crm1p, resulting in the export of Yap1p from the nucleus (Figure 4a).

Delaunay *et al.* (2002) identified the thiol peroxidase Gpx3p, as the hydroperoxide sensor activating Yap1p and renamed it as Orp1p (Oxidant receptor peroxidase). As a result from the presence of oxidants, Yap1p becomes oxidized, through the action of the thiol peroxidase Orp1p that forms a complex with Yap1p, and is important for the H_2O_2 -induced oxidation of the protein (Delaunay *et al.*, 2002). According to this model, Orp1 Cys36 is directly oxidized by H_2O_2 to yield a sulphenic acid Cys36-SOH, which reacts with Yap1p

Cys598 to form the Orp1p-Yap1p disulphide linkage. This disulphide linkage is then transposed to the intramolecular Cys303-S-S-Cys598 disulphide of activated Yap1p, with recycling of reduced Orp1p. Oxidized Yap1p no longer interacts with Crm1p, resulting in the nuclear accumulation of Yap1p (Kuge *et al.*, 1998; Yan *et al.*, 1998). In the nucleus, Yap1p activates the transcription of H_2O_2 -induced genes, such as *TRX2* (Figure 4) (reviewed in Toledano *et al.*, 2004). A recent study characterized Ybp1p (Yap1-binding protein) as a protein that is required for the oxidative response to peroxides, in particular for the H_2O_2 -induced oxidation of Yap1p. Ybp1p forms a stress-induced complex with Yap1p *in vivo* and stimulates the nuclear accumulation in response to H_2O_2, but not in response to diamide. This study also suggests that Ybp1p acts in the same pathway as Orp1p, as the effects of loss of Ybp1p or Orp1p function on the H_2O_2-induced oxidation and nuclear accumulation of Yap1p are very similar, and an *orp1Δybp1Δ* double mutant is no more sensitive to peroxide than either of the single mutants. Ybp1p thus represent a new class of redox regulator proteins (Veal *et al.*, 2003).

The kinetics of Yap1p activation / oxidation by H_2O_2 is very rapid, suggesting that Yap1p is somehow deactivated by reduction (Delaunay *et al.*, 2000). The thioredoxin system plays a role in regulating the Yap1p-dependent stress response, and both Yap1p (Carmel-Harel *et al.*, 2001; Delaunay *et al.*, 2000; Izawa *et al.*, 1999) and Orp1p redox states are coupled to the thioredoxin pathway. Indeed, Yap1p is constitutively active in thioredoxin deficient mutants (*trx1Δtrx2Δ*) (Izawa *et al.*, 1999; Delaunay *et al.*, 2000) and the whole genome analysis of gene expression showed that 70% of the Yap1p targets are upregulated in mutants lacking thioredoxin reductase (*trr1Δ*) under non-stress conditions (Carmel-Harel *et al.*, 2001). Besides, the thioredoxin peroxidase Tsa1p is essential for the transcriptional activation of *TRX2* and *TRR1* genes by Yap1p (Ross *et al.*, 2000). Several evidences suggest that the stronger effects of H_2O_2 on cells lacking thioredoxin reductase may be due to a lower capacity to eliminate the oxidant and / or to reduce Yap1p disulphide bonds (Carmel-Harel *et al.*, 2001). Thus, the reduction of Yap1p disulphide bonds at the CRD by the thioredoxin system exposes the nuclear export signals, and Crm1p exports Yap1p back to the cytoplasm.

Interestingly, different oxidative stress agents appear to regulate Yap1p by different mechanisms. In particular, H_2O_2 and diamide cause oxidation of different cysteine residues in the CRDs of Yap1p (Delaunay *et al.*, 2000; Kuge *et al.*, 2001; Azevedo *et al.*, 2003). A recent study suggests that Yap1p has two distinct molecular redox centers, one triggered by ROS (hydroperoxides and the superoxide anion) and the other by chemicals with thiol reactivity (electophiles and divalent heavy metal cations). Cys303 and Cys598 constitute one redox center that functions in the Yap1p H_2O_2 response, as a disulphide regulatory switch primed for oxidation by the Orp1p H_2O_2 sensor (Delaunay *et al.*, 2002). The C-terminal Yap1p

domain cysteines (Cys598, Cys620, Cys629) constitute the second redox center that functions both as a sensor and as a regulatory switch in the response to thiol-reactive chemicals (Azevedo *et al.*, 2003). The data indicate that yeast cells cannot sense these compounds through the same molecular devices, albeit they are all electrophilic.

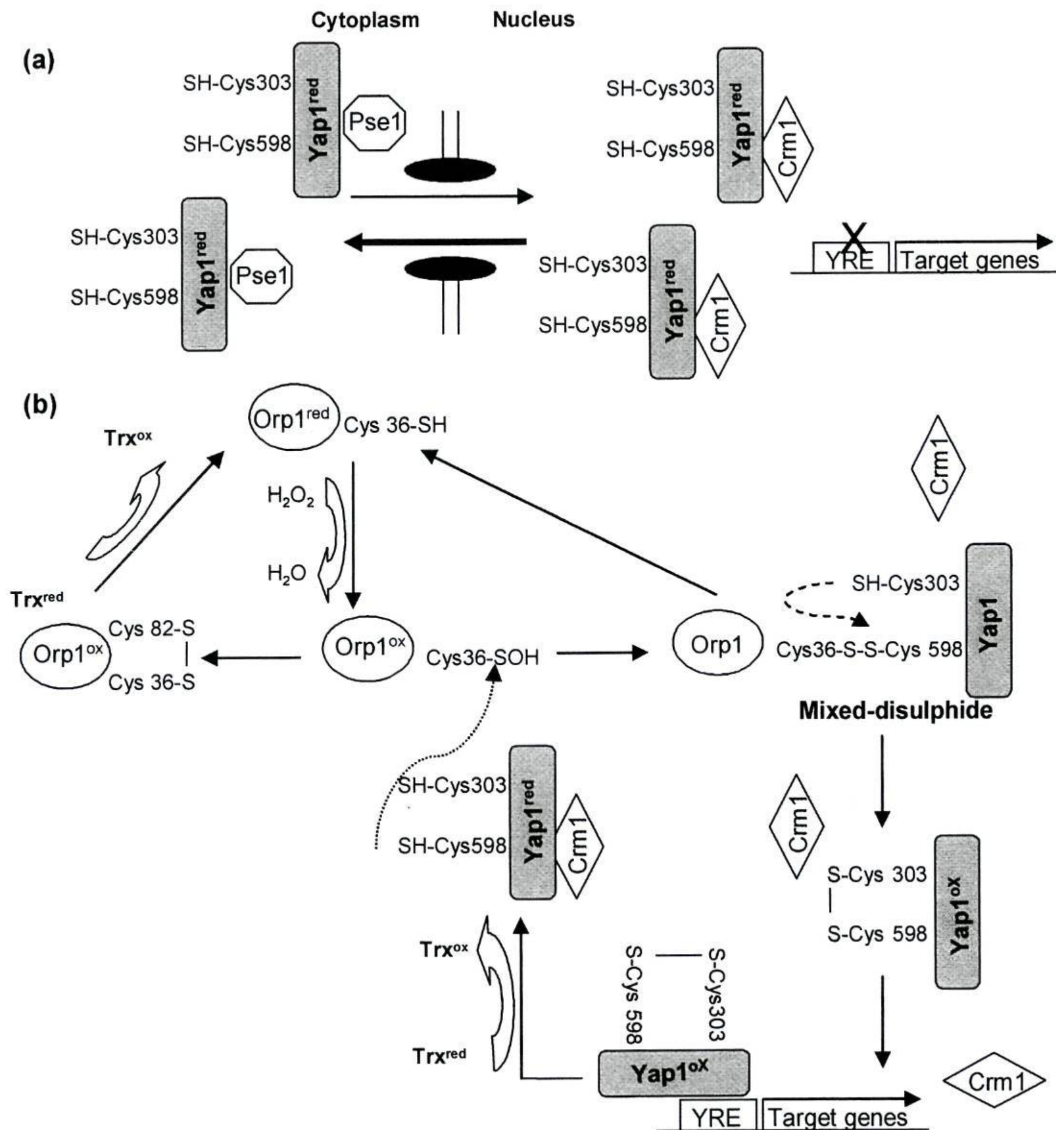


Figure 4.

Regulation of Yap1p in *S. cerevisiae*. Cysteine-rich domains (CRD) in Yap1p, by binding the Crm1p transporter, control Yap1p intracellular localization. **a)** Under non-stress conditions, the Yap1p transported into the nucleus by Pse1p, is therefore enriched in the cytoplasm due to its high rate of Crm1p-catalized nuclear export. **b)** Sensing involves reaction of Orp1p with H_2O_2 to yield a sulphenic acid that reacts with Yap1p to form an Orp1p-Yap1p disulphide linkage. This linkage is then converted into a Yap1p intra-molecular disulphide, concomitantly with Orp1p reduction. Upon oxidation, structural alterations in Yap1p reduce its ability to interact with Crm1p, and the factor accumulates in the nucleus. Oxidised Yap1p can then bind to target genes and stimulate transcription. Yap1p is then reduced by thioredoxin. ^{ox} oxidised; ^{red} reduced.

The budding yeast genome project has uncovered eight potential members of the Yap family (Yap-1/2/3/4/5/6/7/8-p), based on the homology within the B-zip region (Fernandes *et al.*, 1997). The physiological roles of these proteins are unknown, although *YAP2* (equivalent to *CAD1*), when overexpressed, displays similar phenotypes to *YAP1* - pleiotropic drug resistance and resistance to cadmium toxicity (Bossier *et al.*, 1993; Wu *et al.*, 1993). Yap2p clearly plays a role in regulating the H_2O_2 adaptive stress response, since induction of this adaptive response was diminished in the *yap2*Δ null mutant (Stephen *et al.*, 1995). However, the binding sites in the promoters of the Yap1p- dependent genes differ from the sites in the promoters of Yap2p- dependent genes, and DNA microarrays studies showed that Yap1p and Yap2p activate non-overlapping sets of genes (Cohen *et al.*, 2002). Yap1p controls a set of genes involved in detoxifying the effects of ROS, whereas Yap2p controls a set of genes associated with protein stabilization.

1.3.3.1.2 – The Skn7p and Hsf1p transcription factors

The *SKN7/POS9* gene was identified as a high-copy suppressor of a mutation affecting cell wall β-glucan assembly (Brown *et al.*, 1993). Indeed, Skn7p is involved in the regulation of cell wall biosynthesis and cell cycle (Brown *et al.*, 1994; Morgan *et al.*, 1995). Skn7p also operates in the oxidative stress response, as *skn7*Δ mutants are sensitive to several oxidants, including H_2O_2 (Kuge and Jones, 1994; Krems *et al.*, 1996; Morgan *et al.*, 1997; Krems *et al.*, 1995). The receiver domain of Skn7p is essential for its role in the regulation of cell wall biosynthesis and cell cycle, and to the oxidative stress response (Krems *et al.*, 1996; Morgan *et al.*, 1997). The N-terminal region of the Skn7p protein shows similarity to the Hsf1p (heat shock factor) DNA-binding domain. Other regions of the protein show similarity to the receiver domain in two-component signal transduction systems in prokaryotes (Brown *et al.*, 1993). According to the homology between the N-terminal region of Skn7p and Hsf1p, it has been shown that Skn7p is involved, maybe through an interaction with Hsf1p, in the transcriptional activation of heat shock genes (*HSP12*, *HSP26* and *HSP104*) in response to H_2O_2, through binding to heat shock elements (HSE) (Raitt *et al.*, 2000). In addition to its role in the heat shock response, it has been suggested that the activity of the heat-shock factors is modulated by oxidants in eukaryotic cells. Hsf1p has been shown to protect the cell against heavy metals, such as copper and cadmium, through activation of the copper metallothionein gene *CUP1* (Silar *et al.*, 1991, Sewell *et al.*, 1995). A domain rich in basic amino acids at the C-terminus of Hsf1p is essential for oxidative stress-inducible transcription of *CUP1* (Sakurai and Fukasawa, 2001). Hsf1p becomes phosphorylated in response to menadione, and this modification correlates with the transcriptional activation of *CUP1* by

oxidative stress (Liu and Thiele, 1996). Hence, Hsf1p also plays a role in cell defence against oxidative stress.

Evidences indicate that Skn7p and Yap1p are epistatic, acting in the same pathway. Skn7p affects the expression of a seth of genes also regulated by Yap1p, including antioxidant defences (Moradas-Ferreira and Costa, 2000; Lee *et al.*, 1999a) (Table 6). A *skn7Δyap1Δ* double mutant is no more sensitive to H_2O_2 than either single mutant (Krems *et al.*, 1996; Lee *et al.*, 1999a; Morgan *et al.*, 1997). The Skn7p can bind to a specific region within the *TRX2* promoter *in vitro* and may well do so as part of a complex containing Yap1p (Morgan *et al.*, 1997). However, it is not clear whether they physically interact. It has been recently shown that the induction of *GPX2* by oxidative stress is mediated by both Yap1p and Skn7p. In addition, a cis-acting element within the *GPX2* promoter proximal to the functional YRE was identified as being the oxidative stress-responsive Skn7p response element (5'-GGCCC/(T)GGC-3') (Tsuzi *et al.*, 2004).

The mitochondrial function and cytochrome c peroxidase activity are important for signalling to activate both Skn7p and Yap1p, as indicated by the weak induction of *TPX1* (thioredoxin peroxidase) gene expression by H_2O_2 in respiratory deficient mutants and *ccp1Δ* cells, and by the high sensitivity of these mutants to H_2O_2 (Charizanis *et al.*, 1999a,b).

Based on a screening for mutants impairing the activation of Skn7p upon oxidative stress, a protein named Fap7p (factor activating Pos9) was identified. Fap7p is an essential nuclear factor containing a putative ATP/GTP binding motif, and activates the Skn7p factor in cells exposed to H_2O_2. The *fap7-1Δ* mutant strain revealed that the impaired Skn7p induction was specific, as the *TPS2*-lacZ reporter gene expression was not affected upon peroxide stress (Juhnke *et al.*, 2000). However, the mechanism of regulation of Skn7p by Fap7p remains unknown.

1.4 - Sphingolipid metabolism in *S. cerevisiae*

Sphingolipids have been described as important signalling molecules, in addition to being important structural components of cellular membranes. Roles in differentiation, senescence, cell cycle arrest, apoptosis and stress responses, including oxidative stress, have been referred (Hannun and Luberto, 2000; Ohanian and Ohanian, 2001). In mammalian cells, an increase of ceramide levels resulting from sphingomyelinase activity has been observed in response to DNA damage and oxidants (Hannun and Luberto, 2000),

Eukaryotic cell membranes contain sphingolipids composed by a hydrophobic segment, ceramide, coupled to a polar group. The ceramide moiety consists of a sphingoid long chain base (LCB), which is N-fatty acylated. The LCB is usually a linear alkane of 18 or 20

carbons, having hydroxyls on C-1 and C-3 and an amino group on C-2. *S. cerevisiae* makes two types of LCBs, dihydrosphingosine (DHS) and phytosphingosine (PHS), which has an additional hydroxyl on C-4 (Figure 5). Yeast cells are able to synthesize sphingolipids similar to those of mammalian cells, except that phytosphingosine and not sphingosine is the predominant sphingoid base, and phosphoinositol rather than phosphocoline is the polar head group attached to ceramide (reviewed in Le Stunff *et al.*, 2002).

Recent advances in understanding sphingolipid metabolism and function in *S. cerevisiae* have been possible, by the uncovering of genes involved in biosynthesis and breakdown of sphingolipids. *S. cerevisiae* seems on the verge of becoming the first organism in which all sphingolipid metabolic genes are identified.

1.4.1 - Long chain Bases (LCB) and LCB phosphates

In yeast, the first and apparently rate-limiting step in sphingolipid metabolism involves the condensation of serine and palmitoyl-CoA to yield 3-keto DHS, a reaction catalized by the serine palmitoyl transferase encoded by two essential genes, *LCB1* and *LCB2* (Buede *et al.*, 1991; Dickson and Lester, 1999a; Dickson and Lester, 2002). The enzyme 3-keto reductase, encoded by the essential *TSC10* gene, converts 3-keto DHS is into DHS (Beeler *et al.*, 1998), which is then hydroxylated to phytosphingosine, a reaction catalized by the sphingolipid hydroxylase Sur2p (Haak *et al.*, 1997; Grilley *et al.*, 1998).

Both LCBs, DHS and PHS, can be phosphorylated to LCB phosphates by specific kinases (encoded by *LCB4* and *LCB5*) (Nagiec *et al.*, 1998), and LCBPs can be dephosphorylated by the sphigolipid phosphate phosphatases encoded by *YSR2* and *YSR3* genes (Qie *et al.*, 1997; Mao *et al.*, 1997; Mandala *et al.*, 1998) or converted into ethanolamine phosphate and fatty aldehydes by the dihydrosphingosine phosphate lyase, Dpl1p (Saba *et al.*, 1997) (Figure 5).

1.4.2 - Ceramides and complex sphingolipids

Ceramide synthesis involves the acylation of the LCB phytosphingosine to phytoceramide. *S. cerevisiae* almost exclusively uses C_{26} fatty acyl groups in its sphingolipids. Ceramide synthase catalyzes the acylation of these very long-chain fatty acids onto sphingolipids. This activity requires either of two redundant longevity assurance genes, *LAG1* and *LAC1* (Guillas *et al.*, 2001; Jazwinski and Conzelmann, 2002). A full ceramide synthase activity requires the activity of the α' isoform of CK2 kinase, Cka2p (Kobayashi and

Nagiec, 2003). Acylation may also occur on the amide group of DHS to yield dihydroceramide (DH-Cer; ceramide-1), that can then be converted to phytoceramide (PH-Cer; ceramide-2) by hydroxylation at C-4, catalyzed by Sur2p (Haak *et al.*, 1997; Grilley *et al.*, 1998).

When grown aerobically, *S. cerevisiae* cells hydroxylate the fatty acyl group in phytoceramide at C-2 to yield ceramide-3, which can be further hydroxylated at C-3 of the fatty acyl group to produce ceramide-4 (Lester and Dickson, 1993). Scs7p catalyzes the C-2 and perhaps the C-3 hydroxylations (Haak *et al.*, 1997; Dunn *et al.*, 1998; Mitchell and Martin, 1997). Some reports also indicate that the C-3 hydroxylation requires Cu^{2+} and the Golgi copper transporter encoded by *CCC2* (Puoti *et al.*, 1991; Beeler *et al.*, 1997).

S. cerevisiae uses ceramide to make three types of complex sphingolipids. The addition of *myo*-inositol phosphate to ceramides, to form inositolphosphoceramide (IPC), is catalyzed by IPC synthase, encoded by the essential *AUR1* gene (Nagiec *et al.*, 1997). IPC is mannosylated to yield mannose-inositolphosphoceramide (MIPC), by Csg1p and Csg2 (Beeler *et al.*, 1994; Beeler *et al.*, 1997). Deletion of either gene reduces the level of MIPC and mannose-(inositol-P)$_2$-ceramide (M(IP)$_2$C) (Daum *et al.*, 1999; Stock *et al.*, 2000) and causes IPC accumulation. Csh1p is a protein with strong similarity to Csg1p and with redundant functions in MIPC synthesis. In addition, Csg2p interacts with both Csg1p and Csh1p, and deletion of the *CSG2* gene reduced the Csg1p activity and abolished the Csh1p activity. These results suggest that two distinct inositolphosphoceramide mannosyl-transferase complexes, Csg1p-Csg2p and Csh1p-Csg2p, exist (Uemura *et al.*, 2003). The addition of inositol phosphate to MIPC to yield (M(IP)$_2$C), requires the non essential *IPT1* gene (Dickson *et al.*, 1997b).

Enzymes involved in the breakdown of sphingolipids have recently been identified and characterized. The yeast *ISC1* gene encodes an inositolphosphosphingolipid-phospholipase C (IPS-PLC), which has homology to the bacterial and mammalian neutral sphingomyelinase (SMase). Isc1p hydrolyses inositol phosphosphingolipids (IPS), namely IPC, MIPC and M(IP)$_2$C, releasing ceramide and the polar head group. Cells deficient in Isc1p are viable and accumulate IPC and M(IP)$_2$C, but not MIPC (Sawai *et al.*, 2000). Isc1p requires Mg^{2+} for optimal activity and is activated by anionic phospholipids, such as phosphatidylserine (PS), cardiolipin (CL) and phosphatidylglycerol (PG) (Okamoto *et al.*, 2002).

Isc1p contains a domain that is conserved in the entire family of SMases, the P-loop-like domain. This domain has high homology to P-loop domains found in nucleotide-binding proteins (Sanchez *et al.*, 1999). The primary structure of the P-loop domain typically consists of a glycine-rich region followed by a conserved lysine and a serine/threonine (Saraste *et al.*, 1990), and is located in the catalytic region of Isc1p. Recent results indicate that the P-loop-like domain might be essential in the catalysis of Isc1p and may be involved in Mg^{2+} binding

(Okamoto *et al.*, 2003). The second transmembrane domain (TMII) and the C terminus of Isc1p function as anionic phospholipids-selective binding domains and the positively charged amino acid residues within the C terminus are necessary for the cooperative activation of Isc1p by phosphatidylserine. The C terminus interacts with the remainder of the enzyme, and it was proposed that this interaction plays a critical role in enzyme function, through a novel tethering mechanism of enzyme activation by lipid cofactors (Okamoto *et al.*, 2002).

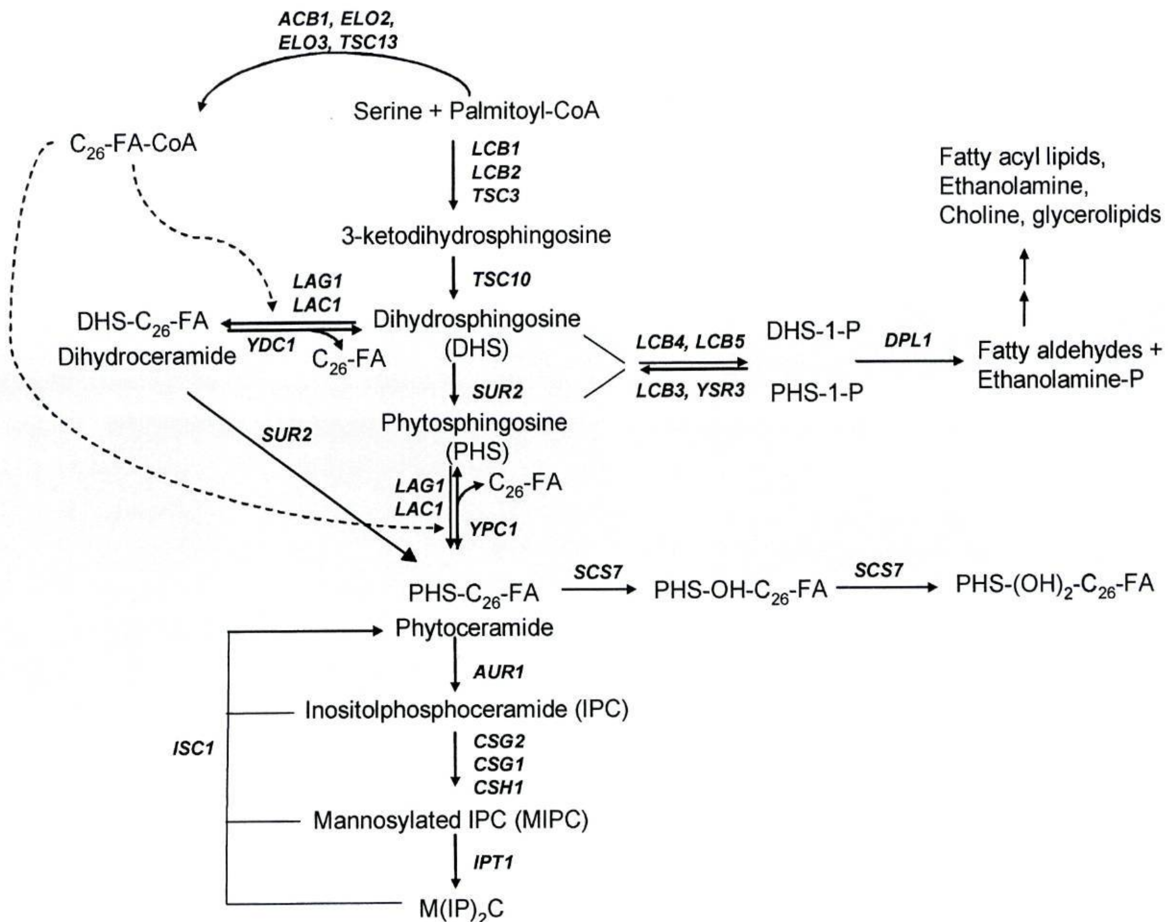


Figure 5.

Overview of yeast sphingolipid metabolism. In *S. cerevisiae*, ceramides are synthesized *de novo* from palmitoyl-CoA and serine. The intermediates in ceramide synthesis, dihydrosphingosine (DHS) and phytosphingosine (PHS), can be phosphorylated to yield DHS-P and PHS-P. Four types of ceramides exist in yeast (ceramide-1 = dihydroceramide; ceramide-2 = phytoceramide; ceramide-3 = PHS-OH-C_{26}-FA; ceramide-4 = PHS-$(OH)_2$-C_{26}-FA). Ceramides can be modified by addition of *myo*-inositol phosphate to form inositolphosphoceramide (IPC), which in turn can be mannosylated to form mannose-inositol-phosphoceramide (MIPC), that is converted to $M(IP)_2C$ by addition of inositol phosphate. Inositol sphingolipids are hydrolyzed by Isc1p to phytoceramide.

Although deletion of *ISC1* renders viable cells, these cells grow slowly. A recent report indicated that Isc1p is activated during growth. This increase in specific activity is not due to transcriptional / translational activation. During exponential growth Isc1p colocalizes with the endoplasmic reticulum, but translocates to the mitochondria during the late logarithmic and postdiauxic phases of growth, suggesting a new mechanism for the activation of Isc1p (Vaena de Avalos *et al.*, 2004). Interestingly, mitochondria are rich in phosphatidylglycerol and cardiolipin, which may act as "activators" of Isc1p, and inositol phosphosphingolipids were also found in the mitochondria (Zinser and Daum, 1995). How ceramide functions to regulate yeast cell growth remains unknown.

Yeast ceramides in turn are broken down by Ypc1p or Ydc1p. Ypc1p is a phytoceramidase that preferentially hydrolyzes phytoceramide, but has significant reverse activity and acts as a ceramide synthase under certain conditions (Mao *et al.*, 2000a). Its homologue, Ydc1p, is a dihydroceramidase with modest reverse activity (Mao *et al.*, 2000b). Mutants deficient in *YPC1*, *YDC1* or both exhibit no growth defect. Whether LCBs are primarily generated by ceramidases or by *de novo* synthesis remains to be established.

1.4.3 - Sphingolipid signalling

Sphingolipids have acquired a novel role as signalling molecules of cell metabolism. Indeed, ceramide was first reported to be a signalling molecule in mammals, controlling cellular processes, such as apoptosis, differentiation, cell cycle arrest and senescence (Riboni *et al.*, 1997; Levade *et al.*, 1999; Kolesnick and Kronke, 1998; Hannun and Luberto, 2000; Guzman *et al.*, 2001; Ohanian and Ohanian, 2001).

The conversion of sphingomyelin into ceramide can play a membrane structural role, with consequences for membrane microdomains function, membrane vesiculation, fusion / fission and vesicular trafficking (Simons and Ikonen, 1997; Simons and Toomre, 2000; Galbiati *et al.*, 2001). All these events contribute to cellular signalling. Indeed, sphingolipids and cholesterol tend to associate in eukaryotic membranes to form domains that are referred as lipid rafts (reviewed in Harder *et al.*, 1998; Simons and Ikonen, 1997). Lipid rafts play key roles in signal transduction and membrane trafficking in mammals (Simons and Ikonen, 1997). At the cell surface, protein clustering in lipid rafts and the formation of endosomes can be facilitated by transient ceramide formation catalysed by acidic sphingomyelinase. Lipid rafts have also been identified in *S. cerevisiae* (Kubler *et al.*, 1996) and shown to be important for delivering specific proteins, including Gas1p, Pma1p and Nce2p, to the plasma membrane (Bagnat *et al.*, 2000).

Ceramide may also act as a second messenger when generated in the membrane inner leaflet by the neutral SMase. Ceramide forms large channels in artificial planar membranes and mitochondria, increasing the permeability of the mitochondria outer membrane for a number of small proteins, including cytochrome c. The cytochrome c released from mitochondria plays a key role in apoptosis (van Blitterswijk *et al.*, 2003).

Ceramide signals are known to be generated in response to a wide range of initiating events that trigger or indicate cellular stress including cytokines, ultraviolet light, ionizing radiation, heat, DNA damage and chemotherapeutic agents (Riboni *et al.*, 1997; Levade *et al.*, 1999; Kolesnick and Kronke, 1998; Hannun and Luberto, 2000). Several studies indicate that ceramide can accumulate via i) activation of *de novo* synthesis mediated by serine-palmitoyl transferase and ceramide synthase; ii) activation of sphingomyelin hydrolysis; iii) inhibition of ceramide hydrolysis; iv) stimulation of glucosylceramide hydrolysis or inhibition of its synthesis (reviewed in Hannun and Luberto, 2000).

Exactly how ceramide activates signal transduction pathways is not well understood. It is reported that ceramide is an inhibitor of protein kinase C, and is known to activate protein phosphatases named as ceramide activated protein phosphatases (CAPP) - protein phosphatase 2A (*PP2A*), and protein phosphatase 1 (*PP1*) (Chalfant *et al.*, 2001; van Blitterswijk *et al.*, 2003). In yeast, both *PKC1* (Madden *et al.*, 1997) and *PP2A* (Ronne *et al.*, 1991) have been implicated in the regulation of cell proliferation. In cerebellar granule cells, the activation of *PPA2* by C2-ceramide induces the dephosphorylation of both protein kinase B (*PKB*) and glycogen synthase kinase-3 (*GSK3*) (Mora *et al.*, 2002). In mammals, *PP2A* also mediates thiol-alkylation dependent redox regulation of Akt (protein kinase B - *PKB*) and cell survival (Yellaturu *et al.*, 2002). Aktp has been implicated in insulin signalling, apoptosis and proliferation in mammals (Kandel and Hay, 1999), and functions in a pathway that regulates ageing and stress resistance in *C. elegans* (Guarente and Kenyon, 2000; Paradis *et al.*, 1999) and other yeast species (Fabrizio *et al.*, 2001). Whether this pathway is regulated by the yeast protein phosphatase 2A, namely *PP2A* and *SIT4*, has not been reported, but some evidences suggest that *PP2A* and *SIT4* might represent ceramide signalling targets (Zabrocki *et al.*, 2002).

Sphingosine-1-phosphate (S1P) also functions as a signalling molecule in a wide range of events, including cellular proliferation, survival, motility and cytoskeletal mechanics (van Brocklyn *et al.*, 1998; Pyne and Pyne, 2000). S1P activates signal transduction pathways through stimulation of its G-protein coupled receptor (Brindley *et al.*, 2002), and the activation of sphingosine kinases (that results in accumulation of S1P) suppresses ceramide-mediated processes. Due to their opposite roles, the dynamic balance between intracellular S1P *versus* ceramide is an important factor that determines the cell fate (Spiegel and Milstien, 2000).

The recognition of sphingolipids as regulatory molecules in *S. cerevisiae* is recent, and evidences have emerged associating sphingolipids with optimal life-span (Jazwinski and Conzelmann, 2002) and stress responses, including heat, osmotic and low pH stress (Vaena de Avalos *et al.*, 2004; Dickson and Lester, 1999b; Jenkins and Hannun, 2001). Indeed, roles for sphingolipids in regulation of the transient cell cycle arrest, control of putative signalling pathways that govern cell integrity, endocytosis, exocytosis, movement of the cortical actin cytoskeleton and regulation of protein breakdown have been reported.

1.4.3.1 – Role of sphingolipids on yeast cell growth

Inactivation of sphingolipid biosynthesis leads to lethality of yeast cells: i) *lcb1Δ* and *lcb2Δ* cells are unviable, unless an exogenous LCB (DHS or PHS) is supplied (Dickson *et al.*, 1990); ii) *dpl1Δysr2Δ* cells are inviable due to accumulation of DHS-1-P and PHS-1-P, and both lethality and the accumulation of LCB phosphates are suppressed by deletion of *LCB4*, that accounts for most of the LCB phosphorylation activity (Kim *et al.*, 2000; Zhang *et al.*, 2001); and iii) *aur1Δ* mutant cells, deficient in IPC synthase, fail to synthesize complex sphingolipids and die (Nagiec *et al.*, 1997). On the other hand, some intermediates of sphingolipids have an inhibitory effect on yeast growth: i) C2-ceramide inhibits proliferation of yeast cells by arresting them at the G_1 phase (Nickells and Broach, 1996), in a yeast ceramide activated protein phosphatase dependent manner; ii) exogenous sphingosine and phytosphingosine also induce growth inhibition of yeast cells (Mao *et al.*, 1997; Saba *et al.*, 1997); iii) the increase of endogenous ceramides and / or sphingoid bases synthesis by overexpression of *YSR2* arrests yeast cells in the G_1 phase of the cell cycle (Mao *et al.*, 1999); iv) inhibition of ceramide synthase, that leads to inhibition of ceramides and complex sphingolipids synthesis, also inhibits yeast growth (Wu *et al.*, 1995); and v) DHS and PHS inhibit cell growth by inhibiting tryptophan import (Skrzypek *et al.*, 1998), and PHS inhibits uracil, leucine and histidine import (Chung *et al.*, 2001a).

1.4.3.2 – Role of sphingolipids on heat stress responses

A protective role of sphingolipids in cell growth and survival under heat stress conditions has been described. Yeast cells lacking the ability to synthesize sphingolipids are unable to growth at elevated temperatures (Patton *et al.*, 1992). Indeed, *lcb1Δ* or *lcb2Δ* mutants carrying a point mutation that allows to produce inositol glycerolipids, are able to

grow at 30°C, but are sensitive to heat stress (37-39°C) (Dickson *et al.*, 1990), and if exogenous DHS or PHS are supplied to the medium, these mutant strains can grow at 39°C (Jenkins *et al.*, 1997). LCBs and other intermediates, including LCB phosphates, DH-Cer and PH-Cer, may mediate heat stress responses as signalling molecules. Indeed, DHS, PHS and ceramide levels increase after a shift to an elevated temperature (Jenkins *et al.*, 1997; Dickson *et al.*, 1997a; Skrzypek *et al.*, 1999), and the accumulation of LCB phosphates in *ysr2/lcb3*Δ or *dpl1*Δ cells results in the enhancement of heat shock tolerance (Mao *et al.*, 1999; Mandala *et al.*, 2000; Skrzypek *et al.*, 1999; Lester *et al.*, 1993). However, studies using *lcb4*Δ*lcb5*Δ cells, deficient in LCB kinase activity, suggest that DHS-1-P and PHS-1-P are not necessary for heat stress resistance in logarithmic or stationary phase cells, while they may play a small role during induced thermotholerance (Fergunson-Yankey *et al.*, 2002; Nagiec *et al.*, 1998).

The transient arrest of the cell cycle at G_1 that occurs after heat shock requires DHS or PHS (Johnston and Singer, 1980). It appears that LCBs are acting through an undetermined pathway that regulates the Cln3p cyclin (Jenkins and Hannun, 2001), which has been demonstrated to block the heat stress- and the sphingoid base-induced cell cycle arrest (Rowley *et al.*, 1993) (Figure 6).

The pathways for the generation of sphingoid bases, ceramide and sphingoid base phosphates upon heat stress have not been well defined. However, the increased ceramide levels in response to heat stress appear to result from *de novo* biosynthesis (Wells *et al.*, 1998) and not from the breakdown of inositolphosphoceramide (Jenkins *et al.*, 1997; Wells *et al.*, 1998). In addition, it has been recently suggested that hydrolysis of dihydroceramide to yield DHS, in contrast to phytoceramide, has an important role in heat stress responses (Mao *et al.*, 2000a; Mao *et al.*, 2000b).

It has been reported that sphingoid bases can induce various components of stress responses (Jenkins *et al.*, 1997; Jenkins and Hannun, 2001). Trehalose is known to be important for the survival under conditions of heat stress, and sphingolipids are necessary for trehalose accumulation upon heat stress (Dickson *et al.*, 1997a). Treating wild type cells with DHS mimics the effect of heat and causes trehalose to accumulate, by activating the transcription of *TPS2*, encoding a subunit of trehalose synthase (Dickson *et al.*, 1997a) (Figure 6). Although sphingolipids do not induce genes encoding the major heat shock proteins such as *HSP104*, *HSP90*s, *HSP70*s, *HSP48* and *HSP26* (Dickson *et al.*, 1997a), a recent study, using the mutant strain *lcb1-100* that is unable to produce sphingolipids in response to heat, indicated that several stress-responsive genes are partially dependent on sphingolipid generation for their induction. These genes include the HSP70 chaperonin *SSA4*, and genes encoding two proteins involved in responses to osmotic stress, *HOR2* and *SIP18*. In addition, a variety of cell functions were found to be dependent on sphingolipid

biosynthesis for heat adaptation, including regulation of message for metabolic enzymes, proteins involved in cell cycle control, aminoacid metabolism, protein synthesis, and cell wall organization and biogenesis (Cowart *et al.*, 2003).

Heat stress was found to cause the inactivation of uracil permease, Fur4p, inhibiting the uracil uptake. Fur4p is known to be ubiquitinated, endocytosed and degraded in the vacuole in response to various stresses (Volland *et al.*, 1994), including heat stress. The heat-induced degradation of nutrient permeases is dependent on sphingolipid biosynthesis. Indeed, inactivation of *LCB1* gene prevents proteolysis during heat stress (Chung *et al.*, 2001b). Furthermore, in absence of heat, exogenously applied PHS inhibits aminoacid import and induces the degradation of Fur4p and of the general permease Gap1p, through a ubiquitination dependent process (Chung *et al.*, 2001a,b) (Figure 6).

1.4.3.3 – Role of sphingolipids on endocytosis and regulation of the actin cytoskeleton

Recent studies supported a role of sphingolipids in endocytosis, in the vesicular transport of GPI-anchored proteins out of the ER and in the proper actin organization (Grote *et al.*, 2000; Zanolari *et al.*, 2000). Studies using the temperature-sensitive serine palmitoyltransferase strain, *lcb1-100*, have shown a role for *de novo* sphingolipid synthesis in endocytosis and demonstrated possible downstream targets for the sphingoid bases in yeast. At a restrictive temperature of 37ºC (heat stress), the *lcb1-100* strain was found to be defective in both the internalization step of endocytosis and in the organization of the actin cytoskeleton (Zanolari *et al.*, 2000).

The role of LCBs in the regulation of endocytosis is mediated by the control of protein phosphorylation (Zanolari *et al.*, 2000; Friant *et al.*, 2000). Indeed, overexpression of two protein kinase genes, *YCK2*, a caseine kinase family member (Robinson *et al.*, 1993), or *PKC1* in the *lcb1-100* strain, suppressed the endocytic and actin defects at 37ºC, but did not suppress strain *lcb1-100*'s temperature-sensitive growth phenotype (Friant *et al.*, 2000). In addition, the loss of the protein phosphatase function of *PP2A* through inactivation or deletion of *CDC55* (the regulatory subunit of *PP2A*) or *PPH21/22/23* (the catalytic subunits of *PP2A*), reversed the endocytic defect at 37ºC and like exogenous LCBs, restored polarization (Friant *et al.*, 2000), but did not reverse the temperature-sensitive phenotype. Thus, LCBs seem to regulate actin polarization during heat stress and perhaps in unstressed cells. More recent data support the idea that LCBs regulate endocytosis and organization of the actin cytoskeleton by a signalling pathway that includes two kinases, Pkh1p and Pkh2p. Overexpression of *PKH1* or *PKH2* also overcomes the endocytic and actin defects of the *lcb1-100* strain at 37ºC, as described for *PKC1* or *YCK2* overexpression. These kinases

phosphorylate Pkc1p in a DHS dependent manner (Friant *et al.*, 2001), thus implicating this kinase as a downstream effector of this signalling cascade involved in endocytosis. Sphingoid bases are thus likely to have roles in the regulation of specific kinases and phosphatases reactions that may be important in both endocytosis and possibly in the yeast heat stress responses (Figure 6). However, no yeast protein has been shown to directly bind either DHS or PHS, so one or more protein components of these pathways may be missing.

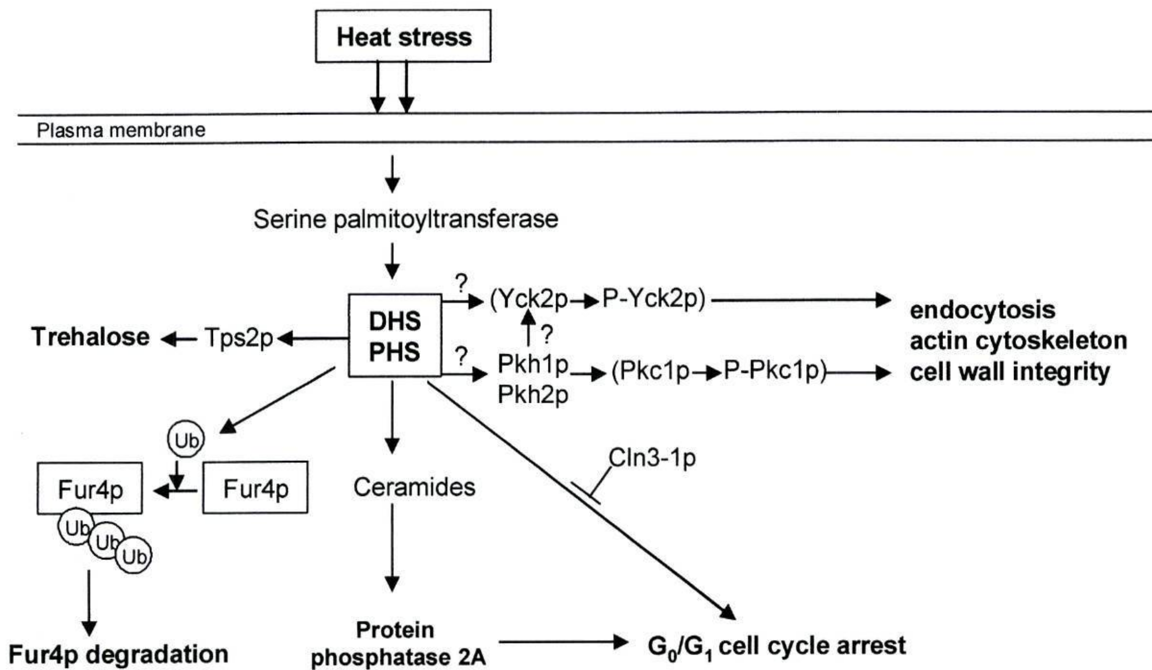


Figure 6.

Signalling mediated by sphingolipids and the heat stress response. Sphingoid bases are involved in the regulation of trehalose levels, endocytosis, and in the heat-induced transient G_0/G_1 cell cycle arrest. Phytosphingosine down-regulates uracil uptake by stimulating Fur4p degradation via the ubiquitin proteasome system. DHS, dihydrosphingosine; PHS, phytosphingosine; Tps2p, trehalose-6-phosphate phosphatase; Yck2p, casein kinase I homolog; P-Yck2p, phosphorylated Yck2p; Pkh1p, 3-phosphoinositide-dependent kinase-1 homolog;P-Pkh1p, phosphorylated Pkh1p; Pkh2p, 3-phosphoinositide-dependent kinase-1 homolog; P-Pkh2p, phosphorylated Pkh2p; Cln3-1p, G_1 cyclin; Fur4p, uracil permease; Ub, ubiquitin.

1.4.3.4 – Role of sphingolipids in the transport of GPI-anchored proteins from ER to the Golgi and in the regulation of vacuolar ATPases

The role of ceramide / sphingolipid synthesis in the efficient vesicular transport of GPI-anchored proteins from the ER to the Golgi apparatus is indicated by several lines of

evidence: i) in *lcb1-100* cells the precursor form of Gas1p, a well characterized GPI-anchored protein, accumulates in the ER, indicating failure to reach the Golgi, where it would be further glycosylated (Sutterlin *et al.*,1997; Horvath *et al.*, 1994); ii) the induction of *MCD4* gene, encoding a protein required for the synthesis of GPI anchors (Gaynor *et al.*, 1999) in response to heat stress, depends on *de novo* sphingolipid synthesis (Cowart *et al.*, 2003); and iii) the strain deleted in ceramide synthases *LAG1* and *LAC1* cannot transport GPI-anchored proteins from ER to the Golgi (Schorling *et al.*, 2001; Guillas *et al.*, 2001; Barz and Walter, 1999). Ceramide is not required for GPI anchor attachment and seems to function in the fusion of GPI-containing vesicles with the Golgi (Sutterlin *et al.*, 1997; Barz and Walter, 1999). In addition, a recent work indicates that protein sorting in the late Golgi does not require production of mannosylated sphingolipids (Lisman *et al.*, 2004).

It has been shown that sphingolipids are also involved in the regulation of vacuolar ATPases (V-ATPases). *S. cerevisiae* V-ATPase contains two domains, V_1 and V_o, which associate to form an active V_1V_o complex on the vacuolar membrane that establishes a proton gradient necessary for vacuolar transport proteins to drive ions and small molecules, aminoacids and metabolites in the vacuole (reviewed in Graham *et al.*, 2000). The V_1 domain has ATPase activity, whereas the V_o domain serves as a proton pore. Sphingolipids with a C_{26} acyl group were found to be essential for the generation of a fully functional V_1 domain, able to hydrolyze ATP. Indeed, V_1 domains in *sur4/elo3Δ* cells lack ATPase activity even though they associate with V_o domains in the vacuolar membrane (Chung *et al.*, 2003).

1.4.3.5 – Role of sphingolipids on longevity and cellular ageing

Sphingolipids are also related to other cellular processes, including longevity and ageing. The yeast *S. cerevisiae* has a finite life span that is measured by the number of times that individual cell divides (Kok *et al.*, 1997). Progress through this replicative life span is associated with morphologic and physiologic changes, which are often decremental (Jazwinski, 1999). Longevity-assurance gene 1 (*LAG1*) was the first of several longevity genes discovered in yeast (D'mello *et al.*, 1994; reviewed in Jazwinski, 1999) that are differentially expressed during the replicative life-span. *LAG1* transcript levels decreased with replicative age of yeast cells, and gene deletion in haploid cells resulted in pronounced increase in mean and in maximum life span, indicating a role in determining yeast longevity (D'mello *et al.*, 1994). The participation of Lag1p in ceramide synthesis places the *LAG1* gene in a pivotal position for affecting life span by modulating metabolism and resistance to stress. Sphingolipids may also be involved in the increase in longevity that results from a transient sublethal heat stress, delivered early in life (Shama *et al.*, 1998).

1.5 – Final remarks

The adaptive response of *Saccharomyces cerevisiae* cells to oxidative stress has been extensively analysed, and is known to involve sensing, transcription regulators, changes in the metabolic flux and antioxidant defence mechanisms. Most of these adaptation mechanisms are associated with the repair or removing of oxidatively damaged molecules, as well as with the restablishment of the redox homeostasis.

The adaptive response to hydrogen peroxide is quite well characterized, namely the mechanisms of H_2O_2 sensing. However, the response of yeast cells when facing lethal concentrations of H_2O_2 has not been uncovered. The challenge for the studies presented in this dissertation was to elucidate the molecular mechanisms underlying the recovery of *S. cerevisiae* cells from a lethal H_2O_2 stress.

Sphingolipids have emerged as important signalling molecules in mammalian cells. Specifically ceramide has been shown to have roles in differentiation, senescence, cell cycle arrest and apoptosis, leading to the proposal of ceramide as a stress-responsive lipid (Hannun, 1996; Riboni *et al.*, 1997; Levade *et al.*, 1999; Kolesnick and Kronke, 1998; Hannun and Luberto, 2000; Guzman *et al.*, 2001; Ohanian and Ohanian, 2001). Lipids are target molecules for ROS and beside its structural role, some lipid molecules act as signal pathway members. The involvement of sphingolipids in signal transduction during stress responses, namely oxidative stress, was demonstrated. Indeed, an increased on ceramide levels resulting from sphingomyelinase activity has been observed in mammalian cells, in response to several stimuli, namely to DNA damage and oxidants (Hannun and Luberto, 2000).

In *S. cerevisiae*, the Isc1p, in addition to the inositolphosphosphingolipid-phospholipase C activity, has sphingomyelinase activity and 30% identity with mammalian neutral sphingomyelinase. So far, the involvement of ceramide and other sphingolipids in the yeast stress response is not characterized. The fact that cells lacking Isc1p activity are more sensitive towards hydrogen peroxide, led us to clarify the role of Isc1p in the regulation of oxidative stress resistance.

CHAPTER 2

Transcriptome and proteome analysis during recovery of Saccharomyces cerevisiae cells from hydrogen peroxide stress

2.1. INTRODUCTION

Reactive oxygen species (ROS), such as superoxide radicals, hydroxyl radicals and H_2O_2, are produced as normal by-products of aerobic metabolism, and cause oxidative damage to lipids, carbohydrates, proteins and nucleic acids, affecting the integrity of cell membranes and inactivating key cellular functions (Halliwell and Gutteridge, 1999). Under normal physiological conditions, cellular damages are prevented by primary antioxidant defences that neutralise the ROS, and therefore prevent oxidative damages. If damage occurs, secondary defences repair or degrade oxidised molecules, and have a key role in cellular recovery to physiological conditions (Jamieson, 1998; Moradas-Ferreira and Costa, 2000). However, the constitutive levels of antioxidant defences confer a limited capacity to resist to a sudden oxidative aggression and, therefore, their induction is essential for cell survival.

In yeast, as in other cell types, an oxidative stress response is triggered when cells are exposed to low concentrations of H_2O_2, leading to the acquisition of cellular resistance to a subsequent lethal stress (Godon *et al.*, 1998; Collinson and Dawes, 1992; Jamieson, 1992; Davies *et al.,* 1995; Flattery-O'Brien *et al.,* 1993). The adaptive response to H_2O_2 has been extensively analysed and most of the data were obtained from the effect of a transient stress induced with sublethal doses. Upon H_2O_2 challenge, the adaptive response involves changes in gene expression, within minutes of exposure to H_2O_2. In response to H_2O_2, carbohydrate metabolism appears to be diverted to the generation of the cellular reducing power, NADPH. Enzymes of the pentose phosphate pathway, trehalose synthesis, glycerol cycle and glutamate catabolism are up-regulated, whereas glycolytic activity is decreased, leading to decreased ATP levels. Several antioxidant defences and heat shock proteins are also induced in response to H_2O_2, as well as subunits of the proteasome along with enzymes of the ubiquitin pathway, mitochondrial and vacuolar proteases (Godon *et al.,* 1998; Lee *et al.,* 1999a; Gash *et al.,* 2000).

H_2O_2 *per se* is a week oxidant, but through its conversion into the highly reactive hydroxyl radicals, is able to induce oxidative damages, namely DNA damage, lipid peroxidation and specific protein carbonylation (Halliwell and Gutteridge, 1999). In yeast, glycolytic enzymes, mitochondrial enzymes and Cu,Zn-superoxide dismutase were identified as major targets oxidatively inactivated by H_2O_2 (Cabiscol *et al.*, 2000; Costa *et al.*, 2002). It is likely that proteins irreversibly inactivated by formation of carbonyl derivatives are targeted to proteolytic pathways. In fact, the increase in protein carbonyl content is one of the factors that have been implicated in the increase of the rate of proteolysis and in the decrease of the half-life of oxidised proteins (Dunlop, 2002). The proteasome has been suggested as the major pathway for degradation of oxidised proteins. The catalytic core of the proteasome is

the 20S form, whereas the 26S proteasome is formed by addition of two 19S particles containing ATPase and ubiquitin associating subunits. The 19S particles are responsible for the recognition and binding of substrate proteins, deubiquitination, unfolding and translocation into the 20S core. However, it has been suggested that protein ubiquitination is not required for the degradation of oxidised proteins. Althought *ubi4Δ* mutants have an increased sensitivity to H_2O_2 (Cheng *et al.,* 1994), protein multiubiquitination has not been observed in H_2O_2 treated cells (Inai and Nishikimi, 2002). In addition, exposure to H_2O_2 increases the activity of the 20S proteasome, which degrades oxidised proteins in an ATP- and ubiquitin-independent manner. Concomitantly, the activity of the 26S proteasome is repressed. The key role of the 20S proteasome in the degradation of oxidised proteins is further supported by the fact that yeast cells deficient in the Rpn9p subunit of the 19S regulatory complex exhibit a higher activity of the 20S proteasome and are able to degrade carbonylated proteins more efficiently than wild type cells (Inai and Nishikimi, 2002). In contrast, the role of vacuolar proteases in the degradation of oxidised proteins in not characterized.

In this study, the changes in gene expression at the genome-wide level during exposure to high concentrations of H_2O_2 and during recovery of yeast cells after H_2O_2 stress were analysed. In addition, the role of the ubiquitin-26S-proteasome system and vacuolar proteolysis in the degradation of carbonylated proteins was assessed using mutants deficient in Doa4p, a deubiquitination enzyme (Swaminathan *et al.,* 1999), or Pep4p, a vacuolar protease.

2.2. MATERIALS AND METHODS

2.2.1. YEAST STRAINS AND PLASMIDS

The strains of *Saccharomyces cerevisiae* and the plasmids used in this study are listed in Tables 2.1 and 2.2.

Table 2.1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Reference/Source
W303	Matα, *ade2-1, can1-100, trp1-1, ura3-1, his3-11,15, leu2-3,112*	Wallis *et al.*, 1989
*doa4*Δ	[W303] *doa4*Δ::*LEU2*	Present work
*pep4*Δ	[W303] *pep4*Δ::*HIS3*	Present work

Table 2.2. Plasmids used in this study.

Plasmid	Reference
pKS-*PRA*ΔEN::*HIS3*	Hirsch *et al.*, 1992
p*DOA4-8*	Papa and Hochstrasser, 1993

2.2.2. GROWTH CONDITIONS

Yeast cells were grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose) or in minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with the appropriate amino acids (40 mg/l tryptophan, 40 mg/l histidine, 80 mg/l leucine, 40 mg/l methionine) and nucleotides (40 mg/l adenine, 40 mg/l uracil), to early exponential phase (OD_{600}=0.6), in an orbital shaker, at 26ºC and 120 rpm, with a ratio of flask volume / medium volume of 5:1. Cellular growth was determined by spectrophotometrical measurement of $O.D._{600}$.

Growth in solid medium was performed at 26ºC, in YPD or minimal medium containing 1.5% (w/v) agar.

2.2.3. HYDROGEN PEROXIDE TREATMENT AND CELL VIABILITY

Yeast cells were treated with 1.5 mM H_2O_2 for 30 min, centrifuged at 4500 rpm for 5 min, ressuspended in minimal medium and allowed to recover. Cell viability was determined by standard dilution plate counts on YPD medium containing 1.5% agar. Colonies were counted after growth at 26ºC for 3 days. Viability was expressed as the percentage of the colony-forming units of non-stressed cells.

2.2.4. GLUCOSE ASSAY

Samples (2 ml) of cultures were taken and centrifuged at 4500 rpm for 5 min. Supernatants were stored at -20°C until use. Glucose in the growth medium was quantified by the glucose / peroxidase method: 2,5 ml TGO reagent [0.5 M Tris / HCl pH 7.0, 20 U glucose oxidase ml^{-1}, 0.38 U peroxidase ml^{-1}, 0.05 mg o-dianisidine hydrochloride ml^{-1}, 1% Triton X-100] was added to 0.5 ml of sample, the mixture was incubated for 20 min at 37°C and the absorbance at 420 nm was determined. Glucose was estimated by reference to a standard curve prepared with known amounts of glucose.

2.2.5. PROTEOME ANALYSIS

For protein synthesis analysis, samples (50 ml) of culture were taken, centrifuged at 4500 rpm for 5 min, ressuspended in 2 ml of fresh minimal medium, and incubated with 10μCi/ml [^{35}S]-methionine for 60 min. Yeast extracts were prepared in 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, containing a protease inhibitor cocktail (Complete™, Mini, EDTA-free Protease Cocktail Inhibitor Tablets, Boehringer Mannhein), by vigorous shaking of the cell suspension, in the presence of glass beads, for 5 min. Short pulses of 1 min were used, with 1 min intervals on ice. Protein content was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. Radiolabel incorporation was measured by scintillation counting. Proteins (1.000.000 cpm) were solubilised in 9 M Urea, 2% CHAPS, 2% β-mercaptoethanol, 0.8% Pharmalytes pH 3-10. Proteins were mixed with three volumes of a gel slurry (30 mg Sephadex IEF / ml solubilisation solution) and loaded on a 13 cm immobilised pH 3-10 linear gradient (IPG) dry strip (Pharmacia), previously re-hydrated in 8 M Urea, 0.5% CHAPS, 0.2% dithiothreitol (DTT), 0.25% Pharmalytes pH 3-10, 0.5% Triton X-100 (first dimension). Isoelectric focusing

was performed in 4 phases: 1- 30 min at 150V, 2- 60 min at 300V, 3- 90 min at 3500V, 2mA, 7W, 2850V/h, 4- 245 min at 3500V, 2mA, 7W, 14150V/h.

In the second dimension, SDS-PAGE (sodium dodecyl sulphate- polyacrilamide gel electrophoresis), IPG-strips were incubated in equilibration buffer (6M Urea, 2% SDS, 0.1mM EDTA, 30% glycerol, 0.01% bromophenol blue, 50mM Tris pH 6.8) containing 10 mg DTT ml^{-1} (first 15 min), or 48.1 mg Iodoacetamide ml^{-1} (second 15 min), and loaded on a 12.5% polyacrylamide gel. Following 2D-electrophoresis, protein fixation was performed in 30% (v/v) methanol, 10% (v/v) acetic acid for 30 min, incubated with Amplify (Amersham) for 30 min, dried and exposed ON to an X-ray film (Kodak), at -70°C. The films were scanned using a densitometer. The analysis of the protein pattern and quantifications were performed using the PD-Quest (BioRad) software. All spot intensities were normalised, by dividing sampled intensities by the mean sampled intensities of all spots. To determine the –fold induction, the relative protein levels were expressed as the ratio recovery / control. All proteins whose expression increased at least 2 fold were listed, because these values were found to be reproducible. All values are means of at least three independent experiments.

2.2.6. mRNA PREPARATION, SYNTHESIS OF cDNA, GENEFILTERS® HYBRIDIZATION AND DATA ANALYSIS

Total RNA was isolated by the acid phenol method (Ausubel *et al.,* 1998). [^{33}P]CTP – labelled cDNA was synthesized in the following way. 5 µg of total RNA and 2 µg of oligo(dT) (10-20-mer mixture, Research Genetics) were mixed with 8 µl of water, heated for 10 min at 70°C, and then chilled on ice. The following components were added: first strand buffer (Life Technologies, Inc.), DTT (3.3 mM), dATP, dGTP, and dTTP (1 mM each), Superscript II reverse Transcriptase (300 units; Life Technologies, Inc.), and [^{33}P]CTP (100 µCi/mmol). The mixture (30 µl volume) was incubated at 37°C for 90 min. The probe was then purified by passage through a Sephadex G-50 column. Approximatelly 60-70% of the label was incorporated in high molecular weight products. Genefilters® (Research Genetics) were prehybridized for 2h with 5 ml of MicroHyb solution (Research Genetics) at 42°C in a roller oven (Hybaid). The purified cDNA probe was denatured for 3 min at 100°C and added to the pre-hybridization mixture. After overnight hybridization at 42°C, filters were rinsed with 2x SSC, 0.1% SDS and incubated in the same buffer at 50°C for 45 min. Filters were then rinsed with 0.5x SSC, 0.1% SDS, transferred to a plastic box, and washed with 0.5x SSC, 0.1% SDS at room temperature for 15 min.

A Molecular Imager® FX (BioRad) was used to obtain a digital image of the filters. Filters were kept moist to facilitate stripping between hybridizations, and stripping was done with 0.5% SDS, pre-heated to 100°C. Filters were rinsed and submerged in this solution for 15 min on a shaker, while the solution was allowed to cool. More than 95% of the signal was consistently removed by this procedure. Images were converted to TIFF and imported into the Pathways4® Software (Research Genetics). Pathways4® was used to compare gene filter images. Prior to determination of induction or repression of gene expression, all spot intensities were normalised, by dividing sampled intensities by the mean sampled intensities of all clones. To determine the –fold induction or repression, the relative mRNA levels were expressed as the ratio H_2O_2 / control (untreated) or recovery / H_2O_2. All genes whose mRNA levels increased at least 1.8 fold or decreased 2-fold were listed, because these values were found to be reproducible. All values are means of at least three independent experiments.

2.2.7. DNA MANIPULATION AND CLONING TECHNIQUES

Restriction enzymes were used according to manufactory instructions. Cloning and DNA manipulation techniques were in general performed according to reference protocols (Sambrook *et al.*, 1989).

Oligonucleotides used in this work are described in table 2.3.

Table 2.3. Oligonucleotides used in this work. # means the Crick strand.

Oligo	Oligonucleotide sequence
LEU-2A#	5' – TGTCGCCGAAGAAGTTAAGA – 3'
PEP4-N#	5' – TCTCACCCTACTGTATTCATA – 3'
PEP4-C	5' – GGCGGAGAAGTAAGAAAAGTT – 3'
DOA4-N#	5' – TACGATAGCACAACTAACCGC – 3'
DOA4-C	5' – GCACAACCATTGCTCATCC – 3'
PEP4R	5' – ACACAGGAAACAGCTATGAC – 3'
PEP4F#	5' – AGGGTTTTCCCAGTCACGAC – 3'

2.2.8. DISRUPTION OF *DOA4* AND *PEP4* GENES

Disruption of *DOA4* and *PEP4* genes was performed by one step gene disruption (Rothstein *et al.*, 1983).

For disruption of *DOA4* gene, a 2.5 kb deletion fragment containing *LEU2* and the flanking regions of *DOA4* was obtained from p*DOA4-8*, using DOA4-C and DOA4-N primers.

For disruption of *PEP4* gene, a 3.0 Kb deletion fragment containing *HIS3* and the flanking regions of *PEP4* was obtained from pKS-*PRA1*ΔEN::*HIS3*, using PEP4R and PEP4F primers.

*S. cere*visiae W303 cells were transformed using the lithium acetate protocol (Gietz and Woods, 1994). For selection after transformation, cells were grown in 10 ml of selective minimal medium, at 26°C, and plated in the appropriate minimal medium containing 1.5 % agar. *doa4*Δ and *pep4*Δ mutants were selected in minimal medium lacking leucine and histidine, respectively. Gene disruption was confirmed by PCR analysis.

2.2.9. PROTEIN CARBONYLATION ANALYSIS

Yeast extracts were prepared as described in 2.2.5. For 1D analysis, proteins were derivatised with 2,4-dinitrophenylhydrazine (Levine *et al.*, 1994). An aliquot of each sample (15 μg) was loaded in a polyacrilamide gel (12.5%) and visualized by silver staining (O'Connel and Stults, 1997) or blotted onto nitrocellulose membranes (Hybond-C, Amersham Biosciences Europe, GmbH). Slot-blot assays were performed by loading an aliquot of derivatised protein (0.05 μg) into polyvinylidene fluoride membranes (Hybond PVDF, Amersham Biosciences Europe, GmbH). The nitrocellulose or PVDF membranes were probed with rabbit IgG anti-DNP (Dako, Glostrup, Denmark) at a 1:5000 dilution, as the primary antibody, and goat anti-rabbit IgG-peroxidase (Sigma, St. Louis, MO, USA) at a 1:5000 dilution, as the secondary antibody. Immunodetection was performed by chemiluminescence, using a kit from Amersham (RPN 2109). The membranes were exposed to a Hybond-ECL film (Amersham Biosciences Europe, GmbH) for 15 seconds to 1 min, and the film was developed. Band intensities were quantified by densitometry.

2.3. RESULTS

2.3.1. CELLULAR GROWTH AND GLUCOSE CONSUMPTION DURING RECOVERY FROM HYDROGEN PEROXIDE STRESS

Aiming to elucidate the molecular mechanisms involved in cellular recovery from oxidative stress, yeast exponentially grown cells were exposed to 1.5 mM H_2O_2 for 30 minutes and allowed to recover in fresh minimal medium without H_2O_2. The analysis of cellular viability showed that 50% of the cells survived and were able to recover after exposure to H_2O_2 The analysis of cellular growth and the rate of glucose consumption, during recovery from H_2O_2 stress showed that cellular growth was almost completely absent during the first 4-6 hours of recovery, becoming subsequently similar to the rate observed in control cells. Concomitantly, the rate of glucose consumption decreased but was not impaired, indicating that cells remained metabolically active (Figure 2.1).

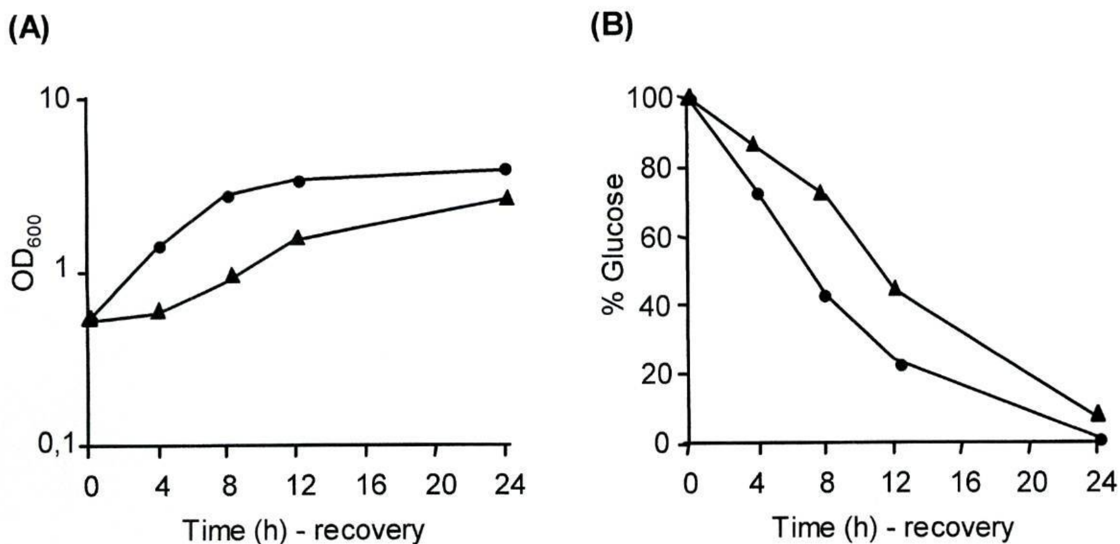


Figure 2.1.

Cellular growth and glucose consumption during recovery of *S. cerevisiae* cells from H_2O_2 stress. *S. cerevisiae* W303 cells were grown to the exponential phase ($OD_{600} \approx 0.6$). Control (●) and cells treated with 1.5 mM H_2O_2 for 30 min (▲) were centrifuged and ressuspended in minimal medium without H_2O_2. Cell density (A) and % of glucose remaining in the growth medium (B) were determined during cellular recovery up to 24 h. A representative experiment is shown (out of three independent experiments with similar results).

2.3.2. PROTEOME ANALYSIS DURING RECOVERY FROM HYDROGEN PEROXIDE STRESS

To analyse the changes in the proteome associated with cellular recovery from H_2O_2 stress, yeast cells were labelled with [^{35}S]-Met. Protein synthesis rate initially decreased during recovery, however, after 2 hours of recovery it became similar to control, and after 4 hours, increased to levels exceeding those of control cells (Figure 2.2).

Aiming to investigate if yeast cells induce an adaptive response during the lag phase that precedes the resumption of normal protein synthesis rate, the changes in the proteome were analysed by 2D – gel electrophoresis (Figure 2.3).

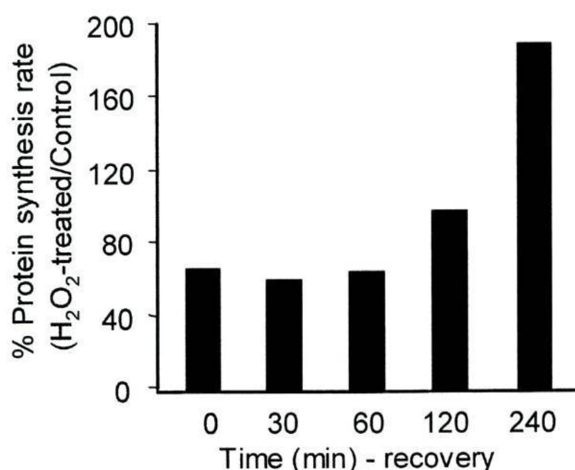


Figure 2.2.
Rate of protein synthesis during recovery of *S. cerevisiae* cells from H_2O_2 stress. *S. cerevisiae* W303 cells were grown to the exponential phase ($OD_{600} \approx 0.6$). Control and cells treated with 1.5 mM H_2O_2 for 30 min were centrifuged, ressuspended in minimal medium without H_2O_2, and labelled with [^{35}S]-methionine for 60 min at time 0, 30, 60, 120 and 240 min of recovery. Radiolabel incorporation was measured by scintillation counting. Protein synthesis rate was expressed as cpm (mg protein)$^{-1}$. The ration H_2O_2 treated / Control is shown. Values are means ± SD of three independent experiments.

Cell recovery involves the up-regulation of several proteins (Figure 2.3). The expression of 15 proteins increased 2-4 fold, 10 proteins increased 4-6 fold, and 3 proteins were found to be expressed only during recovery (Table 2.4). These results suggest that yeast cells induce an adaptive response that may help in cellular recovery to physiological conditions.

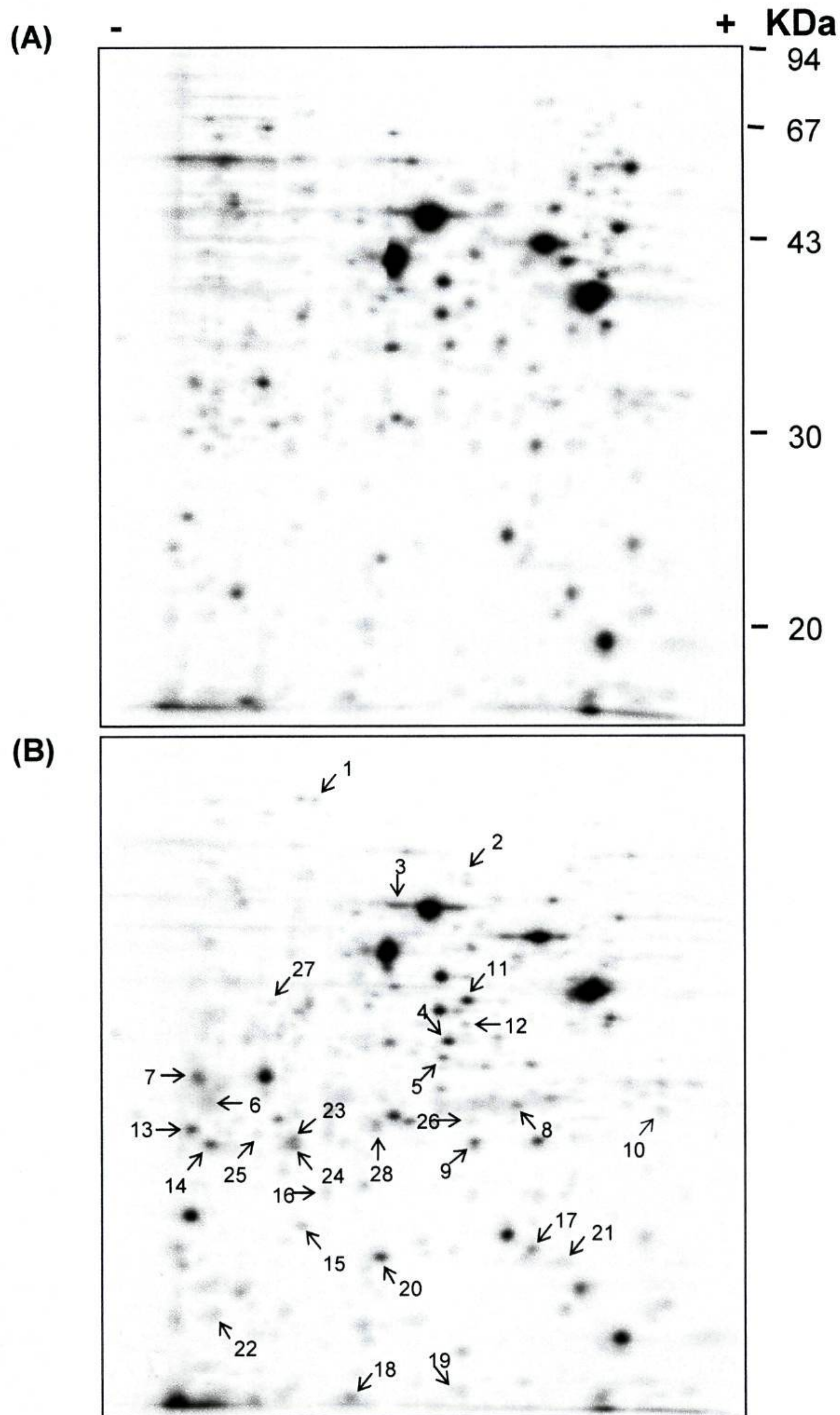


Figure 2.3.

Proteome analysis during recovery of *S. cerevisiae* cells from H_2O_2 stress. *S. cerevisiae* W303 cells were grown to the exponential phase. Control (A) and cells recovering for 1 hour after exposure to 1.5 mM H_2O_2 (B) were labelled with [^{35}S]-methionine. Two-dimensional gels were performed with total yeast soluble extracts (1.000.000 cpm), and exposed to an X-ray film. A representative experiment is shown (out of three independent experiments).

Table 2.4. Proteins whose expression is up-regulated during recovery from H_2O_2 stress.

Protein number[a]	Fold induction
1	2,3
2	2,7
3	3,7
4	2,6
5	2,7
6	3,9
7	2,6
8	6,0
9	4,2
10	4,9
11	3,1
12	5,1
13	6,4
14	4,6
15	2,8
16	3,3
17	3,1
18	4,7
19	2,7
20	3,5
21	5,6
22	4,3
23	3,6
24	4,2
25	2,8
26	new
27	new
28	new

[a] According to Figure 2.3.

2.3.3. TRANSCRIPTOME ANALYSIS DURING RECOVERY FROM HYDROGEN PEROXIDE STRESS

To identify the cellular functions associated with recovery from H_2O_2 stress, the changes in the gene expression at the genome-wide level were analysed. RNA was isolated from control cells, from cells exposed to 1.5 mM H_2O_2 for 30 min, and from H_2O_2-treated cells allowed to recover for 60 min in H_2O_2-free medium. Hydrogen peroxide triggers a stress response that is well characterised, both at the proteome and transcriptome level (Godon *et al.*, 1998; Lee *et al.*, 1999; Gasch *et al.*, 2000). Major alterations in the transcriptome of

H_2O_2-treated cells were also observed (data not shown). During recovery, 21 of the genes induced by H_2O_2 were further induced. Of the genes repressed by H_2O_2 only 1 gene was further downregulated (Table 2.5). Specific changes associated with cellular recovery after H_2O_2 stress (recovery *vs* H_2O_2) were considered as those not observed in H_2O_2-treated cells (H_2O_2 *vs* control). The results showed that the mRNA level of 68 genes increased (Table 2.6), whereas that of 144 genes (including 76 encoding ribosomal proteins) was diminished (Table 2.7). These genes encode proteins involved in cell rescue and defence, metabolism (carbohydrate, lipid, aminoacid and nucleotide), energy, protein folding and stabilisation, protein modification, proteolytic degradation, protein synthesis, cell cycle and DNA processing, transcription regulation, ionic homeostasis, transport, cell wall metabolism and cytoskeleton organisation. Most of the genes down-regulated were associated with aminoacid (8%) and protein (57%) biosynthesis. Of the genes specifically upregulated during cellular recovery, 40% were related with redox homeostasis and protein/aminoacid metabolism: 7% encoded dehydrogenases, 10% were associated with protein folding and stabilization, 12% with proteolysis (ubiquitin-proteasome system), and 7% with amino acid catabolism.

Interestingly, both the vacuolar proteases *PEP4* and *LAP4* genes were induced during exposure to 1.5 mM H_2O_2 (2-2.5 fold; H_2O_2 *vs* control) and further induced during recovery (2-2.5 fold; recovery *vs* H_2O_2). Furthermore, the expression of 3 genes associated with protein traffic into the vacuole (*YKS5*, *YKT6* and *MVP1*; Table 2.6) was specifically induced during recovery.

Table 2.5. Genes induced / repressed both during exposure to 1.5 mM H_2O_2 stress and during recovery of yeast cells. Yeast cells were exposed to 1.5 mM H_2O_2 for 30 min and allowed to recover during 60 min in minimal medium. Genes upregulated / downregulated by H_2O_2 stress (H_2O_2 *vs* control) and during cellular recovery (recovery *vs* H_2O_2) and sorted into functional categories (according to MIPS) are shown.

Gene	Gene product	H_2O_2/control		Recovery/H_2O_2	
		Log₂ (ratio) mean	SD	Log₂ (ratio) mean	SD
Cell rescue and defence					
TRX2	thioredoxin 2	1,44	0,89	0,89	0,44
PRX1	thiredoxin peroxidase activity	1,18	0,56	1,43	0,42

Table 2.5. (cont.)

Gene	Gene product	H_2O_2/control		Recovery/H_2O_2	
		Log_2 (ratio) mean	SD	Log_2 (ratio) mean	SD
C-compound and carbohydrate metabolism					
EMI2	early meiotic induction; similar to glucokinase I	1,54	0,75	1,21	0,42
GRE3	aldose reductase	0,85	0,68	1,90	0,64
ALD4	acetaldehyde dehydrogenase (mitochondria)	1,23	0,59	1,67	0,58
ROM1	GDP/GTP exchange protein for Rho1p	1,61	0,62	2,29	0,97
Protein folding and stabilisation					
HSP42	42KDa heat-shock protein	3,39	3,26	1,16	0,75
ERO1	FAD-dependent oxidase of protein disulphide isomerase	1,60	0,78	0,86	0,66
Protein activity regulation					
FES1	Hsp70 nucleotide exchange factor	1,46	1,06	0,94	0,62
Proteolytic degradation					
PEP4	vacuolar proteinase A	1,02	0,54	1,47	0,65
LAP4	vacuolar aminopeptidase ysc1	1,48	0,83	1,34	1,09
Amino acid metabolism					
CYS3	cystathionine gamma-lyase	1,18	0,86	1,05	0,67
Nucleotide metabolism					
PNC1	nicotinamidase (NAD^+ salvage pathway)	1,75	0,75	0,90	0,73
Cell cycle and DNA processing					
RFA1	heterotrimeric RPA (RF-A) single stranded DNA binding protein 69kDa subunit RF-A	1,07	0,50	1,26	0,56
Transcription					
DAL80	transcriptional repressor of multiple nitrogen regulated genes	1,02	0,41	1,19	0,45
Ionic homeostasis					
FET3	multicopper oxidase	1,60	0,67	0,85	0,65
Transport facilitation					
ENB1	enterobactin transport	1,06	0,65	1,46	1,29

Table 2.5. (cont.)

Gene	Gene product	H_2O_2/control		Recovery/H_2O_2	
		Log₂ (ratio) mean	SD	Log₂ (ratio) mean	SD
Cell wall					
SPI1	cell wall protein; stationary phase-induced, Msn2/4p-dependent	2,68	2,50	1,15	0,66
Miscellaneous and uncharacterised					
HMX1	heme oxigenase (putative)	1,13	0,65	1,51	0,92
MSC1	meiotic sister-chromatid recombination	1,71	1,01	0,89	0,41
YMR173W-A	uncharacterised	1,84	1,57	0,95	0,86
Protein biosynthesis					
RPS10A	ribosomal protein, small subunit	-0,99	0,40	-1,78	0,16

Table 2.6. Genes induced during recovery of yeast cells from 1.5 mM H_2O_2 stress

Yeast cells were exposed to 1.5 mM H_2O_2 for 30 min and allowed to recover during 60 min in minimal medium. Genes up-regulated (recovery *vs* H_2O_2) and sorted into functional categories (according to MIPS) are shown.

Gene	Gene product	Recovery/H_2O_2	
		Log₂ (ratio) mean	SD
Cell rescue and defence			
DDI1	SNARE-binding protein; vesicle-mediated transport;ubiquitin-dependent protein catabolism	1,2	0,8
C-compound and carbohydrate metabolism			
HXK1	hexokinase I	2,0	1,1
AAD6	aryl-alcohol dehydrogenase (putative)	2,4	1,1
AAD16	aryl-alcohol dehydrogenase	1,9	1,7
GUT2	glycerol-3-phosphate dehydrogenase	1,1	0,6
ARO10	pyruvate decarboxylase (leucine catabolism)	1,6	0,6
BDH1	(R,R)-butenadiol dehydrogenase activity	1,4	0,6
SOR2	oxireductase activity	1,6	0,8
SOR1	sorbitol dehydrogenase	1,5	0,5
Energy			
COX18	cytochrome c oxidase biogenesis	1,5	0,7
PET10	uncharacterised	1,1	0,5
YML125C	uncharacterised	1,5	0,8

Table 2.6. (cont.)

Gene	Gene product	Recovery/H_2O_2	
		Log_2 (ratio) mean	SD
Protein folding and stabilisation			
PDI1	protein disulphide isomerase	1,2	0,6
CPH1	cyclophilin, peptidyl-prolyl cis-trans isomerase	1,6	0,6
FPR1	peptidyl-prolyl cis-trans isomerase	1,0	0,5
HSP26	heat shock protein 26	1,9	1,7
SSA4	70kDa heat shock protein	1,3	1,0
AHA1	Hsp90 system cochaperone	1,4	0,5
CCT4	chaperone activity	1,4	0,8
Proteolytic degradation			
UBA1	ubiquitin activating enzyme	1,2	0,6
UBC1	ubiquitin conjugating enzyme	1,2	0,4
RAD6	ubiquitin conjugating enzyme	1,1	0,6
UMP1	20S proteasome maturation factor	1,8	0,6
PRE6	20 S proteasome subunit, proteasome endopeptidase	1,1	0,6
PRE7	20 S proteasome subunit, proteasome endopeptidase	1,1	0,5
PRE9	20 S proteasome subunit, proteasome endopeptidase	1,2	0,8
YTA2	ATPase and endopeptidase activity	1,3	0,6
Protein modification			
YDR140W	S-adenosylmethionine dependent methyltransferase activity	1,6	0,5
Amino acid metabolism			
ARO9	aromatic aminoacid aminotransferase II	1,7	0,8
YLR089C	transaminase activity	1,6	1,0
TWT2	branched-chain amino acid aminotransferase	1,5	0,6
GDH2	NAD-dependent glutamate dehydrogenase	1,9	1,8
PUT1	proline oxidase	2,0	2,0
Lipid, fatty-acid and isoprenoid metabolism			
ACB1	acyl-CoA-binding protein	1,0	0,4
ERG13	3-hydroxy-3-methylglutaryl coenzyme A synthase	0,9	0,4
ERG5	cytochrome P450	1,3	0,9
YDC1	alkaline dihydroceramidase	1,6	0,8
OPI3	unsaturated phospholipid N-methyltransferase	1,2	0,8
Nucleotide metabolism			
RNR4	ribonucleotide reductase, small (r2) subunit	2,2	0,6
ADE6	5'-phosphoribosylformyl glycinamidine synthetase	1,5	0,6

Table 2.6. (cont.)

Gene	Gene product	Recovery/H_2O_2	
		Log$_2$ (ratio) mean	SD
Cell cycle and DNA processing			
RHC21	mitotic chromosome condensation	1,0	0,6
NAP1	nucleosome assembly protein I	1,5	1,0
MAG1	3-methyladenine DNA glycosylase	1,0	0,5
Transcription			
TFC4	131 kDa transcription factor tau (TFIIIC) subunit	1,2	0,4
RPC31	HMG-like protein, RNA polymerase III © 31 kDa subunit	1,2	0,7
YNT20	RNA exonuclease	1,0	0,4
Ionic homeostasis			
SLF1	copper sulphide mineralization	1,3	0,5
FRE1	cupric reductase; ferric reductase	1,2	0,5
Cellular transport and transport mechanisms			
YKS5	clathrin assembly complex beta adaptin component (putative)	1,0	0,8
YKT6	v-SNARE	1,3	0,4
MVP1	protein vacuolar targeting	1,3	0,8
Cytoskeleton			
APP1	actin cytoskeleton organization and biogenesis	1,0	0,5
Miscellaneous and uncharacterised			
YHR138C	endopeptidase inhibitor activity	1,1	0,6
SGT2	small glutamine-rich tetracopeptide repeat containing protein	1,2	0,7
GIR2	uncharacterised	1,4	0,5
YGR073C	uncharacterised	1,0	0,5
YGR226C	uncharacterised	1,3	0,8
YBR062C	uncharacterised	0,8	0,3
YCR102C	uncharacterised	1,4	0,3
YDL046W	uncharacterised	1,2	0,8
YLR302C	uncharacterised	1,5	1,1
YNL335W	uncharacterised	1,0	0,6
YOR285W	uncharacterised	1,0	0,4
BRE5	uncharacterised	1,4	0,6
YLR327C	uncharacterised	2,1	0,7
YLR257W	uncharacterised	2,0	0,9
YML048W-A	uncharacterised	1,0	0,6
YKR032W	uncharacterised	1,2	0,9

Table 2.7. Genes repressed during recovery of yeast cells from 1.5 mM H_2O_2 stress.

Yeast cells were exposed to 1.5 mM H_2O_2 for 30 min and allowed to recover during 60 min in minimal medium. Genes down-regulated (recovery *vs* H_2O_2) and sorted into functional categories (according to MIPS) are shown. Genes (n=76) encoding ribosomal proteins were omitted.

Gene	Gene product	Recovery/H_2O_2	
		Log_2 (ratio) mean	SD
Cell rescue and defence			
TPO2	spermine transporter activity	-1,24	0,15
SRL1	uncharacterised	-1,40	0,07
YDR033W	membrane protein related to Hsp30p	-3,69	0,04
Aminoacid metabolism			
LEU1	isopropylmalate isomerase	-2,49	0,02
LEU2	beta-isopropylmalate dehydrogenase	-2,44	0,05
LYS1	saccharopine dehydrogenase	-2,36	0,04
LYS9	saccharopine dehydrogenase	-2,34	0,04
LYS12	homoisocitrate dehydrogenase	-1,53	0,12
LYS20	homocitrate synthase	-1,10	0,11
LYS21	homocitrate synthase	-2,27	0,06
ARO8	aromatic a.a. aminotransferase	-1,12	0,18
THR1	homoserine kinase	-1,21	0,10
ARG1	argininosuccinate synthetase	-3,03	0,03
AAT2	aspartate aminotransferase	-1,05	0,13
ARO4	2-dehydro-3-deoxyphosphoheptonate aldolase	-1,89	0,09
Protein synthesis			
EFT2	translation elongation factor 2 (EF-2)	-2,36	0,08
EFT1	translation elongation factor 2 (EF2)	-1,91	0,05
TEF4	translation elongation factor EF-1 gamma	-1,28	0,18
ASC1	uncharacterised; involved in translation	-2,44	0,03
SSB1	heat shock protein of HSP70 family	-1,53	0,21
SSB2	heat shock protein of HSP70 family	-2,13	0,08
C-compound and carbohydrate metabolism			
CDC19	pyruvate kinase	-1,32	0,14
PDC1	pyruvate decarboxylase	-1,65	0,08
CIT2	citrate synthase	-1,00	0,12
GPD2	glycerol-3-phosphate dehydrogenase (NAD^+)	-1,23	0,10
PMI40	mannose-6-phosphate isomerase	-1,02	0,11
SDH2	succinate dehydrogenase (ubiquinone) iron-sulphur protein subunit	-1,23	0,34
Energy			
QCR9	ubiquinol citochrome c oxireductase complex 7.3 kDa subunit 9	-2,05	0,05

Table 2.7. (cont.)

Gene	Gene product	Recovery/H_2O_2	
		Log_2 (ratio) mean	SD
Nucleotide metabolism			
URA5	orotate phosphoribosyltransferase I	-1,77	0,11
ADO1	adenosine kinase	-1,22	0,11
Cell cycle and DNA processing			
YRF-1	Y'-helicase protein 1	-1,00	0,11
Transcription			
HTA2	histone H2A	-1,38	0,07
HTA1	histone H2A	-1,01	0,14
HTB2	histone H2B	-1,56	0,09
HHT2	histone H3	-1,21	0,09
RME1	zinc finger protein, negative regulator of meiosis	-1,35	0,16
FZF1	transcription factor containing 5 zinc fingers	-1,10	0,14
MGA1	similar to heat shock transcription factor	-1,01	0,15
TYE7	33 kDa protein, potential member of the bHLM/leucine zipper protein family	-1,37	0,10
Transport facilitation			
HXT1	high afinity hexose (glucose) transporter	-1,83	0,10
HXT3	low afinity glucose transporter	-1,75	0,07
CTP1	citrate transporter	-1,12	0,12
OAC1	oxaloacetate transport protein	-1,45	0,15
PMA1	plasma membrane H^+-ATPase	-1,72	0,10
GAP1	general amino acid permease	-1,51	0,09
Cell wall			
CCW12	cell wall mannoprotein	-1,60	0,05
Miscellaneous and uncharacterised			
STP4	involved in pre-tRNA splicing and in uptake of branched-chain amino acids	-1,24	0,12
FMP48	kinase activity	-1,24	0,11
USE1	SNARE protein	-1,08	0,10
PHO3	acid phosphatase	-1,16	0,14
YBL109W	uncharacterised	-1,17	0,11
YDR417C	uncharacterised	-2,24	0,10
SNT2	uncharacterised	-2,03	0,06
YGL102C	uncharacterised	-1,44	0,08

Table 2.7. (cont.)

Gene	Gene product	Recovery/H_2O_2	
		Log$_2$ (ratio) mean	SD
Miscellaneous and uncharacterised (cont.)			
YBR147W	uncharacterised	-1,79	0,04
YDR544C	uncharacterised	-1,12	0,13
YDL228C	uncharacterised	-1,90	0,08
YCR025C	uncharacterised	-1,01	0,17
YPL142C	uncharacterised	-1,86	0,07
YPR044C	uncharacterised	-1,39	0,15
YNL338W	uncharacterised	-1,57	0,08
YOL092W	uncharacterised	-1,66	0,07
TOS11	uncharacterised	-1,48	0,08
YPL197C	uncharacterised	-1,37	0,14
BUD19	uncharacterised	-1,70	0,09
YJR115W	uncharacterised	-1,86	0,08
YLL044W	uncharacterised	-1,89	0,03
YKL030W	uncharacterised	-1,44	0,09

2.3.4. TURNOVER OF PROTEIN CARBONYLS IN *doa4*Δ AND *pep4*Δ MUTANTS

The transcriptome analysis revealed that genes encoding enzymes involved in proteolytic degradation are up-regulated during recovery. The increase in proteolysis is certainly related to the accumulation of carbonylated proteins that are inactivated.

The involvement of the ubiquitin-proteasome system and vacuolar proteolysis in the degradation of carbonylated proteins during recovery from H_2O_2 stress was assessed. For that end, the experiments were carried out using mutant cells, deficient in a deubiquitination enzyme, Doa4p, important for ubiquitin recycling, (Swaminathan *et al.*, 1999), or in the vacuolar protease Pep4p.

2.3.4.1. DISRUPTION OF *DOA4* AND *PEP4* GENES

Gene disruption was performed by homologous recombination, using the one step gene disruption protocol (Rothstein *et al.*, 1983), as described in 2.2.8. A 2.5 Kb fragment containing *LEU2* gene and the flanking regions of *DOA4* deletion, obtained from p*DOA4-8* (Figure 2.4), was used to disrupt *DOA4* gene.

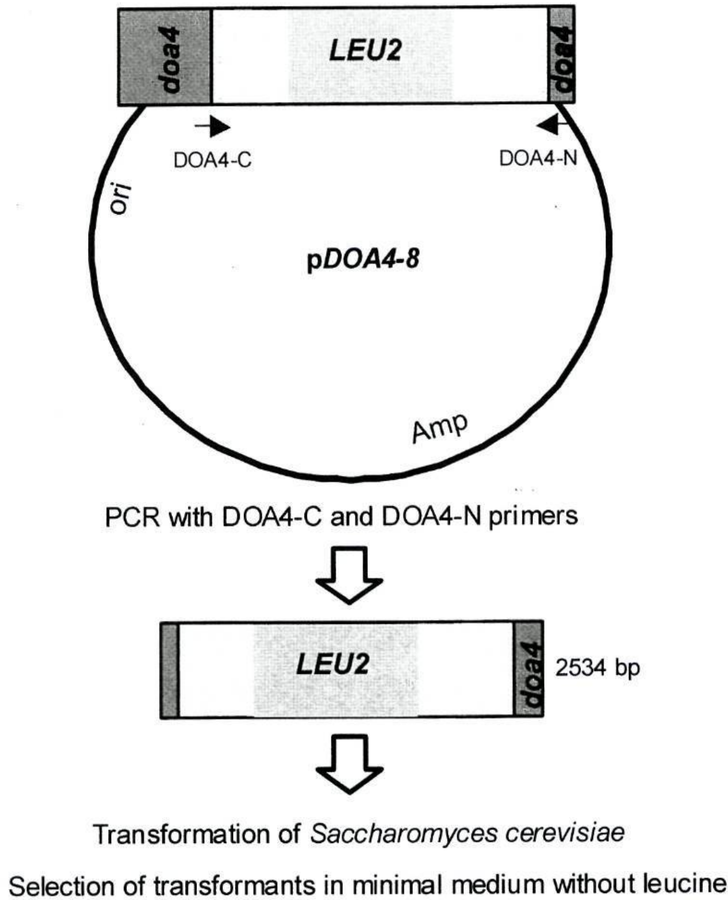


Figure 2.4.
Strategy for *DOA4* disruption in *S. cerevisiae* W303 with *LEU2* gene, using a fragment isolated from p*DOA4-8* (Papa and Hochstrasser, 1993).

Genomic DNA was isolated from wild type (W303 strain) and *doa4*Δ mutant cells. The disruption of *DOA4* gene was confirmed by PCR analysis, using DOA4-C primer from *DOA4* gene and LEU2-A primer from *LEU2* gene (Figure 2.5). A 720 bp fragment was expected for the disrupted strain (according to the integration of *LEU2* in the genome), and no fragments were expected for the wild type strain. PCR analysis revealed a fragment with the expected size for the disrupted strain.

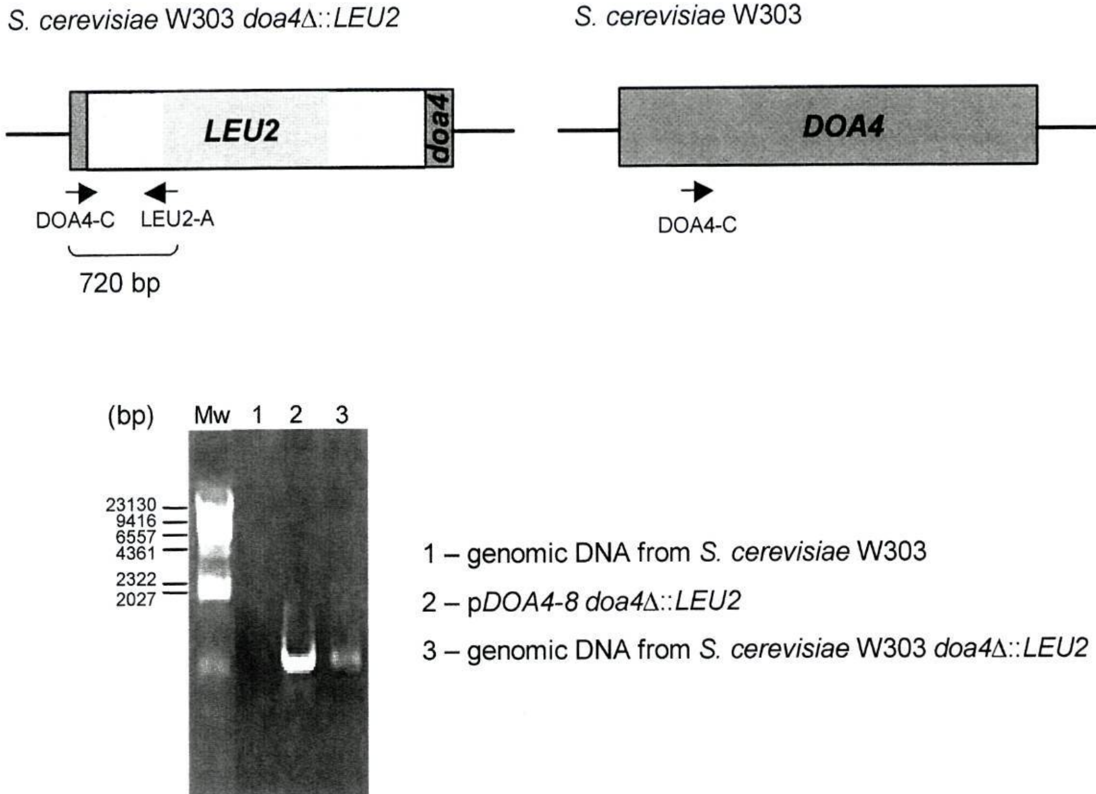


Figure 2.5.

Confirmation of *DOA4* disruption by PCR. PCR products using genomic DNA from wild type (W303) or *doa4Δ* mutant cells, and the primers LEU2-A and DOA4-C, were analysed in a 0.8% agarose gel. p*DOA4-8* was used as a positive control. The expected fragment sizes for the disrupted strain and for the wild type strain are indicated. The molecular weight marker (Mw) was phage λ digested with *Hin*dIII.

A 3.0 Kb fragment containing *HIS3* gene and the flanking regions of *PEP4* deletion, was obtained from pKS-*PRA1*ΔEN::*HIS3* (Figure 2.6), and was used to disrupt *PEP4* gene.

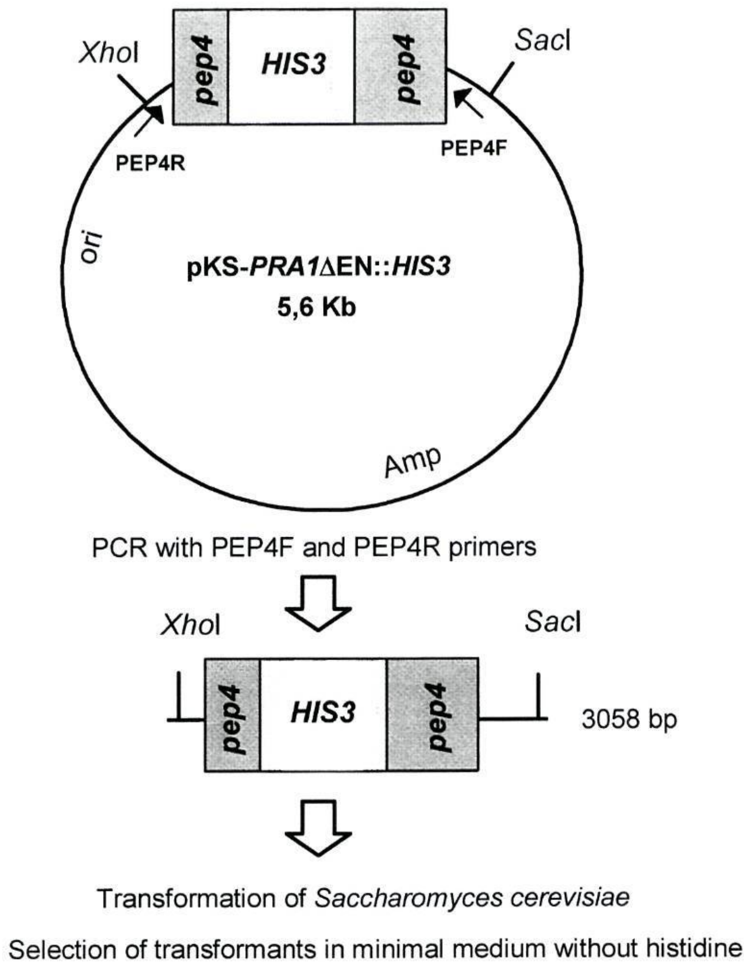
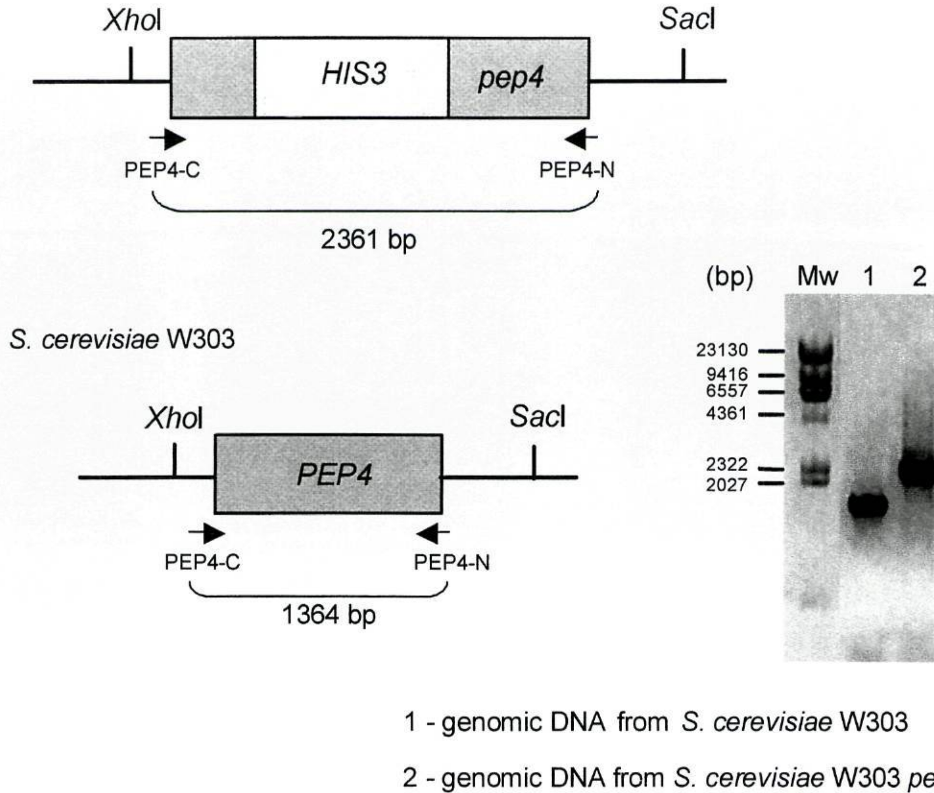


Figure 2.6.

Strategy for *PEP4* disruption in *S. cerevisiae* W303 with *HIS3* gene, isolated from pKS-*PRA*ΔEN::*HIS3* (Hirsch *et al.*, 1992).

The disruption of *PEP4* gene was confirmed by PCR analysis using primers PEP4-C and PEP4-N from *PEP4* gene. A 1.4 Kb fragment was expected for the wild type strain, and a 2.4 Kb fragment was expected for the disrupted strain (according to the integration of *HIS3* in the genome). The analysis revealed fragments with the expected size for both strains (Figure 2.7).

S. cerevisiae W303 *pep4*Δ::*HIS3*



1 - genomic DNA from *S. cerevisiae* W303

2 - genomic DNA from *S. cerevisiae* W303 *pep4*Δ::*HIS3*

Figure 2.7.

Confirmation of *PEP4* disruption by PCR. PCR products using genomic DNA from wild type (W303) or *pep4*Δ mutant cells, and the primers PEP4-N and PEP4-C, were analysed in a 0.8% agarose gel. The expected fragment sizes for the disrupted strain and for the wild type strain are indicated. The molecular weight marker (Mw) was phage λ digested with *Hin*dIII.

2.3.4.2. CELLULAR GOWTH AND RESISTANCE TO HYDROGEN PEROXIDE OF *doa4*Δ AND *pep4*Δ MUTANTS

The effect of the disruption of *DOA4* or *PEP4* genes regarding H_2O_2 resistance or resumption of cellular growth during recovery of yeast cells from the exposure to 1.5 mM H_2O_2 for 30 minutes was analysed. In wild type strain, 55.8 ± 1.9 cells survived to H_2O_2 stress, and similar results were obtained with *doa4*Δ or *pep4*Δ mutants (data not shown). In addition, the resumption of cellular growth after H_2O_2 stress was similar in wild type and *pep4*Δ cells, whereas that of *doa4*Δ cells occurred at a slower rate (Figure 2.8).

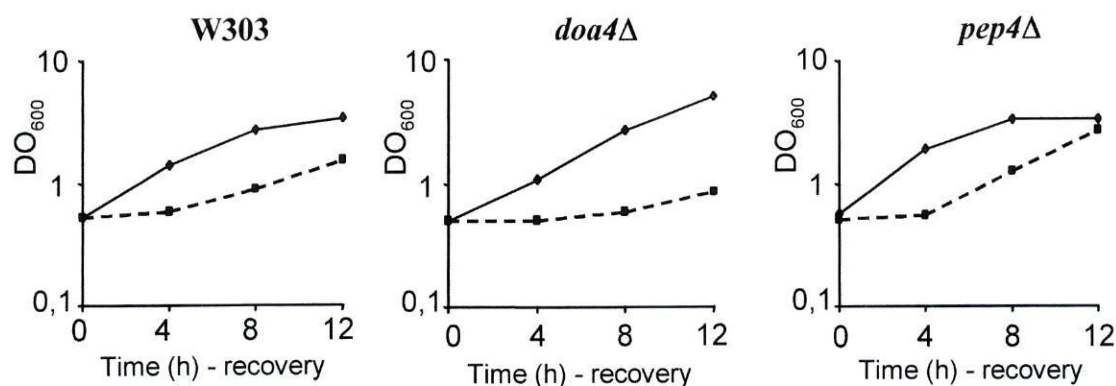


Figure 2.8.

Cellular growth during recovery of *S. cerevisiae* W303 *doa4*Δ and *pep4*Δ mutant cells from H_2O_2 stress. Yeast cells were grown to the exponential phase ($OD_{600} \approx 0.6$). Control (straight line) and cells treated with 1.5 mM H_2O_2 for 30 min (stripped line) were centrifuged, ressuspended in minimal medium without H_2O_2 and allowed to recover up to 12 hours. Cell density was determined. A representative experiment is shown (out of three independent experiments with similar results).

2.3.4.3. PROTEIN CARBONYLS IN *doa4*Δ AND *pep4*Δ MUTANTS

To identify the main proteolytic system involved in the degradation of oxidised proteins, carbonyl content was assayed during recovery of *doa4*Δ and *pep4*Δ mutants from a 30 minutes exposure to 1.5 mM H_2O_2. Consistent with previous studies (Inai and Nishikimi, 2002), in the wild type strain protein carbonylation increased during exposure to 1.5 mM H_2O_2 and decreased as cells were allowed to recover (Figure 2.9). In the *doa4*Δ mutants, both basal (data not shown) and H_2O_2-induced carbonyl levels were similar to wild type, and the decrease of carbonyl content after removal of H_2O_2 occurred at similar rate (Figure 2.9A). Notably, the disruption of *PEP4* gene increased the basal levels of protein carbonyls (146 ± 14%, $p<0.001$; see also Fig. 2.9B). Nevertheless, H_2O_2-induced protein carbonylation in the pep4Δ mutants was of the same order of magnitude compared with the wild type strain. However, the decrease of carbonyl content during cellular recovery occurred at lower rate (Figure 2.9A and B). It was previously shown that H_2O_2 induces specific protein carbonylation, and glyceraldehyde-3-phosphate dehydrogenase (Tdh) is a major target inactivated by H_2O_2 (Cabiscol *et al.*, 2000; Costa *et al.*, 2002). The analysis of carbonyls in proteins separated by 1D-gel electrophoresis showed that the effect of Pep4p deficiency was not specific, being similar for Tdh and other proteins oxidised during H_2O_2 stress (Figure 2.9B).

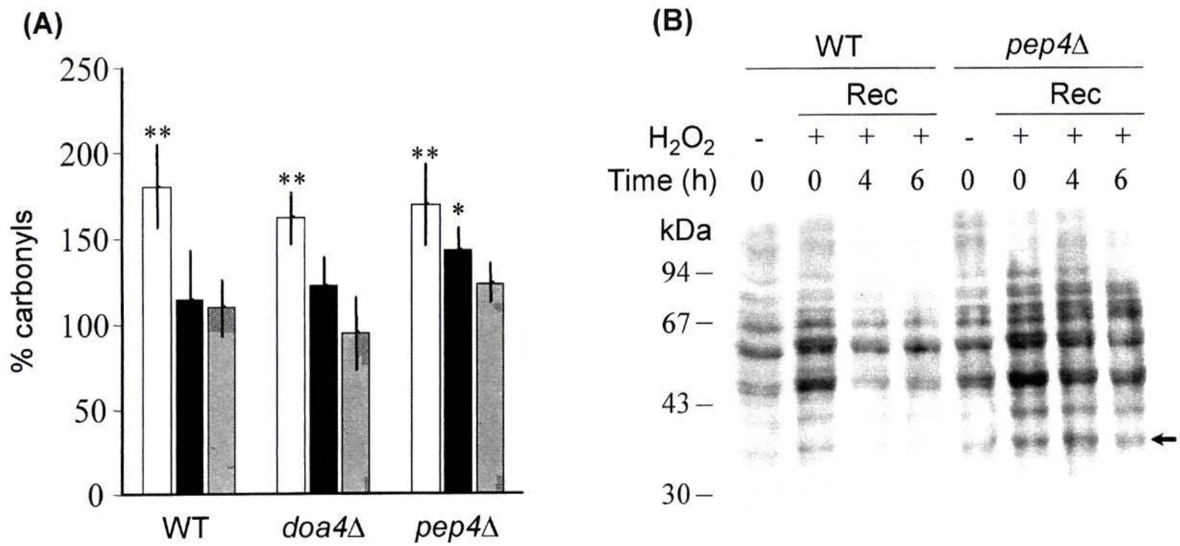


Figure 2.9.

Protein carbonyl content during recovery of *doa4*Δ and *pep4*Δ mutants from H_2O_2 stress. *S. cerevisiae* W303 (WT), *doa4*Δ and *pep4*Δ cells were treated with 1.5 mM H_2O_2 for 30 min (open bars) and allowed to recover (Rec) in H_2O_2 -free minimal medium for 4h (closed bars) or 6 h (stripped bars). Proteins were isolated and derivatised with DNPH. (A) Proteins were slot-blotted into a PVDF membrane. Immunodetection was performed using anti-DNP antibodies as described in Methods. Quantitative analysis of carbonyls was performed by densitometry using data taken from the same membrane. Values are means ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ (H_2O_2 (t0) or recovery (t4, t6) *vs* control). The basal levels of protein carbonyls in wild type and *doa4*Δ cells were similar, but increased to 146 ± 14% in the *pep4*Δ mutants ($p<0.001$) (data not shown). (B) Proteins from WT and *pep4*Δ cells were derivatised with DNPH, separated by SDS-PAGE and blotted into a Hybond-ECL membrane. Immunodetection was performed using anti-DNP antibodies as described in Methods. Tdh band, identified using a rabbit anti-Tdh antibody, is indicated with an arrow.

2.4. DISCUSSION

The accumulation of oxidised molecules is a characteristic feature of cells facing an oxidative stress. To recover, cells have to repair or degrade the damaged molecules. Aiming to study the molecular mechanisms involved in the recovery from oxidative damage, changes in gene expression at the genome-wide level were analysed during recovery from H_2O_2 stress.

Most of the genes specifically repressed were related to aminoacid (arginine, leucine, lysine, aromatic and branched-chain amino acids) and protein biosynthesis (Table 2.7). The reduced expression of these genes, together with the repression of genes related with aminoacid uptake (Gap1p) and transcription, was correlated with growth arrest (Figure 2.1A). It is likely that these changes help to preserve mass and energy to repair activities that are critical to restore the internal homeostasis. Indeed, H_2O_2 depletes the cell ATP pool (Osorio *et al.*, 2003) and the induction of genes associated with ATP production in the mitochondrial respiratory chain (*COX18*) and ATP/ADP exhange (*PET10*) can contribute to restore the ATP levels. Superoxide radicals are released from the respiratory chain as by-products of aerobic metabolism and converted into H_2O_2 by superoxide dismutases. In the presence of metal ions, H_2O_2 can be reduced into the highly reactive hydroxyl radicals via the Fenton reaction (Halliwell and Gutteridge, 1999). The increased expression of genes related with scavenging of reactive oxygen species (*YML125C*), control of redox potential (several dehydrogenases) and ionic homeostasis (*SLF1* and *FRE1*) during recovery from H_2O_2 stress (Table 2.7), probably aims to decrease the levels of reactive oxygen species and restore the redox homeostasis.

Regarding carbohydrate metabolism, it is clear that the induction of hexokinase 1 results in an increased synthesis of glucose-6-phosphate. As pyruvate kinase (*CDC19*) and *TYE7*, a positive regulator of glycolysis, were downregulated (Table 2.6), the rate of glycolysis remains low, which is consistent with the decreased rate of glucose consumption in the first hours of recovery (Figure 2.1B). The slow rate of glycolysis is associated with the inactivation of glyceraldehyde-3-phosphate dehydrogenase by oxidation of Tdh2p and Tdh3p isozymes (Grant *et al.*, 1999; Cabiscol *et al.*, 2000; Costa *et al.*, 2002). In contrast, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are not inhibited and the pool of glucose-6-phosphate is utilized to generate NADPH in the pentose-phosphate pathway (Shenton and Grant, 2003).

The repair and degradation of oxidised molecules, together with *de novo* biosynthesis to replace them, require ATP and NADPH and play a key role in cellular recovery from oxidative stress. Indeed, H_2O_2 induces lipid peroxidation and NADPH provides the reducing power for glutathione / glutathione peroxidases and thioredoxin / thioredoxin peroxidases

systems that reduce lipid hydroperoxides (Moradas-Ferreira and Costa, 2000), preventing lipid fragmentation and, therefore, cell lysis. In addition, NADPH is required for lipid biosynthesis and, in agreement, genes associated with ergosterol (*ERG13* and *ERG5*), GPI anchor (*GPI12*), phosphatidylcholine (*OPI3*) and sphingolipids (*ACB1* and *YDC1*) biosynthesis were induced during cellular recovery after H_2O_2 stress (Table 2.6).

The expression of genes encoding enzymes involved in DNA repair (*MAG1*) and genes associated with nucleotide metabolism (*RNR4*), cell cycle and DNA processing (*RHC21* and *NAP1*), rRNA and tRNA transcription (*TFC4* and *RPC31*), and cytoskeleton organization (*APP1*) also increased.

Genes associated with proteolysis were also specifically induced, namely genes encoding the ubiquitin-activating enzyme Uba1p, the ubiquitin-conjugating enzymes Ubc1p and Rad6p, one component of the proteasome regulatory particle, and several subunits of the 20S core proteasome. The enhanced proteolytic activity during recovery leads to an increased pool of amino acids, and was associated with the specific upregulation of 5 genes encoding enzymes of the amino acid catabolic pathway (Table 2.6). In addition, genes related with aminoacid uptake and biosynthesis or protein synthesis were downregulated (Table 2.7). The induction of molecular chaperones and auxiliary cochaperones aims to assist proteins unfolded by oxidative modifications. Besides, chaperones also have an important role in transcriptional regulation, chromatin structure and assembly of actin and tubulin (Martin, 2000; Trott and Morano, 2003). In agreement, the expression of genes encoding proteins with chaperone activity (*AHA1* and *CCT4*) was induced.

The proteasome has been implicated as the main pathway involved in the degradation of oxidised proteins. However, experimental evidences suggest that the 20S proteasome, rather than the ubiquitin-26S proteasome pathway, is responsible for the degradation of oxidised proteins: ubiquitin-activating and conjugating enzymes are inhibited by oxidative stress; purified 20S proteasome preferentially degrades oxidised proteins in an ATP- and ubiquitin-independent manner (Shringarpure *et al.*, 2003). Recent studies using yeast cells also support this hypothesis (Inai and Nishikimi, 2002). The results showed that, although the resumption of cellular growth after H_2O_2 stress occurred at a slower rate, the rate of turnover of oxidised proteins was not affected in Doa4p deficient cells (Figure 2.9) that contain reduced levels of free ubiquitin (Swaminathan *et al.*, 1999). Thus, these results rule-out the involvement of the 26S-proteasome in the proteolysis of oxidised proteins.

It has been suggested in higher eukaryotes that lysosomal proteases may operate sequentially or in parallel with the proteasomal system to degrade certain oxidatively modified proteins (Dunlop, 2002). In this study, it is reported the induction of *PEP4* and *LAP4* vacuolar protease genes, both during exposure to H_2O_2 and during recovery from oxidative stress (Table 2.5), that was correlated with the specific induction of genes associated with

protein traffic into the vacuole: *YKS5*, *YKT6* and *MVP1*. Consistent with a role of vacuolar proteolysis in cellular recovery from oxidative damage, protein carbonyl content decreased at lower rate in *pep4*Δ mutants (Figure 2.9). Furthermore, the level of oxidised proteins was constitutively higher in *pep4*Δ mutants, indicating that Pep4p plays a major role in the turnover of proteins oxidised by endogenously generated or exogenously added reactive oxygen species.

In summary, these studies show that cell recovery from H_2O_2 stress involves global reprogramming of gene expression, and includes major changes in the expression of genes encoding chaperones, DNA and lipid repair enzymes, and proteins involved in cell rescue and defence, transcription, redox control, ionic homeostasis and cell wall, and cytoskeleton organization. The reprogramming of these metabolic pathways and cell functions permits the yeast cells to resume growth. In addition, the results provide evidences of the critical role of the vacuolar protease Pep4p in the turnover of proteins oxidised by H_2O_2 or ROS generated as by-products of normal aerobic metabolism.

CHAPTER 3

Dynamic of glucose-6-phosphate dehydrogenase (Zwf1) and glyceraldehyde-3-phosphate dehydrogenase (Tdh) during recovery of Saccharomyces cerevisiae cells from hydrogen peroxide stress

3.1. INTRODUCTION

In yeast, it was previously shown that H_2O_2 oxidises methionine and cysteine residues. Methionine sulphoxide reductase reduces methionine sulphoxide (MetSO) groups (Moskovitz *et al.*, 1997), while protein-Cys-SOH (cysteine-sulphenic acid) derivatives are readily S-thiolated with reduced glutathione. Protein S-glutathionilation was described for glycolytic enzymes, including glyceraldehyde-3-phosphate dehydrogenase Tdh3p isoenzyme, as well as for translation factors and the 20S proteasome. In most cases, it affects enzyme activity, but prevents the irreversible oxidation of cysteine residues to the Cys-SO_2H (cysteine sulphinic acid) and –SO_3H (cysteic acid) forms (Grant *et al.*, 1999; Demasi *et al.*, 2003; Shenton and Grant, 2003). Indeed, S-glutathionilation of the Tdh3p isoenzyme is correlated with the capacity of yeast cells to restore Tdh3p activity after removal of H_2O_2, by a mechanism that involves dethiolation mediated by the monothiol glutaredoxin Grx5p (Shenton *et al.*, 2002). In agreement, the Tdh2p isoform is not S- thiolated and its activity is only partially restored during recovery from H_2O_2 stress (Grant *et al.*, 1999), and Tdh is irreversibly inhibited in yeast cells deficient in glutathione (Shenton and Grant, 2003). It was recently shown that cysteine-sulphinic acid derivatives can be reduced by sulphiredoxin, a highly conserved protein that is important for the antioxidant function of peroxiredoxins (Biteau *et al.*, 2003).

H_2O_2 also induces specific protein carbonylation, probably due to its conversion into hydroxyl radicals, catalysed by redox active metal ions, such as copper and iron (Halliwell and Gutteridge, 1999). Glyceraldehyde-3-phosphate dehydrogenase was also identified as a major target oxidatively carbonylated during H_2O_2 stress (Cabiscol *et al.*, 2000; Costa *et al.*, 2002). Proteins irreversibly inactivated by formation of carbonyl derivatives are targeted to proteolytic pathways. Indeed, the degradation of carbonylated proteins depends both on the 20S proteasome (Inai and Nishikimi, 2002) and the vacuolar protease Pep4 (see Chapter 2).

The inactivation of glycolytic enzymes favours the production of NADPH via the pentose phosphate pathway. In agreement, the pentose phosphate pathway plays an essential role in oxidative stress resistance, namely glucose-6-phosphate dehydrogenase (Zwf1p), which appears to play important roles in the adaptive response to H_2O_2 besides providing metabolic intermediates (Izawa *et al.*, 1995, 1998; Kuge and Jones, 1994; Morgan *et al.*, 1997).

In this study, Zwf1p and Tdh activity, as well as Tdh degradation / synthesis *de novo* and carbonyl content were analysed during cellular recovery from H_2O_2 stress.

3.2. MATERIALS AND METHODS

3.2.1. YEAST STRAINS

The strains of *S. cerevisiae* used in this study are listed in table 3.1.

Table 3.1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Reference/Source
W303	Matα, *ade2-1*, *can1-100*, *trp1-1*, *ura3-1*, *his3-11,15*, *leu2-3,112*	Wallis *et al.*, 1989
By4741	Matα; $his3\Delta_1$, $leu2\Delta_0$, $met15\Delta_0$, $ura3\Delta_0$	EUROSCARF
tdh2Δ	[By4741] *tdh2*Δ::*KanMx4*	EUROSCARF
tdh3Δ	[By4741] *tdh3*Δ::*KanMx4*	EUROSCARF

3.2.2. GROWTH CONDITIONS, HYDROGEN PEROXIDE TREATMENT AND CELL VIABILITY

Yeast cells were grown as described in section 2.2.2. Hydrogen peroxide stress and recovery after stress were performed as described in 2.2.3. Cell viability was determined as described in 2.2.3.

3.2.3. ENZYME ACTIVITIES

Yeast extracts were prepared in 100 mM potassium phosphate buffer (pH 7.4) containing a protease inhibitor cocktail (Complete^{TM}, Mini, EDTA-free Protease Cocktail Inhibitor Tablets, Boehringer Mannhein), by vigorous shaking of the cell suspension, in the presence of glass beads, for 5 min. Short pulses of 1 min were used, with 1 min intervals on ice. Protein content was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Glucose-6-phosphate dehydrogenase activity was assayed by incubating 25 μg of total cytoplasmic proteins with 0,5 mmol/L $NADP^+$ in 100 mM phosphate buffer (pH 7.4). The reaction was initiated by addition of 0,7 mmol/L glucose-6-phosphate and the enzymatic

activity was determined spectrophotometrically at 340 nm, by monitoring the reduction of $NADP^+$ at 24°C, according to the manufactory instructions (Glucose-6-phosphate dehydrogenase Kit, 345-B, Sigma Diagnostics), and expressed as U (mg protein)$^{-1}$ or as percentage of control.

Glyceraldehyde-3-phosphate dehydrogenase activity was assayed using 80 mM phosphate buffer (pH 7.4) containing 10 mM EDTA, 1 mM β-NAD^+, 0.1 mM DTT, 8 mM frutose-1,6-biphosphate and 0.03 U/ml aldolase. 25 μg of total cytoplasmic proteins were used for the enzymatic assay. Tdh activity was determined spectrophotometrically at 340 nm, by monitoring the reduction of NAD^+ at 24°C, and expressed as U (mg protein)$^{-1}$ or as percentage of control (modified from Holland and Weshead, 1973).

3.2.4. NORTHERN-BLOT

RNA was isolated by the acid phenol method (Ausubel *et al.*, 1998). Total RNA (30 μg) was denaturated with glyoxal and dimethyl sulphoxide, blotted by capillarity into Hybond membranes (Amersham Biosciences Europe, GmbH) and probed as described (Ausubel *et al.*, 1998). The membrane was washed with 6x SSC for 5 min and fixed at 80°C for 2 hours. Filters were pre-hybridised for 3 hours at 65ºC, using hybridization buffer (5x SSC, 5x Denhardt, 1% SDS, 0.1 mg/ml Herring Sperm DNA). After pre-hybridization, 100 ng of denaturated and radiolabelled probes were added. The following probes were used: a 1 Kb *Hin*dIII-*Eco*RI fragment of *ACT1* gene, a 2 Kb *Hin*dIII-*Hin*dIII fragment of *TDH1* gene and 3.8 Kb *Cla*I-*Cla*I fragment of *ZWF1* gene. *ACT1* gene (Gallwitz and Sures, 1980) was used as RNA loading control. Probes were labelled with [α^{32}]P-dCTP using the Multiprime DNA Labelling System Kit (Amersham RPN 1601Z) or using the ECL™ random prime labelling system, version II (Amersham RPN 3040). The membrane was exposed to an X-ray film (Kodak), at -70°C. Alternatively, a digital image was obtained using a Molecular Imager (Typhoon, Amersham). Band intensities were evaluated by densitometry.

3.2.5. PROTEIN DEGRADATION AND TDH IMMUNODETECTION

Yeast cells (5 ml / sample) were incubated with 10μCi/ml [^{35}S]-methionine for 30 min, centrifuged and ressuspended in 5 ml of minimal medium supplemented with methionine. *S. cerevisiae* By4741 and its isogenic strains were incubated in minimal medium without methionine for 20 min, before labelling. Yeast extracts were prepared as described in 2.2.5.

Proteins (15 μg) were separated by SDS-PAGE, using the method described by Laemmli *et al.* (1970), and visualized by silver staining (O'Connell and Stults, 1997), or exposed to an X-ray film (Kodak). For Tdh immunodetection, proteins were blotted into a nitrocellulose membrane (Hybond C, Amersham Biosciences Europe GmbH) and probed with rabbit IgG anti-Tdh at a 1:2500 dilution, as the primary antibody, and goat anti-rabbit IgG-peroxidase (Sigma, St. Louis, MO, USA) at a 1:5000 dilution, as the secondary antibody. Immunodetection was performed by chemiluminescence, using a kit from Amersham (RPN 2109). The membranes were exposed to a Hybond-ECL film (Amersham Biosciences Europe GmbH) for 15 seconds to 1 min, and the film was developed. Band intensities were quantified by densitometry.

3.2.6. PROTEIN CARBONYLATION ANALYSIS

Yeast extracts were prepared as described in 2.2.5. Proteins were derivatised with 2,4-dinitrophenylhydrazine and carbonylated proteins were analysed by 1D-gel or Slot blot, as described in 2.2.9. For 2D analysis, an aliquote of each sample (100 μg proteins) was mixed with 1ml of 10 mM 2.4-dinitrophenylhydrazine in 2M HCl, and incubated 60 min at room temperature, in the dark. Proteins were precipitated with trichloroacetic acid (10% v/v, final concentration), 10 min on ice. The protein pellets, obtained after centrifugation at 14000 rpm for 5 min, were washed twice with 1 ml of ethanol:ethyl acetate (1:1), and once with acetone, and solubilised as described in 2.2.5. Sample preparation, isoelectric focusing and second-dimension (SDS-PAGE) were performed as described in 2.2.5. Following 2D-gel electrophoresis, proteins were visualised by silver staining, or blotted into Hybond-C. Immunodetection of carbonylated proteins was performed using a rabbit IgG anti-DNP, as described in 2.2.9.

3.3. RESULTS

3.3.1. GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY AND *ZWF1* EXPRESSION DURING RECOVERY FROM HYDROGEN PEROXIDE STRESS

Aiming to elucidate the importance of NADPH production for cell recovery from H_2O_2 stress, glucose-6-phosphate dehydrogenase (Zwf1p) activity was analysed in cells exposed to 1.5 mM H_2O_2 for 30 min and during recovery. The activity Zwf1p was not affected by exposure to H_2O_2, but increased during cell recovery Figure 3.1(A). The analysis of *ZWF1* expression showed that mRNA-*ZWF1* levels decreased 50% after exposure to H_2O_2, and increased during the first 2 hours of recovery, becoming similar to control after 4 hours (Figure 3.1(B, C)). These results suggest that NADPH generated in the pentose phosphate pathway may indeed be needed for cellular recovery, namely for the activity of antioxidant defences (*e.g.* for glutathione reductase), and to the biosynthetic processes, necessary to replace oxidised molecules.

3.3.2. GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ACTIVITY AND *TDH* EXPRESSION AND TURNOVER DURING RECOVERY FROM HYDROGEN PEROXIDE STRESS

In contrast to glucose-6-phosphate dehydrogenase, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Tdh) was identified as a major target inactivated during exposure to H_2O_2, both by S-glutathionilation and carbonylation (Grant *et al.*, 1999; Cabiscol *et al.*, 2000). Protein carbonylation, in contrast to S-glutathionilation, is irreversible. Thus, Tdh dethiolation contributes, but may be insufficient to fully restore Tdh activity after H_2O_2 has been removed. The mechanisms associated with the recovery of Tdh activity after H_2O stress were assessed. Our data shows that Tdh activity increased almost to control levels during cellular recovery (Figure 3.2), and this increase is mediated by different mechanisms. Indeed, Tdh dethiolation *in vitro* by treating cellular extracts from cells exposed to H_2O_2 with the reducing agent DTT (25mM), led to a partial increase of Tdh activity - from 41.1 ± 0.7 to 56.6 ± 6.0 % of untreated cells ($p<0.01$). Therefore, the fully recovery of Tdh activity may also involve protein degradation and synthesis *de novo*.

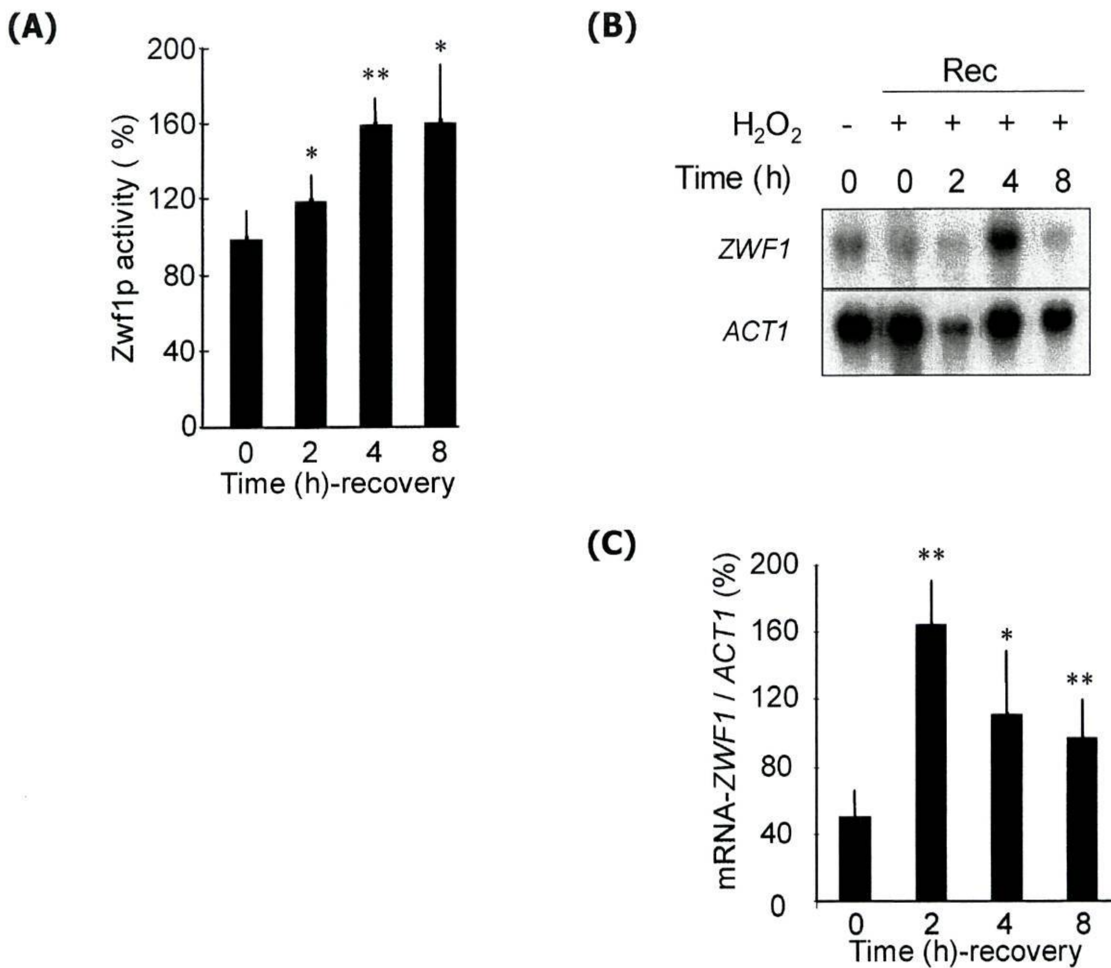


Figure 3.1.

Glucose-6-phosphate dehydrogenase (Zwf1p) activity and mRNA-*ZWF1* levels during recovery of *S. cerevisiae* cells from H_2O_2 stress. (A) *S. cerevisiae* W303 cells treated with 1.5 mM H_2O_2 for 30 min were allowed to recover in H_2O_2-free minimal medium up to 8 hours. Zwf1p activity was determined as described in methods and expressed as % activity of untreated cells. Basal Zwf1p activity was 0.381 ± 0.033 U (mg protein)$^{-1}$. Zwf1p activity in untreated cells remained unchanged up to 8 hours. Values are means ± SD of three independent experiments. *$p<0.05$, **$p<0.01$. (B) mRNA-*ZWF1* levels in yeast cells exposed to 1.5 mM H_2O_2 for 30 min and allowed to recover (Rec) in H_2O_2-free minimal medium up to 8h were analysed by Northern Blot. A representative experiment is shown. (C) mRNA-*ZWF1* band intensities were quantified, corrected for *ACT*1 (RNA loading control) and expressed as % of untreated cells. Values are means ± SD of three independent experiments. *$p<0.05$, **$p<0.01$.

To confirm this hypothesis, Tdh activity was assayed in cells allowed to recover up to 6 hours, either in the absence or in the presence of cycloheximide (100 μg/ml). The results obtained indicate that the recovery of Tdh activity decreased in the presence of

cycloheximide: Tdh activity increased 1.90- and 1.39- fold, respectively, in the absence and presence of cycloheximide (Figure 3.2). It should be noted that the activity of Tdh restored *in vivo* in the presence of cycloheximide after 6 h of recovery and *in vitro* by dethiolation mediated by DTT, was similar.

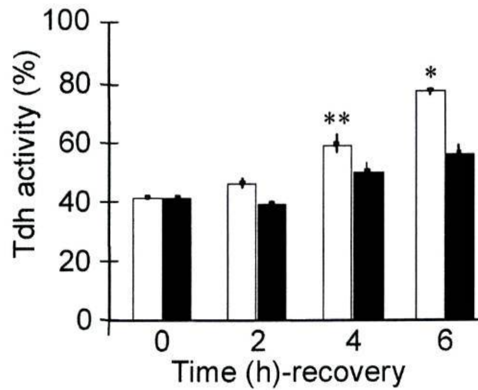


Figure 3.2.

Glyceraldehyde-3-phosphate dehydrogenase (Tdh) activity during recovery of *S. cerevisiae* cells from H_2O_2 stress. *S. cerevisiae* W303 cells treated with 1.5 mM H_2O_2 for 30 min were allowed to recover in H_2O_2-free minimal medium up to 6 h, in the presence (closed bars) or absence (open bars) of cycloheximide (100 μg/ml). Tdh activity was determined as described in methods and expressed as % activity of untreated cells. Basal Tdh activity was 0.246 ± 0.051 U(mg protein)$^{-1}$. Tdh activity in untreated cells remained unchanged up to 6 h. Values are means ± SD of three independent experiments. *$p<0.05$, **$p<0.01$ (± cycloheximide *vs* t_o of recovery).

The analysis of *TDH* gene expression showed that mRNA-*TDH* levels were not affected by H_2O_2 and slightly decreased during the first 2 hours of recovery, becoming similar to control after 4 hours (Figure 3.3). Thus, synthesis *de novo* of Tdh contributed to the recovery of Tdh activity, and this increase cannot be accounted from the up-regulation of *TDH* genes, as already suggested by proteome and transcriptome analysis (Chapter 2).

Protein degradation was assessed following the fate of [^{35}S]-methionine newly labelled proteins during recovery from H_2O_2 stress. The results obtained show that 25% of [^{35}S]-Tdh was degraded after 6 hours of recovery, whereas total Tdh protein levels remained identical (Figure 3.4).

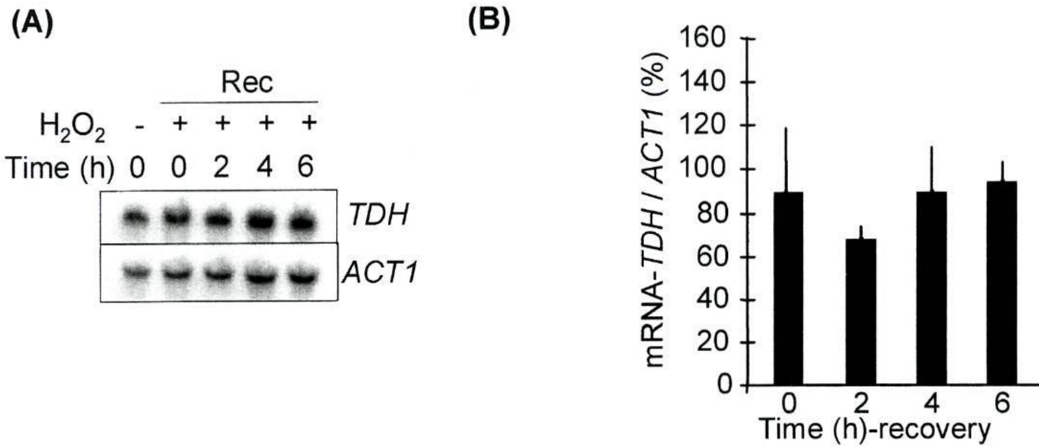


Figure 3.3.

***TDH* expression during recovery of *S. cerevisiae* cells from H_2O_2 stress.** (A) mRNA-*TDH* levels in yeast cells exposed to 1.5 mM H_2O_2 for 30 min and allowed to recover (Rec) in H_2O_2-free minimal medium up to 6 h were analysed by Northern Blot. A representative experiment is shown. (B) mRNA-*TDH* band intensities were quantified, corrected for *ACT1* (RNA loading control) and expressed as % of untreated cells. mRNA-*TDH* levels in untreated cells remained unchanged up to 6 h. Values are means ± SD of three independent experiments.

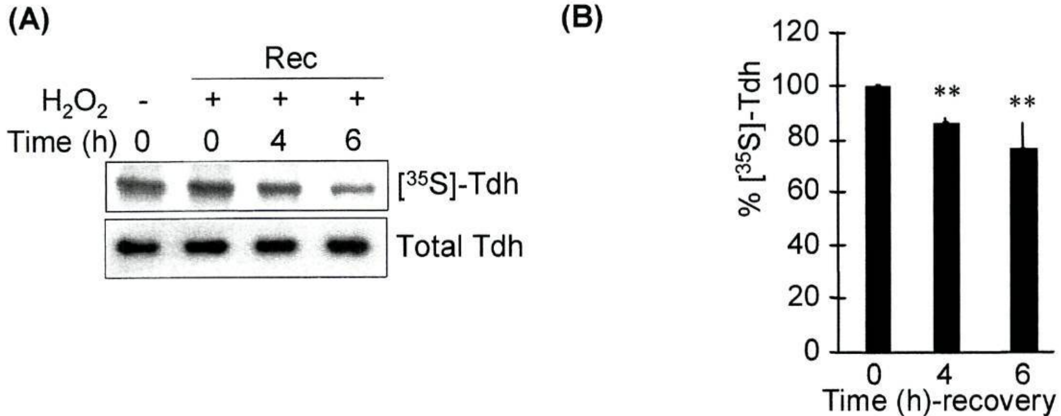


Figure 3.4.

Glyceraldeyde-3-phosphate turnover during recovery of *S. cerevisiae* from H_2O_2 stress. *S. cerevisiae* By4741 cells were labelled with [^{35}S] methionine, exposed to 1,5 mM H_2O_2 for 30 min, and allowed to recover (Rec) in H_2O_2-free minimal medium supplemented with methionine up to 6 h. A) Turnover of [^{35}S]-Tdh and immunodetection of Tdh. For protein degradation analysis, proteins (15 µg) were separated by SDS–PAGE and exposed to an X–ray film. Tdh band is shown. For immunodetection, proteins (2.5 µg) were separated by SDS-PAGE, and blotted into a nitrocellulose membrane. Tdh was detected using a rabbit IgG anti-Tdh polyclonal antibody. A representative experiment is shown. B) Quantification of [^{35}S]-Tdh band intensities from labelled cells treated with 1.5 mM H_2O_2 for 30 min and allowed to recover in H_2O_2-free minimal medium supplemented with methionine up to 6 hours. Values are means ± SD of three independent experiments. **$p < 0.01$.

3.3.3. CONTRIBUTION OF TDH2 AND TDH3 GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ISOZYMES TO THE RECOVERY OF TDH ACTIVITY AFTER HYDROGEN PEROXIDE STRESS

Glyceraldehyde-3-phosphate dehydrogenase is encoded by three genes designated *TDH1*, *TDH2* and *TDH3*, which encode structural and functional related polypeptides. In the conditions that these experiments were performed, only Tdh2p and Tdh3p isozymes are detected, as Tdh1p is only synthesized in stationary phase or heat-shocked cells (Boucherie *et al.*, 1995). Tdh3p, in contrast with Tdh2p, is S-thiolated during oxidative stress, and Tdh3p S-thiolation seems to have a protective effect (Grant *et al.*, 1999). However, both Tdh2p and Tdh3p are carbonylated (Costa *et al.*, 2002). To analyse if the lack of Tdh2p S-thiolation affects protein turnover, studies were performed, using *tdh2Δ* and *tdh3Δ* mutant strains.

To analyse if the cellular recovery after a H_2O_2 stress was affected by disruption of *TDH2* or *TDH3* genes, *tdh2Δ* and *tdh3Δ* mutant cells were treated with H_2O_2 and allowed to recover in H_2O_2-free minimal medium. The disruption of either *TDH2* or *TDH3* gene did not affect H_2O_2 stress resistance (data not shown). In addition, cell growth during recovery was similar in the wild type strain and in the *tdh2Δ* and *tdh3Δ* mutants (Figure 3.5).

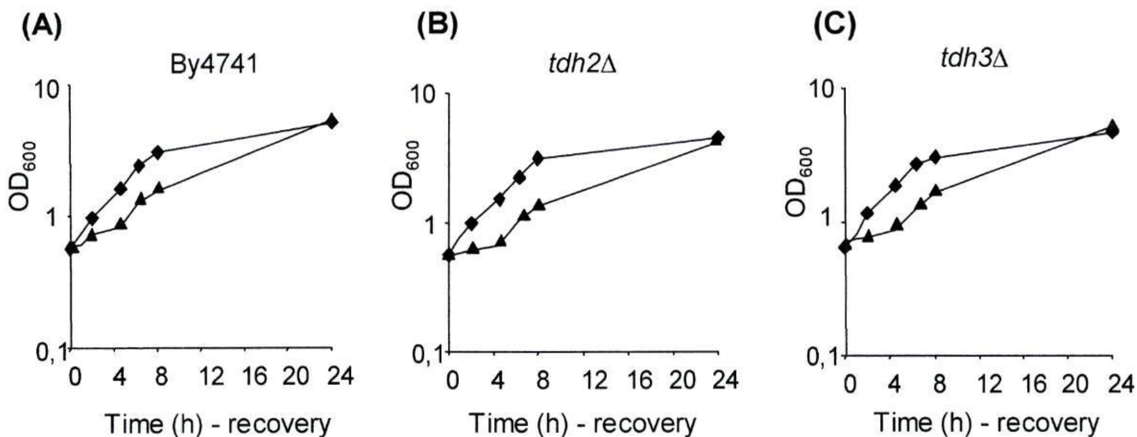


Figure 3.5.

Cellular growth during recovery of *S. cerevisiae* *tdh2Δ* and *tdh3Δ* mutant cells from H_2O_2 stress. *S. cerevisiae* By4741 (wild type) (A), *tdh2Δ* (B) and *tdh3Δ* C) mutant cells were grown to the exponential phase ($OD_{600} \approx 0.6$). Control (♦) and cells treated with 1.5 mM H_2O_2 for 30 min (▲) were centrifuged and ressuspended in minimal medium without H_2O_2. Cell density was determined during cellular recovery up to 24h. A representative experiment is shown (out of three independent experiments with similar results).

Tdh activity was analysed during recovery of *tdh2Δ* and *tdh3Δ* mutant strains from H_2O_2 stress. Exposure to H_2O_2 resulted in a different degree of enzyme inhibition of Tdh2p and Tdh3p, 72% and 58%, respectively (Figure 3.6). After 4 hours of recovery, Tdh3p activity was almost completely restored, while Tdh2p activity was partially restored and only exhibited values similar to control levels after 6 hours of recovery.

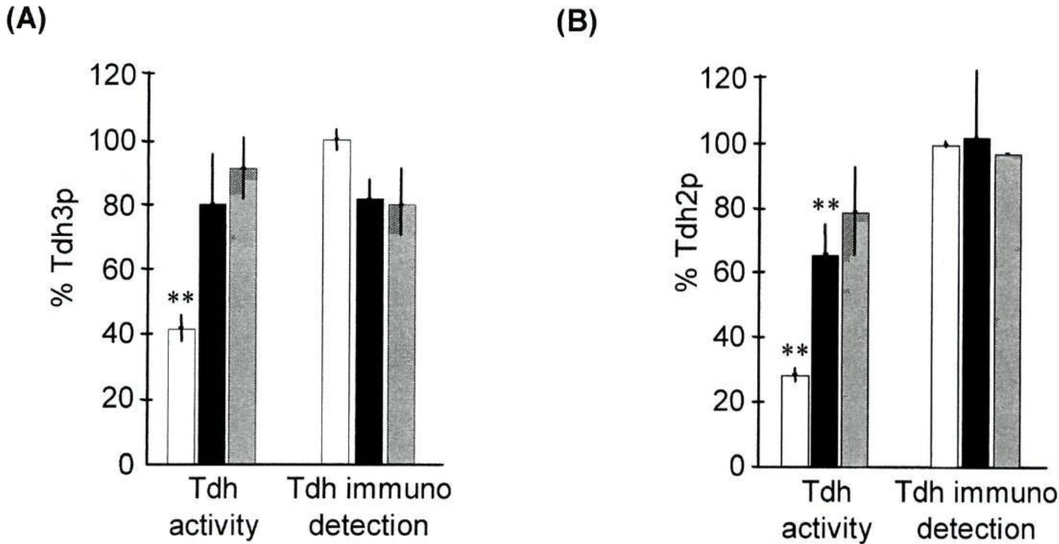


Figure 3.6.

Tdh2p and Tdh3p activity during recovery of *Saccharomyces cerevisiae* cells from H_2O_2 stress. *S. cerevisiae tdh2Δ* (A) and *tdh3Δ* (B) mutant cells were treated with 1.5 mM H_2O_2 for 30 min (open bars) and allowed to recover in H_2O_2-free minimal medium for 4h (closed bars) and 6h (stripped bars). Glyceraldehyde-3-phosphate dehydrogenase (Tdh) activity was expressed as percentage of control cells. Basal activity levels of *tdh2Δ* and *tdh3Δ* mutants were, respectively, 94.0 ± 6.1 % and 37.3 ± 5.5 %** of control wild type cells (By4741 strain). Total Tdh levels were analysed by immunodetection, as described in Figure 3.4, and quantified by densitometry using data taken from the same membrane. Values are means ± SD of three independent experiments. **$p < 0.01$.

The analysis of protein turnover showed that 30% of [^{35}S]-Tdh3p (in the *tdh2Δ* mutant) and [^{35}S]-Tdh2p (in the *tdh3Δ* mutant) were degraded after 6 hours of recovery (Figure 3.7), as observed in the wild type strain (Figure 3.4). In both mutants, total Tdh protein levels remained constant during recovery (Figure 3.6). These results indicate that both Tdh2p and Tdh3p may contribute to the recovery of Tdh activity in wild type cells, although Tdh2p recovered at a lower rate.

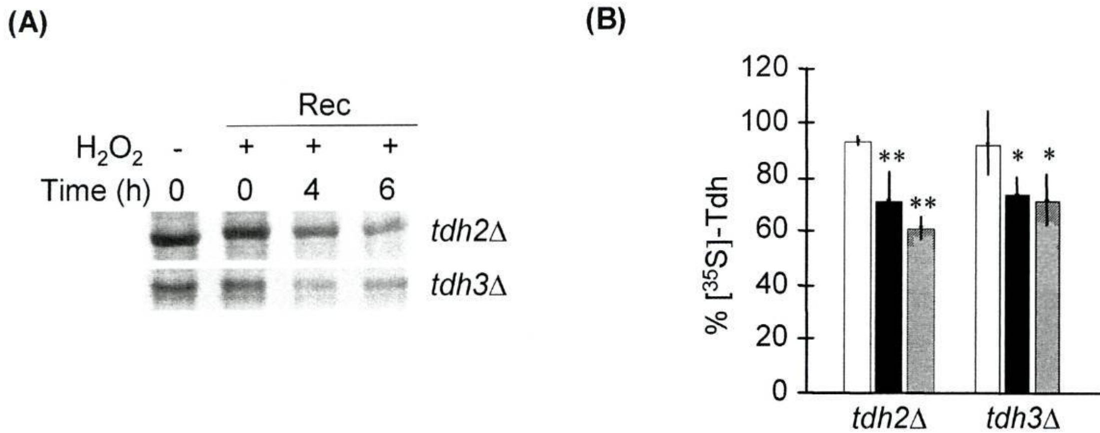


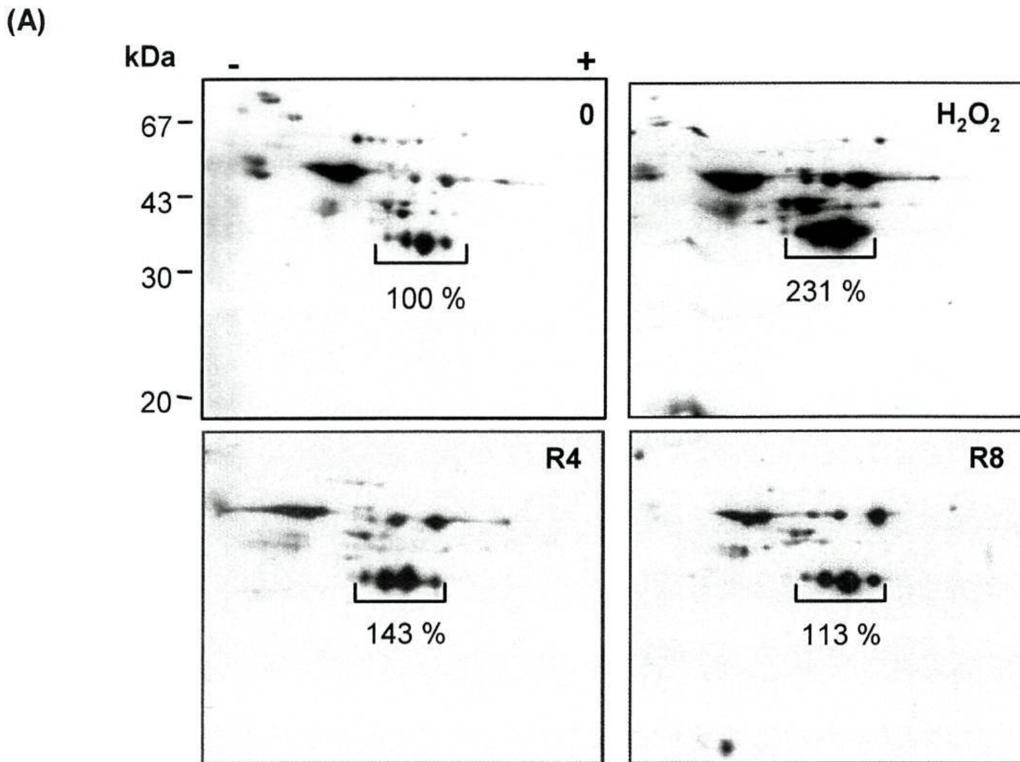
Figure 3.7.
Tdh2p and Tdh3p turnover during recovery of *Saccharomyces cerevisiae* cells from H_2O_2 stress. *S. cerevisiae* By4741 (wild type), *tdh2*Δ and *tdh3*Δ mutant cells were labelled with [^{35}S] methionine, exposed to 1.5 mM H_2O_2 for 30 min, and allowed to recover in H_2O_2-free minimal medium supplemented with methionine up to 6 h. (A) Turnover of [^{35}S]-Tdh. Proteins (15 μg) were separated by SDS–PAGE and exposed to an X–ray film. Tdh band is shown. A representative experiment is shown. (B) Quantification of [^{35}S]-Tdh band intensities from cells treated with 1.5 mM H_2O_2 for 30 min (open bars) and allowed to recover in H_2O_2-free minimal medium for 4h (closed bars) or 6 h (stripped bars). Values are means ± SD of three independent experiments. *$p<0.05$, **$p < 0.01$.

3.3.4. GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE CARBONYL CONTENT DURING RECOVERY FROM HYDROGEN PEROXIDE STRESS

To investigate if Tdh turnover involves the degradation of the carbonylated isozymes, the protein carbonyl content was analysed during recovery from H_2O_2 stress. The results obtained in wild type cells, show that the Tdh carbonyl levels increased 2.3 fold during exposure to H_2O_2, and timely decreased to normal levels during cellular recovery (Figure 3.8(A)). Besides, studies using the *tdh2*Δ and *tdh3*Δ mutant strains showed that the carbonyl content of both Tdh2p (in the *tdh3*Δ mutant) and Tdh3p (in the *tdh2*Δ mutant) increased during exposure to H_2O_2, as previously shown (Costa *et al.*, 2002), and decreased as cells were allowed to recover (Figure 3.8(B)).

Total carbonyl content was also analysed in these mutants, by slot blot assays, and the results showed that both basal and H_2O_2–induced total protein carbonyls were similar to that observed in the wild type cells. In addition, the decrease of carbonyl content after removal of H_2O_2 was similar to that observed in wild type cells (Figure 3.9). The overall results suggest that only a small fraction of Tdh molecules are irreversibly inactivated by carbonylation and subsequently targeted for proteolysis.

(A)



(B)

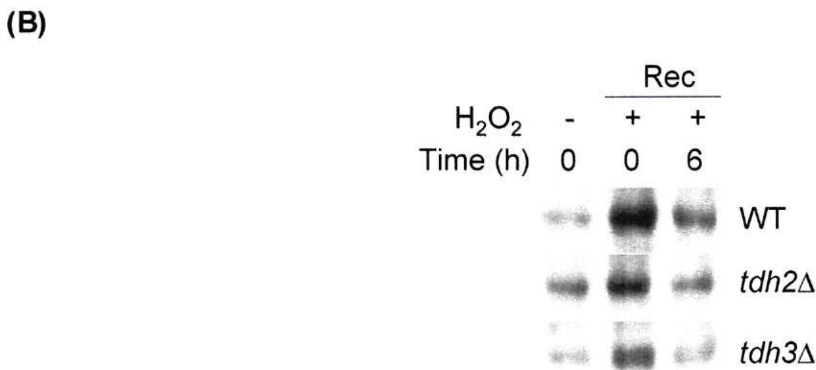


Figure 3.8.

Tdh2p and Tdh3p carbonyl content during recovery of *Saccharomyces cerevisiae* cells from H_2O_2 stress. (A) *S. cerevisiae* wild type cells were treated with 1.5 mM H_2O_2 for 30 min and allowed to recover for 4 (R4) or 8 (R8) hours. Proteins were derivatised with DNPH, separated by 2D-electrophoresis and blotted into Hybond-C. All blots were processed at the same time to allow direct comparisons. Total carbonylated Tdh was quantified and band intensities are shown. A representative experiment is shown (out of three independent experiments). (B) *S. cerevisiae* By4741 (wild type), *tdh2Δ* and *tdh3Δ* mutant cells were treated with 1.5 mM H_2O_2 for 30 min and allowed to recover (Rec) for 6 hours. Proteins were derivatised with DNPH, and slot-blotted into PVDF. Immunodetection of carbonylated proteins was performed using an anti-DNP antibody, as described in Methods. Only Tdh band is shown (Tdh2p and Tdh3p in WT cells; Tdh3p in *tdh2Δ* cells; Tdh2p in *tdh3Δ* cells). A representative experiment is shown (out of three independent experiments).

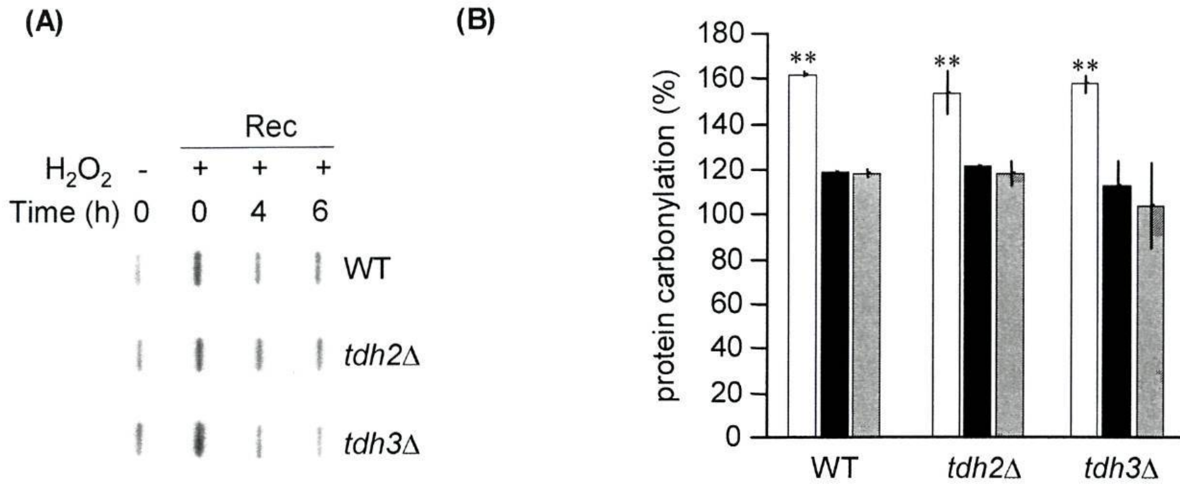


Figure 3.9. Protein carbonylation during recovery of *Saccharomyces cerevisiae* wild type, *tdh2Δ* and *tdh3Δ* mutant cells from H_2O_2 stress. *S. cerevisiae tdh2Δ* and *tdh3Δ* mutant cells were treated with 1.5 mM H_2O_2 for 30 min (open bars) and allowed to recover in H_2O_2-free minimal medium for 4h (closed bars) and 6h (stripped bars). (A) Total protein carbonyl content. Proteins derivatised with DNPH were slot-blotted into a PVDF membrane. Carbonylated proteins were detected using an anti-DNP antibody. A representative experiment is shown (out of three independent experiments). (B) Quantitative analysis of total protein carbonyl content was performed by densitometry using data taken from the same membrane. Values are means ± SD of three independent experiments. **$p < 0.01$.

3.4. DISCUSSION

The maintenance of a redox balance and the degradation of oxidised molecules is essential for cellular recovery after an oxidative challenge. It was previously shown that the pentose phosphate pathway plays a key role in cellular protection during oxidative stress, by providing NADPH for antioxidant defences (Godon *et al.*, 1998; Junhke *et al.*, 1996). Consistently, the flux of carbohydrate metabolism to the pentose phosphate pathway seems to be favoured by the down-regulation of genes encoding glycolytic enzymes (Godon *et al.*, 1998) and the oxidative inactivation of glycolytic enzymes (Costa *et al.*, 2002; Cabiscol *et al.*, 2000). Our data show that glucose-6-phosphate dehydrogenase, the rate-limiting enzyme of the pentose phosphate pathway, is not oxidatively inactivated during exposure to H_2O_2 (in agreement with Cabiscol *et al.*, 2000). Furthermore, *ZWF1* gene expression and glucose-6-phosphate dehydrogenase activity increased during cellular recovery from H_2O_2 stress. These results support a key role of this enzyme in NADPH production to restore a reducing environment. It is conceivable that NADPH is also required for *de novo* biosynthesis of fatty acids or ergosterol in order to replace oxidised lipids. In agreement, several genes of lipid metabolism are up-regulated during recovery (see Chapter 2).

The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Tdh) is a main target protein oxidatively inactivated during exposure to H_2O_2 and the decrease of Tdh activity has been attributed both to S-thiolation and carbonylation (Grant *et al.*, 1999; Cabiscol *et al.*, 2000; Costa *et al.*, 2002). Tdh S-thiolation is reversible, and Tdh dethiolation, mediated by Grx5p, is important for enzyme reactivation (Shenton *et al.*, 2002), and requires NADPH, GSH and glutathione reductase activity (Holmgren, 1990). Thus, the increase of glucose-6-phosphate dehydrogenase activity during cellular recovery from H_2O_2 stress may also be associated with Tdh dethiolation. The inactivation due to protein carbonylation is irreversible as there is no repair mechanism of carbonyls. In agreement, our results show that the recovery of Tdh activity was partially dependent on protein synthesis *de novo* (Figure 3.2), in addition to dethiolation. The requirement of protein synthesis *de novo* to restore Tdh activity after H_2O_2 stress was also recently described in *S. aureus* (Weber *et al.*, 2004). Tdh degradation and Tdh synthesis *de novo* occurred at a similar rate (Figure 3.4) and were associated with a decrease of the Tdh carbonyl content (Figure 3.8). Tdh2p and Tdh3p isozymes had similar rates of turnover and the carbonyl content of both isozymes decreased during cellular recovery (Figures 3.7 and 3.8). The turnover of carbonylated Tdh2p and Tdh3p was correlated with the restoration of enzyme activity, although Tdh2p recovered at a slower rate (Figure 3.6). As both Tdh2p and Tdh3p isozymes are carbonylated (Costa *et al.*, 2002) but only Tdh3p is S-thiolated (Grant *et al.*, 1999), these results indicate that the absence of S-thiolation does not affect protein turnover. The increase of protein carbonyls

during exposure to H_2O_2 and the capacity to turnover carbonylated protein was not affected in *tdh3Δ* mutants (Figure 3.9), indicating that protein oxidation cannot account for the increased H_2O_2 sensitivity previously observed in these mutants (Grant *et al.*, 1999).

In summary, the increase of *ZWF1* expression and Zwf1p activity supports the role of NADPH production, required for the activity of antioxidant defences. In addition, this study provide new evidence that protein turnover (degradation of oxidised protein and enzyme synthesis *de novo*) contributes to restore the activity of glyceraldehyde-3-phosphate dehydrogenase, a major target oxidatively inactivated during H_2O_2 stress. The turnover of both Tdh2p and Tdh3p isozymes was similar and associated to a decrease in carbonyl content. Both Tdh2p and Tdh3p contribute to restore Tdh activity. Consistent with these results, genes associated with aminoacid catabolism are induced, and genes related with aminoacid uptake and biosynthesis or protein synthesis are down-regulated during cellular recovery (see Chapter 2).

CHAPTER 4

Role of Isc1p in the oxidative stress resistance

4.1. INTRODUCTION

Sphingolipids are important regulatory molecules in addition to being critical structural components of cellular membranes. In *S. cerevisiae*, sphingolipids are required for viability (Dickson *et al.,* 1990), optimal life-span (Jazwinski and Conzelmann, 2002; D'mello *et al.*, 1994; Jazwinski, 1999; Shama *et al.*, 1998), and for regulation of stress responses including osmotic and low pH stress (Vaena de Avalos *et al.*, 2004; Dickson and Lester, 1999b; Jenkins and Hannun, 2001). Indeed, sphingolipids participate in regulation of the transient cell cycle arrest, control of putative signalling pathways that govern cell integrity, endocytosis, exocytosis, movement of the cortical actin cytoskeleton, vesicular transport of GPI-anchored proteins and regulation of protein breakdown (Johnston and Singer, 1980; Jenkins and Hannun, 2001; Rowley *et al.*, 1993; Grote *et al.*, 2000; Zanolari *et al.*, 2000).

S. cerevisiae cells use ceramide to make three types of complex sphingolipids. Ceramides are modified by the attachment of the polar head group, *myo*-inositol phosphate, yielding inositolphosphoceramide (IPC). IPC is mannosylated to yield mannose-IPC (MIPC) and the addition of inositol phosphate to MIPC yields $M(IP)_2C$ (reviewed in Le Stunff *et al.*, 2002). Yeast *ISC1* encodes inositolphosphosphingolipid-phospholipase C (IPS-PLC), which hydrolyses these inositol phosphosphingolipids (IPS), releasing ceramide and the polar head group. Cells deficient in Isc1p are viable but grow slowly, and accumulate IPC and $M(IP)_2C$, but not MIPC (Sawai *et al.*, 2000). Isc1p also has neutral sphingomyelinase activity and has 30% identity to mammalian neutral sphingomyelinase (nSMase2). Isc1p and nSMase2 share other common features, as both require Mg^{2+} for optimal activity, are activated by anionic phospholipids, such as phosphatidylserine, cardiolipin and phosphatidylglycerol, and contain a P-loop-like domain that seems to be essential for catalysis and Mg^{2+} binding (Okamoto *et al.,* 2002). The interaction of the C terminus, that functions as anionic phospholipids-selective binding, with the remainder of the enzyme, seems to play a critical role in enzyme function through a novel mechanism of enzyme activation by lipid cofactors (Okamoto *et al.*, 2002). A recent report indicated that Isc1p is activated during growth, and this is not due to transcriptional / translational activation. The new model suggests that Isc1p co-localises with the endoplasmic reticulum during exponential growth, but translocates to the mitochondria during the late logarithmic and post-diauxic phases of growth (Vaena de Avalos *et al.*, 2004).

In mammalian cells, the first report on ceramide as a signalling molecule indicated a role in the control of events such as differentiation, cell cycle arrest, apoptosis and senescence (Riboni *et al.,* 1997; Levade *et al.,* 1999; Kolesnick and Kronke, 1998; Hannun and Luberto, 2000; Guzman *et al.,* 2001; Ohanian and Ohanian, 2001). The accumulation of ceramides by activation of sphingomyelinases has been observed in response to a variety of stimuli, including ionising radiation, DNA damage, heat stress, and oxidants (Hannun and

Luberto, 2000). In this study, we analysed the role of Isc1p in oxidative stress resistance. In addition, and to analyse if the localization of Isc1p was effected by exposure to H_2O_2, a quimeric protein was engineered in which GFP was fused to the C terminus of Isc1p.

4.2. MATERIALS AND METHODS

4.2.1. YEAST STRAINS AND GROWTH CONDITIONS

The strains of *Saccharomyces cerevisiae* used in this study are listed in table 4.1.

Table 4.1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Reference/Source
By4741	Matα, *his3*Δ_1, *leu* Δ_0, met15 Δ_0, *ura3*Δ_0	EUROSCARF
isc1Δ	[By4741] *isc1*Δ::*KanMX4*	EUROSCARF
ISC1-GFP3	[By4741] *ISC1-GFP3*	This work

Yeast cells were grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose) to early exponential phase (OD_{600}=0.6), or to post-diauxic-shift phase (OD_{600}=10 for By4741; OD_{600}=6 for *isc1*Δ mutants), in an orbital shaker, at 26ºC, and 120 rpm, with a ratio of flask volume / medium volume of 5:1.

4.2.2. STRESS CONDITIONS AND CELL VIABILITY

Oxidative stress was generated by treating exponentially growing yeast cells with 0.4 mM H_2O_2 or 1.5 mM H_2O_2 for 30 or 60 min. Cellular adaptation was induced by pre-treating exponential phase cells with 0.4 mM H_2O_2 for 30 min prior to a 1.5 mM H_2O_2 stress. Post-diauxic shift cells were exposed to 5 mM H_2O_2 for 60 min. For the other stresses, exponentially growing yeast cells were exposed to 14% ethanol for 1 hour, to 120 mM acetic acid (in YPD, pH 3.0) for 2 hours, or to 50°C for 8 min. Cell viability was determined by standard dilution plate counts on YPD medium containing 1.5% agar. Colonies were counted after growth at 26ºC for 3 days. Viability was expressed as the percentage of the colony-forming units of non-stressed cells.

4.2.3. NORTHERN BLOT AND MICROARRAY ANALYSIS

RNA was isolated by the acid phenol method as described by Ausubel *et al.* (1998). Northern blot analysis was performed as described in 3.2.4. The following probes were used: a 1 Kb *Hin*dIII-*Eco*RI fragment of *ACT1* gene, a 1,5 Kb *Bgl*II-*Bgl*II fragment of *SSA4* gene, a 1,5 Kb *Bgl*II-*Pst*I fragment of *HSP26* gene, a 1,1 Kb *Eco*RI-*Eco*RI fragment of *CTT1* gene, a 0,5 Kb *Hin*dIII- *Hin*dIII fragment of *SOD1* gene and a 470 bp *Bgl*II-*Bgl*II fragment of *SOD2* gene. The *ACT1* gene (Gallwitz and Sures, 1980) was used as RNA loading control. Band intensities were evaluated by densitometry. Microarray analysis were performed as described in 2.2.6. To determine the –fold induction or repression, the relative mRNA level from *isc1*Δ mutant cells was divided by that from wild type cells. All genes whose mRNA level was at least 2-fold higher or 1.8-fold lower in *isc1*Δ mutant cells were listed, because these values were found to be reproducible. All values are means of at least three independent experiments.

4.2.4. PROTEIN CARBONYLATION AND LIPID PEROXIDATION ANALYSIS

Yeast extracts were prepared in 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, containing a protease inhibitor cocktail (Complete^{TM}, Mini, EDTA-free Protease Cocktail Inhibitor Tablets, Boehringer Mannhein), by vigorous shaking of the cell suspension, in the presence of glass beads, for 5 min. Short pulses of 1 min were used, with 1 min intervals on ice. Protein content was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. Protein carbonyl content was determined by Slot-blot analysis, as described in 2.2.9.

For the analysis of lipid peroxidation, yeast extracts were prepared in 20 mM sodium phosphate buffer (pH 7.2), by vigorous shaking of the cell suspension, in the presence of glass beads, for 3 min. Short pulses of 1 min were used, with 1 min intervals on ice. 100% trichloroacetic acid was added and two more short pulses of 1 min, with 1 min interval on ice were performed. Protein content was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. MDA concentration was assayed using 1% TBA, 0.05M NaOH, 0.025% BHT (butylated hydroxytoluene) and 0.1M EDTA. 50 μg of total cytoplasmic proteins were used for each assay. MDA concentration was determined spectrophotometrically at 532 nm, and expressed as nmol MDA (mg protein)$^{-1}$.

4.2.5. ADENINE NUCLEOTIDE DETERMINATION

Yeast cells grown to early exponential phase (OD_{600}=0.6) (1.2 mg wet weight.ml^{-1}), were rapidly collected by centrifugation, and washed once with 5 ml of a mixture of methanol / water (1:1, v/v) at -40ºC. The samples were kept at -70ºC until extraction. To prepare the acidic extracts, 1.2 M $HClO_4$ was added to the frozen yeast (0.4 ml per 100 mg wet weight) and the suspension was frozen and thawed three times to extract metabolites (Weibel *et al.*, 1974). Cell debris was removed by centrifugation and the pellet re-extracted once with 0.2 M $HClO_4$ (0.1 ml per 100 mg wet weight). The supernatants were combined, neutralized with KOH / K_2CO_3 and analysed by HPLC as described by Sillero *et al.*, (1994). The amount of nucleotides was determined from the areas of the corresponding peaks, using the absorption coefficients obtained from standard curves; their intracellular concentration was calculated assuming that 1 g of yeast (wet weight) contains 0.6 ml of intracellular volume (Conway and Downey, 1950). In the assay conditions, the detection limit was 5 nmol per gram of yeast cell dry weight.

4.2.6. *IN SILICO* ANALYSIS

The *in silico* analysis of the promoters was performed using the MEME software (Bailey and Elkan, 1994). The promoter sequences and the consensus binding sequences of the transcription factors were obtained in the Promoter Database of *Saccharomyces cerevisiae* (SCPD) (http://cgsigma.cshl.org/jian/).

4.2.7. EXPRESSION OF A FUSION PROTEIN ISC1-GFP

For expression of the fusion protein ISC1-GFP3, a construct was engineered by the short flanking homology method (as described by Wach *et al.*, 1994; 1998) (Figure 4.1). The fragment used for integration in the genome of the sequence encoding GFP fused to the C terminus of Isc1p was obtained by PCR, from the pUG30.TXT plasmid (Güldener, 2000), using ISC1-GFP-FORW and ISC1-GFP-REV primers. The fragment containing yEGFP3 and KanMX4 sequences and flanked by a region upstream the stop codon and by a region downstream the stop codon of *ISC1* gene was used to transform *S. cerevisiae* cells. Yeast transformation was performed by the lithium acetate method (Sambrook *et al.*, 1989). Transformants were selected in YPD medium supplemented with geneticin (200 ng/ml).

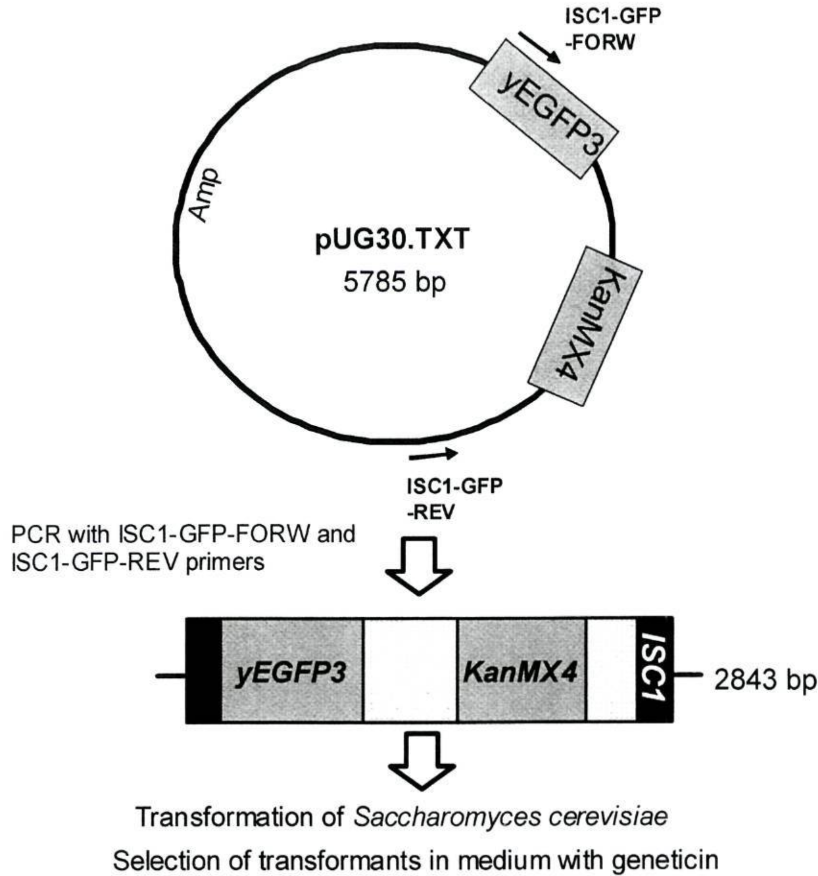


Figure 4.1.

Strategy for constitutive expression in *S. cerevisiae* By4741 of a fusion protein ISC1-GFP3. The fragment used for homolog recombination was obtained by PCR from pUG30.TXT (Güldener, 2000), using ISC1-GFP-FORW and ISC1-GFP-REV primers.

Table 4.2. Oligonucleotides used in this work. # means the Crick strand.

OLIGO	OLIGONUCLEOTIDE SEQUENCE
ISC1-GFP-FORW	5' – GAAGTCGAACAAGAGGTTCTGGACGCGGAG CACCACCTGCAAACTTTCTTGAGCGAGAAATCTA AACCTCAACAATTATTCACTG – 3'
ISC1-GFP-REV #	5' – GAAAAGCAAGCAAAACAAACGCCCACTTCCA CCAGACAGGCTGGTTACGGTGGGTCTTCAGCAT AGGCCACTAGTGGATCTG – 3'
ISC1-FUSION	5' – CCGGTTATGCGGTGATTGTG – 3'
GFP-DO	5' – GGCGTGAATGTAAGCGTGACAT – 3'

4.3. RESULTS

4.3.1. CELL GROWTH AND SENSITIVITY OF *S. cerevisiae isc1*Δ MUTANT CELLS TO HYDROGEN PEROXIDE

The role of the yeast Isc1p, an inositolphosphosphingolipid-phospholipase C (IPS-PLC), in cell resistance to H_2O_2 was assessed using *isc1*Δ mutants. The analysis of cell growth showed that the disruption of *ISC1* gene did not affect the exponential growth rate. However, *isc1*Δ mutants were unable to grow after the diauxic shift phase (Figure 4.2). Thus, our results indicate that Isc1p is required for optimal aerobic growth. In agreement, *isc1*Δ mutants are unable to grow on non-fermentable carbon sources, such as glycerol or ethanol (data not shown).

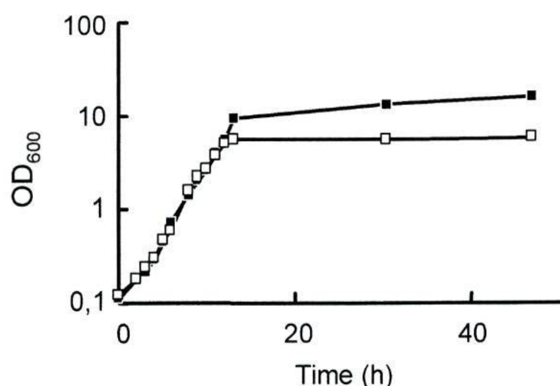


Figure 4.2.

Cellular growth of *S. cerevisiae* By4741 wild type and *isc1*Δ mutant cells. *S. cerevisiae* By4741 (■) and *isc1*Δ mutant (□) cells were grown in YPD medium. Cell density was measured for 48 h. A representative experiment is shown (out of three independent experiments with similar results).

Our data shows that the deficiency in Isc1p increased the sensitivity of yeast cells to H_2O_2: 37% and 5% of *isc1*Δ mutant remained viable, in contrast to 65% and 26% in wild type cells, after exposure to 0.4 or 1.5 mM H_2O_2, respectively. In yeast, as in other cell types, an oxidative stress response is triggered when cells are exposed to low concentrations of H_2O_2, leading to the acquisition of cellular resistance to a subsequent lethal stress (Godon *et al.*, 1998). The lack of Isc1p activity did not impair the acquisition of oxidative stress resistance by pre-exposure of exponential phase cells to 0.4 mM H_2O_2 for 30 min prior to the 1.5 mM H_2O_2 treatment (Figure 4.3). Post-diauxic shift phase cells are also known to display an intrinsically higher stress resistance. The H_2O_2 resistance of *isc1*Δ mutants also increased by growth from the exponential to the post-diauxic shift phase, but it was significantly lower

compared to that of wild type cells: 57 ± 14% (*isc1*Δ) and 84 ± 15% (By4741). In all cases, *isc1*Δ mutants were always more sensitive to H_2O_2.

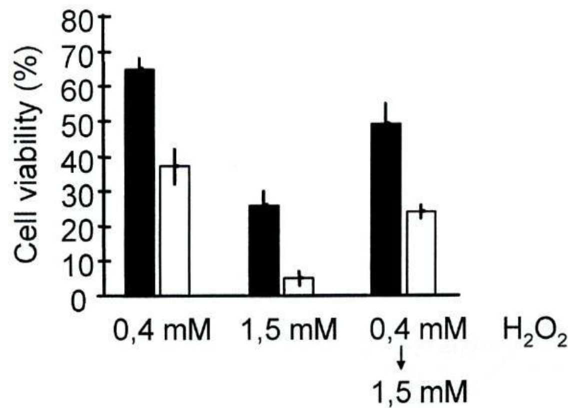


Figure 4.3.

Sensitivity of *S. cerevisiae* BY4741 wild type and *isc1*Δ mutant cells to H_2O_2 stress. *S. cerevisiae* By4741 wild type (closed bars) *and isc1*Δ *(*open bars) cells were grown to the exponential phase (OD_{600} ≈ 0.6), and treated with 0.4 mM H_2O_2 for 30 min, or with 1.5 mM H_2O_2 for 30 min, or with 0.4 mM H_2O_2 followed by 1.5 mM H_2O_2 for 30 min. Cell viability was determined by standard dilution plate counts and expressed as the percentage of the colony-forming units of non-stressed cells. Values are means ± SD of three independent experiments.

4.3.2. ADAPTIVE RESPONSE OF *S. cerevisiae isc1*Δ MUTANT CELLS TO HYDROGEN PEROXIDE: ANTIOXIDANT DEFENCES AND HEAT-SHOCK PROTEINS

To identify molecular features associated with the higher sensitivity of *isc1*Δ mutant cells to H_2O_2, we analysed the expression of stress proteins. Both constitutive and H_2O_2-induced mRNA levels of some antioxidant defences (*SOD1*, Cu,Zn-superoxide dismutase; *SOD2*, Mn-superoxide dismutase; *CTT1*, cytosolic catalase) and heat-shock proteins (*HSP26* and *SSA4*, a HSP70 family member) were quantified. The results obtained show that the expression of these genes under physiological conditions and their induction in response to 0.4 mM H_2O_2 was similar in *isc1*Δ mutants and wild type cells (Figure 4.4). Thus, the higher sensitivity of *isc1*Δ mutants to H_2O_2 is not due to lower levels of these antioxidant defences or heat-shock proteins.

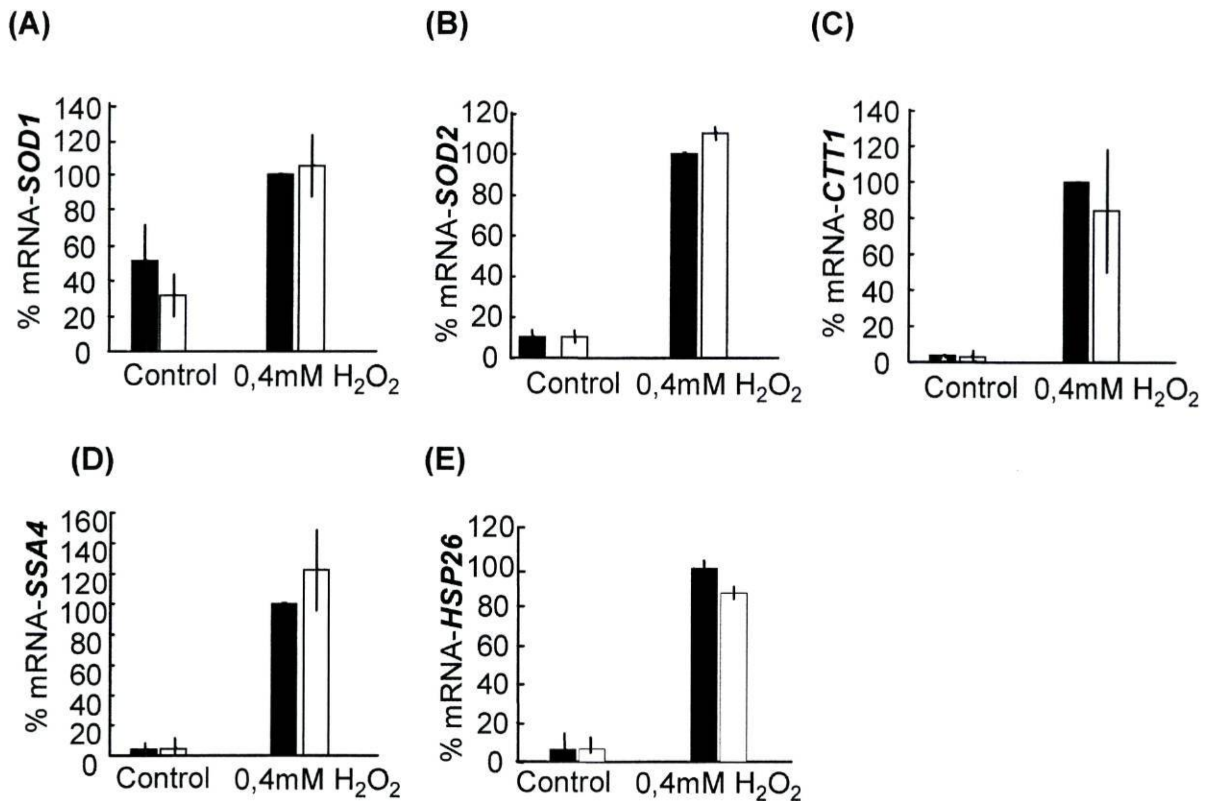


Figure 4.4.

Effect of Isc1p deficiency in the constitutive and H_2O_2 stress-induced mRNA levels for some antioxidant defences and heat shock proteins. *S. cerevisiae* By4741 wild type (closed bars) and *isc1*Δ (open bars) cells were grown to exponential phase ($OD_{600} \approx 0.6$) and treated with 0.4 mM H_2O_2 for 30 min. The mRNA-*SOD1* (A), -*SOD2* (B), -*CTT1* (C), -*SSA4* (D) and -*HSP26* (E) levels were analysed by Northern Blot. Band intensities were quantified and corrected for *ACT1* (RNA loading control). Values are means ± SD of three independent experiments.

4.3.3. PROTEIN CARBONYLATION AND LIPID PEROXIDATION IN *S. cerevisiae isc1*Δ MUTANT CELLS

It is known that H_2O_2 induces DNA damage, lipid peroxidation and specific protein carbonylation (Cabiscol *et al.*, 2000; Costa *et al.*, 2002). To investigate if the higher sensitivity of *isc1*Δ mutant cells could be associated to the levels of damaged molecules, the constitutive and H_2O_2-induced levels of protein carbonylation and lipid peroxidation were analysed. The constitutive carbonyl levels were significantly higher in *isc1*Δ mutant cells

(122% of the observed in wild type cells) (Figure 4.5(A)). In addition, exposure to 1.5 mM H_2O_2 increased carbonyl content of *isc1Δ* mutant cells to 195%, while that for wild type cells increased to 148%.

The analysis of lipid peroxidation shows that the constitutive levels were similar in the *isc1Δ* mutants and wild type cells (Figure 4.5B). However, lipid peroxidation levels increased to 136% in *isc1Δ* cells treated with 0.4 mM H_2O_2, a concentration that did not affect wild type cells. Furthermore, the induction of lipid peroxidation by exposure to 1.5 mM H_2O_2 was higher in *isc1Δ* mutant cells (185% compared to 168% for wild type cells). The overall results suggest that the lower resistance to H_2O_2 in cells deficient in Isc1p is associated with an increased accumulation of oxidised proteins and lipids, exceeding the levels assessed in wild type cells.

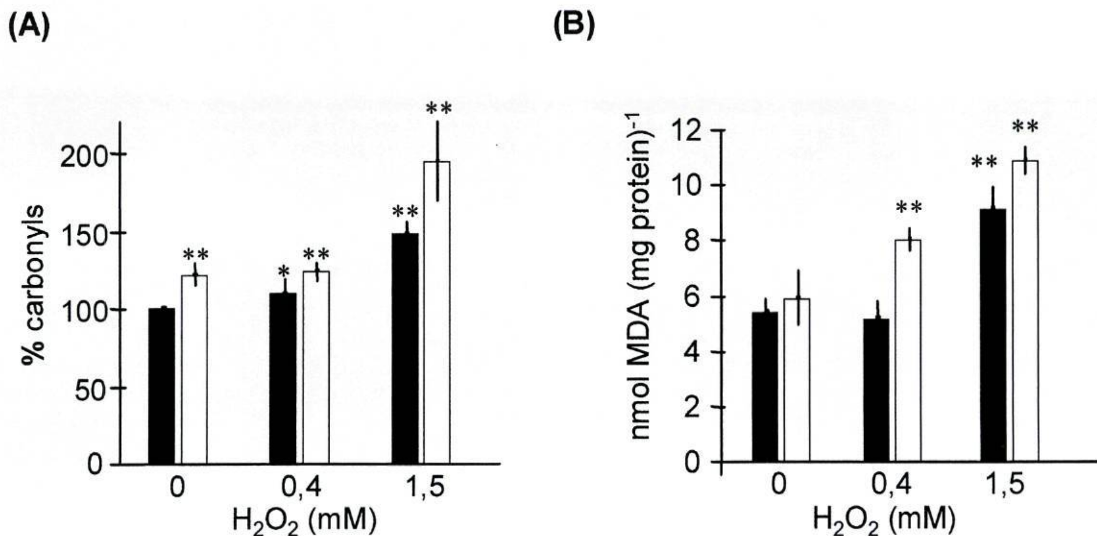


Figure 4.5.

Analysis of oxidative damages in *S. cerevisiae* wild type and *isc1Δ* mutant cells. *S. cerevisiae* By4741 wild type (closed bars) and *isc1Δ* mutant (open bars) cells were grown to exponential phase ($OD_{600} \approx 0.6$) and treated with 0.4 mM H_2O_2 or with 1.5 mM H_2O_2 for 30 min. (A) Protein carbonylation. Proteins derivatised with DNPH were slot-blotted into a PVDF membrane. Carbonylated proteins were detected using an anti-DNP antibody. Quantitative analysis of total protein carbonyl content was performed by densitometry using data taken from the same membrane. (B) Lipid peroxidation. Proteins were isolated and TBARS were determined as described in Methods. Values are means ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$.

4.3.4. ANALYSIS OF ADENINE NUCLEOTIDE LEVELS IN *isc1*Δ MUTANT CELLS

It is known that oxidative stress conditions drastically decrease ATP levels, impairing several metabolic functions that are essential for cell viability and integrity (Cabiscol *et al.*, 2000; Cabiscol and Levine, 1995; Huang and Philbert, 1996; Osório *et al.*, 2003). To test if the higher sensitivity of *isc1*Δ mutant cells could be associated to decreased ATP levels, adenine nucleotide levels were determined under physiological conditions and after exposure to 0.4 mM or 1.5 mM H_2O_2 for 30 min. The constitutive levels of ATP, ADP and AMP were similar in wild type and *isc1*Δ mutant cells. Consistent with previous studies, H_2O_2 decreased ATP levels in a dose-dependent manner, and the decrease in ATP was correlated with an increase of AMP levels. However, the effect of H_2O_2 was similar in wild type and *isc1*Δ cells (Figure 4.6). These results suggest that the higher sensitivity of *isc1*Δ mutants to H_2O_2 was not correlated with decreased ATP levels.

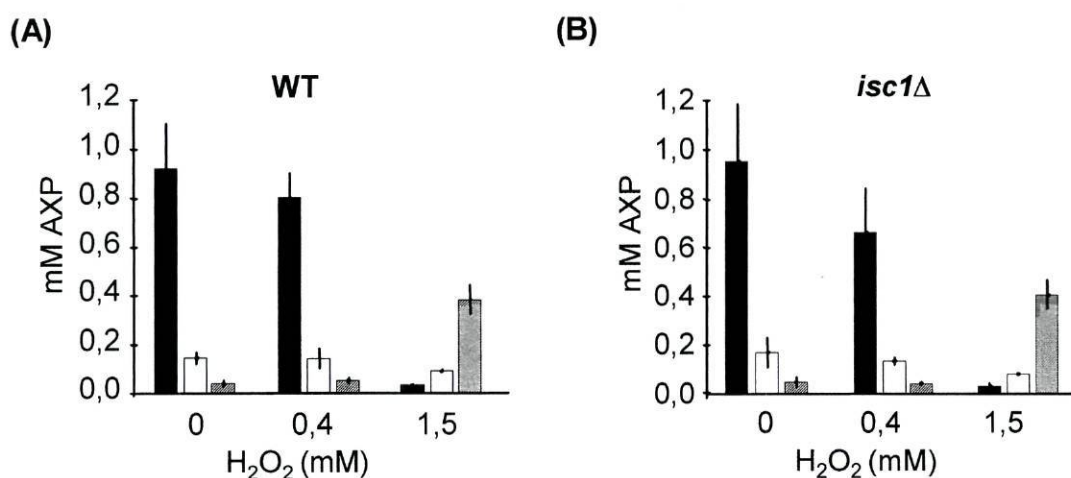


Figure 4.6.

Adenine nucleotide levels in *S. cerevisiae* wild type and *isc1*Δ mutant cells. *S. cerevisiae* By4741 wild type (A) and *isc1*Δ mutant cells (B) cells were grown to exponential phase ($OD_{600} \approx 0.6$) and treated with 0.4 mM H_2O_2 for 30 min or with 1.5 mM H_2O_2 for 10 min. ATP (closed bars), ADP (open bars) and AMP (stripped bars) levels were determined as described in Methods. Values are means ± SD of three independent experiments.

4.3.5. SENSITIVITY OF *S. cerevisiae isc1*Δ MUTANT CELLS TO HEAT-SHOCK, ETHANOL AND ACETIC ACID

To analyse if yeast cells deficient in Isc1p display higher sensitivity towards different stress agents, we analysed the resistance of wild type and *isc1*Δ cells to heat shock, ethanol and acetic acid. Cells were exposed to 50°C for 8 min, to 14% ethanol for 1 hour, or to 120

mM acetic acid for 2 hours. The results revealed that *isc1*Δ mutants were more resistant than wild type cells to all these stress conditions (Figure 4.7): cellular viability after ethanol stress increased from 37% (wild type) to 54% (*isc1*Δ mutants); the induction of thermotolerance and acetic acid resistance was even more pronounced, increasing 5-fold and 4-fold, respectively, in the *isc1*Δ mutants, compared to wild type cells.

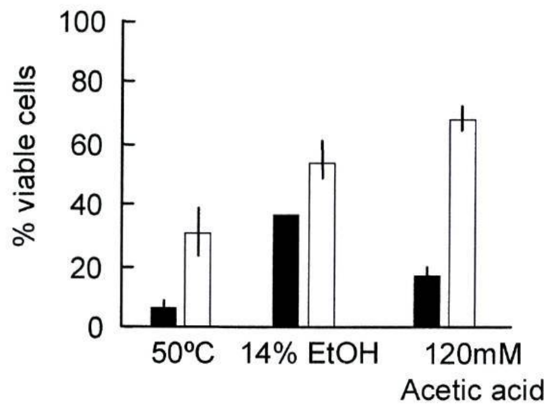


Figure 4.7.

Stress resistance in *S. cerevisiae isc1*Δ mutant cells. *S. cerevisiae* By4741 wild type (closed bars) and *isc1*Δ mutant (open bars) cells were grown to exponential phase ($OD_{600} \approx 0.6$). Yeast cells were heat-shocked at 50ºC for 8 min, treated with 14% ethanol for 1 h, or with 120 mM acetic acid for 2h. Cell viability was determined by standard dilution plate counts and expressed as the percentage of the colony-forming units of non-stressed cells. Values are means ± SD of three independent experiments.

4.3.6. TRANSCRIPTIONAL ANALYSIS OF *isc1*Δ MUTANT CELLS

A large scale analysis of the changes in the transcriptome of *isc1*Δ cells was performed. The results show that the mRNA level of 30 genes was higher in *isc1*Δ mutant cells (Table 4.3), whereas that of 38 genes was diminished (Table 4.4). The up-regulated genes encode proteins involved in cell rescue and defence, metabolism (carbohydrate and nucleotide), proteolytic degradation, cell cycle and DNA processing, transcription regulation and cellular transport. Most of the genes down-regulated in *isc1*Δ mutant cells were associated with protein biosynthesis (89%). Although the expression of ribosomal proteins was markedly decreased in *isc1*Δ cells, genes associated with rRNA processing (*SRP40* and *DRS1*) were up-regulated in these cells.

Of the genes constitutively up-regulated in *isc1*Δ mutant cells, 67% were associated with the secretory pathway, plasma membrane and the cell wall (Figure 4.8). Interestingly,

23% of the up-regulated genes were related with cellular transport: 7% encoded multidrug resistance transporter proteins, 13% were associated with iron transport and one gene was associated with glucose transport.

The expression of several genes involved in transduction of cell wall stress or nutrient deprivation signals (*MTL1*) and cell wall biogenesis (*FKS2* and *ROM1*), or known to be regulated in response to changes in the cell wall (*YGP1*, *TIR4*, and *TSL1*) also increased in *isc1Δ* mutants. The *SIM1* gene, encoding a cell wall protein involved in cell cycle regulation and cell ageing, and the *RSB1* gene, encoding a putative transporter or flippase that translocates long chain sphingoid bases from the cytoplasmic side toward the extracytoplamsic side of the membrane, were also up-regulated.

The disruption of *ISC1* gene also increased the expression of genes associated with oxidative damage. These include the *PEP4* gene that encodes a vacuolar protease involved in the turnover of oxidised proteins (see Chapter 2), the *RNR2* gene that encodes a ribonucleotide reductase subunit derepressed by DNA damage (Zhou and Elledge, 1992; Huang *et al.*, 1998), and the *XBP1* gene that encodes a transcription factor induced by oxidative stress and DNA-damage (Mai and Breeden, 1997). This data suggests that the Xbp1p transcription factor may mediate at least some of alterations in gene expression associated with Isc1p deficiency. Indeed, 70% of the induced genes and 37% of the repressed genes contain Xbp1p-binding sites (consensus sequence CTCGA; Mai and Breeden, 1997) in their promoter (Table 4.5 and 4.6).

Disruption of *ISC1* gene also increased the expression of genes encoding ABC drug efflux pumps, namely *PDR5* and *PDR15*. The *PDR5* gene encodes a plasma membrane pump that seems to have a role in cellular detoxification during exponential growth (Mamnun *et al.*, 2004). The *PDR15* gene is induced by heat-shock, weak acids and low pH and seems to have a role in cellular detoxification during metabolic stress or non-growing cells (Wolfger *et al.*, 2004).

Table 4.3. Genes constitutively up-regulated in *isc1Δ* mutant cells

Yeast By4741 (wild type) and *isc1Δ* mutant cells were grown in YPD medium to early exponential phase ($OD_{600} \approx$ 0.6). Genes up-regulated (*isc1Δ vs* wt) and sorted into functional categories (according to MIPS) are shown. Genes associated with several categories are repeated.

Gene	Gene product	*isc1Δ* / wt	
		Log_2(ratio) mean	**SD**
Transcription			
XBP1	transcriptional repressor	1,30	0,43
SRP40	Nopp 140 homolog, nonribosomal protein of the nucleolus and coiled bodies	2,06	1,56
DRS1	ATP dependent RNA helicase (putative)	1,23	0,47
Protein fate (folding, modification, destination)			
PEP4	vacuolar proteinase A	1,47	0,97
DRS1	ATP dependent RNA helicase (putative)	1,23	0,47
Cellular transport, transport facilitation and transport routes			
FIT2	cell wall protein involved in iron transport	3,21	2,21
FIT3	cell wall protein involved in iron transport	3,46	2,67
ENB1	ferric-enterobactin transport	1,82	0,78
ARN1	iron-siderochrome transport	1,72	0,74
PDR15	multidrug resistance transporter (putative)	1,75	0,79
PDR5	multidrug resistance transporter	2,47	1,56
HXT3	low affinity glucose transporter	1,62	1,12
C-compound and carbohydrate metabolism			
PGK1	3-phosphoglicerate kinase	1,09	0,68
GLK1	glucokinase	1,73	1,57
TDH1	glyceraldehyde-3-phosphate dehydrogenase	1,37	0,64
FKS2	1,3-beta-D-glucan synthase catalytic component	1,99	1,07
ROM1	GDP-GTP Exchange Protein (GEP) for the Rho1p Small GTP-binding Protein	1,42	0,89
TSL1	trehalose-6-phosphate synthase/phosphatase complex 123kDa regulatory subunit	1,38	1,06
Protein activity regulation			
ROM1	GDP-GTP Exchange Protein (GEP) for the Rho1p Small GTP-binding Protein	1,42	0,89

Table 4.3. (cont.)

Gene	Gene product	*isc1*Δ / wt	
		Log_2(ratio) mean	SD
Cell cycle and DNA processing			
SIM1	strong similarity to Sun4p, Uth1p, Nca3p	1,52	0,83
MTL1	acts in concert with Mid2p to transduce cell wall stress signals	2,57	1,47
RNR2	ribonucleotide reductase, small (R2) subunit	1,55	0,65
Cell rescue, defence and virulence			
YGP1	gp37, a glycoprotein synthesised in response to nutrient limitation	1,69	0,6
TIR4	cell wall mannoprotein	3,19	3,96
Cell fate			
ROM1	GDP-GTP Exchange Protein (GEP) for the Rho1p Small GTP-binding Protein	1,42	0,89
SIM1	strong similarity to Sun4p, Uth1p, Nca3p	1,52	0,83
Nucleotide metabolism			
RNR2	ribonucleotide reductase, small (R2) subunit	1,55	0,65
Cell type differentiation			
FKS2	1,3-beta-D-glucan synthase catalytic component	1,99	1,07
ROM1	GDP-GTP Exchange Protein (GEP) for the Rho1p Small GTP-binding Protein	1,42	0,89
Interaction with cellular environment			
MTL1	acts in concert with Mid2p to transduce cell wall stress signals	2,57	1,47
PRY1	homology to the plant PR-1 class of pathogen related proteins	1,26	0,83
Miscellaneous and uncharacterised			
RSB1	phospholipid-translocating ATPase activity	2,06	1,67
PEX8	peroxisome associated protein containing a PTS1 signal	1,50	0,48
SRF4	small hydrophobic protein	0,98	1,14
YHR214W-A	uncharacterised	1,33	0,41
YGR069W	uncharacterised	1,49	0,63
YOR152C	uncharacterised	1,54	0,77
YFR039c	uncharacterised	1,59	0,64

Table 4.4. Genes constitutively down-regulated in *isc1*Δ mutant cells

Yeast By4741 (wild type) and *isc1*Δ mutant cells were grown in YPD medium to early exponential phase ($OD_{600} \approx$ 0.6). Genes down-regulated (*isc1*Δ *vs* wt) and sorted into functional categories (according to MIPS) are shown. Genes associated with several categories are repeated.

Gene	Gene product	*isc1*Δ / wt	
		Log_2(ratio) mean	SD
Protein biosynthesis			
RPS10A	ribosomal protein, small subunit	-1,85	0,06
PLC2	ribosomal protein, small subunit	-1,90	0,04
RPS1A	ribosomal protein, small subunit	-1,59	0,08
SUP46	ribosomal protein, small subunit	-1,62	0,09
RPS18A	ribosomal protein, small subunit	-1,70	0,05
RPS26A	ribosomal protein, small subunit	-1,34	0,1
URP2	ribosomal protein, small subunit	-1,40	0,08
RPS18B	ribosomal protein, small subunit	-1,36	0,07
RPS17B	ribosomal protein, small subunit	-1,51	0,07
RPS28A	ribosomal protein, small subunit	-1,09	0,07
RPS30	ribosomal protein, small subunit	-1,39	0,06
RPL27	ribosomal protein, large subunit	-1,57	0,09
RPL30A	ribosomal protein, large subunit	-1,70	0,06
RPL38	ribosomal protein, large subunit	-1,89	0,04
RPL25	ribosomal protein, large subunit	-1,47	0,12
RPL21B	ribosomal protein, large subunit	-1,56	0,08
RPL9A	ribosomal protein, large subunit	-1,35	0,1
RPL20B	ribosomal protein, large subunit	-1,60	0,05
RPL32	ribosomal protein, large subunit	-1,50	0,06
RPL17B	ribosomal protein, large subunit	-1,57	0,06
SOS1	ribosomal protein, large subunit	-1,56	0,1
SOS2	ribosomal protein, large subunit	-1,77	0,05
RPL30B	ribosomal protein, large subunit	-1,32	0,04
SPB2	ribosomal protein, large subunit	-1,58	0,1
RPS27A	ribosomal protein, large subunit	-1,46	0,07
RPL17	ribosomal protein, large subunit	-1,38	0,09
RPL27B	ribosomal protein, large subunit	-1,44	0,09
RPL16A	ribosomal protein, large subunit	-1,37	0,05
URP1	ribosomal protein, large subunit	-1,52	0,08
RPL13A	ribosomal protein, large subunit	-1,28	0,06
RPL37B	ribosomal protein, large subunit	-1,29	0,05
MAK18	ribosomal protein, large subunit	-1,12	0,09
UBI3	ribosomal protein,small subunit, protein tagging activity	-1,60	0,08
UBI2	ribosomal protein, large subunit, protein tagging activity	-1,62	0,07

Table 4.4. (cont.)

Gene	Gene product	$isc1\Delta$ / wt	
		Log₂(ratio) mean	**SD**
Nucleotide metabolism			
URA1	dihydroorotate dehydrogenase	-1,29	0,12
Protein fate (folding, modification, destination)			
UBI3	ribosomal protein,small subunit, protein tagging activity	-1,60	0,08
UBI2	ribosomal protein, large subunit, protein tagging activity	-1,62	0,07
Miscellaneous and uncharacterised			
BUD19	uncharacterised	-1,35	0,1
BUD28	uncharacterised	-1,56	0,03
YDR442W	uncharacterised	-1,47	0,11

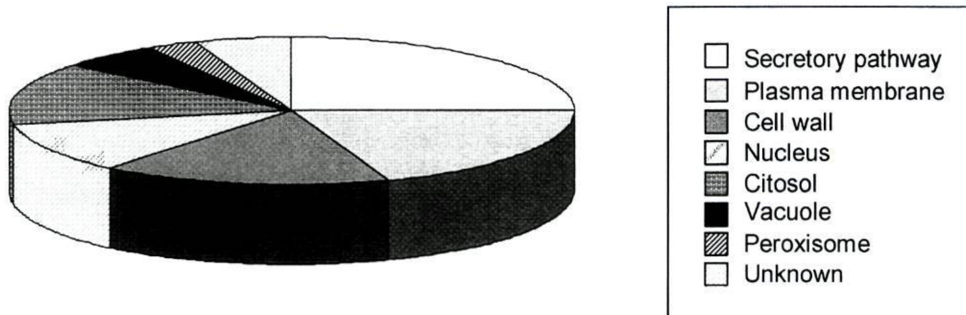


Figure 4.8.

Distribution of the genes up-regulated in *isc1*Δ mutant cells, accordingly to the cellular localization of their gene product.

Table 4.5. Genes constitutively up-regulated in *isc1*Δ mutant cells that contain Xbp1-binding sites.

Position[a]	*Sequence*	*Gene*
-392	gcCTCGAaccgc	*XBP1*
-300	aaCTCGAacgaa#	*SRP40*
-121	cgCTCGAgcata	*SRP40*
-197	acCTCGAaacag#	*FIT2*
-158	aaCTCGAttaca#	*FIT2*
-148	atCTCGAacgat#	*FIT3*
-358	gtCTCGAgcggg	*PDR15*
-636	tgCTCGAaggcg#	*PDR5*
-104	caCTCGActttg	*PDR5*
-524	ttCTCGAtaaga#	*PGK1*
-452	cgCTCGActtcc	*PGK1*
-153	gtCTCGAaatca	*TDH1*
-432	aaCTCGAaataa#	*FKS2*
-393	taCTCGAtcttc	*ROM1*
-342	ttCTCGAaattt#	*TSL1*
-280	tgCTCGAaagtg	*TSL1*
-285	ttCTCGAtgcta	*YGP1*
-474	aaCTCGAttatg#	*TIR4*
-226	aaCTCGAaatga	*RNR2*
-52	ccCTCGAttggc	*RNR2*
-616	ctCTCGAattcc	*SIM1*
-578	tgCTCGAccccc#	*MTL1*
-406	atCTCGAaagtc	*MTL1*
-703	tcCTCGAtctac	*PRY1*
-548	tcCTCGAacaac	*PRY1*
-403	tgCTCGAagggg	*PRY1*
-117	gcCTCGAtgcca#	*YFR039C*
-359	gcCTCGAaaaaa	*YDL023C*
-739	ccCTCGAttgtt	*YGR069W*
-562	ccCTCGAaaggg	*YGR069W*
-465	ggCTCGAaagtg	*YGR069W*
-107	ggCTCGAcatta	*YOR152C*
-90	tcCTCGAtttga#	*YOR152C*

[a] Position relative to the ATG; The consensus binding site for Xbp1p is GC**CTCGA**(G/A)G(C/A)g(a/g) (Mai and Breeden, 1997). # Means the Crick strand.

Table 4.6. Genes constitutively down-regulated in *isc1Δ* mutant cells that contain Xbp1-binding sites.

Position [a]	*Sequence*	*Gene*
-458	tcCTCGAtctta	*URA1*
-696	ctCTCGAtaaag	*BUD28*
-509	gcCTCGAaaaga#	*BUD28*
-40	taCTCGAcctta#	*RPS10A*
-538	taCTCGAcctta#	*PLC2*
-502	tgCTCGAggagc	*RPS1A*
-890	ttCTCGAtcttt#	*RPS26A*
-515	aaCTCGAagtgt	*RPS26A*
-173	atCTCGAaacgg#	*RPS17B*
-147	acCTCGAtgcct#	*RPS17B*
-183	caCTCGAacgta#	*RPL38*
-697	agCTCGAtcctg	*RPL9A*
-124	cgCTCGAgttgg	*RPL20B*
-130	ttCTCGAcagcc#	*SPB2*
-128	gtCTCGAtttgc	*SP17A*
-4	ttCTCGAtttgt#	*SP16A*
-392	atCTCGAtaata	*URP1*

[a] Position relative to the ATG; The consensus binding site for Xbp1p is GC**CTCGA**(G/A)G(C/A)g(a/g) (Mai and Breeden, 1997). # Means the Crick strand.

4.3.7. *IN SILICO* ANALYSIS OF THE PROMOTERS OF GENES DIFFERENTIALLY EXPRESSED IN *isc1Δ* MUTANT CELLS

Aiming to understand how gene expression is reprogrammed in cells deficient in Isc1p, we searched *in silico* for highly conserved regions (motifs) in the promoter region of the genes differentially expressed in these mutants. Using the MEME software (Bailey and Elkan, 1994), 10 motifs were found for the up-regulated genes, and 8 motifs for the down-regulated genes (see tables 4.7 and 4.8 to 4.25, in Appendix). Four of the motifs retrieved for the up-regulated genes include in the sequence, a consensus binding site for a known transcription factor. Indeed, one of the motifs includes the binding sequence for the Uasphr transcription factor (Table 4.11, in Appendix). Seventeen genes, previously described as being regulated by the Uasphrp transcription factor, are mainly involved in DNA damage repair, namely in the nucleotide excision repair pathway, in dsDNA repair, and include glycosylase, methyltransferase and ribonucleotide-diphosphate reductase activities. Another motif includes the binding sequence for that Gcr1p transcription factor (Table 4.14, in Appendix), which is described as a transcriptional activator of genes involved in glycolysis, namely the genes encoding triose-phosphate isomerase, phosphoglycerate kinase and

piruvate kinase (Haw *et al.*, 2001). A third motif containing the binding sequence for the Swi5p transcription factor was also retrieved (Table 4.16, in Appendix). This transcription factor activates the transcription of genes expressed in G_1 phase and at the G_1/M boundary, namely the HO endonuclease gene (Dohrmann *et al.*, 1992). Finally, one of the motifs includes the binding site for Pdr1/Pdr3p transcription factors (Table 4.17, in Appendix) which are known to regulate the transcription of genes involved in cellular detoxification, namely *PDR5* and *PDR15* genes (Mamnun *et al.*, 2004; Wolfger *et al.*, 2004).

Table 4.7. Motifs discovered by the *in silico* analysis (using the MEME software) of the promoters of genes differentially expressed in *isc1*Δ mutant cells.

Motifs	*Number*[a]
in up-regulated genes	
C(C/T)C(T/C)(T/C)T(C/T)T(T/C)	20
(G/A)CAA(C/G)A(A/C)(C/A)A	14
TTTCTTC	11
A(G/A)(A/G)G(G/C)AAG(G/C/A)	12
(A/T)(A/G)GA(G/A)AA(G/A)A(A/G)	22
C(T/A)A(T/C)(C/T)TT(T/C)(T/G)C	24
CTT(C/G)CT(C/T)(G/T)(T/G)T	21
(T/C)T(C/G)(C/G)N(C/T)CCT	21
T(C/G)(C/A)(G/T)CG(G/C)(A/C)	22
(G/A)A(A/C)A(C/G)(C/A)AGCA	21
in down-regulated genes	
(T/A)(C/G)TGC(C/T)(T/G)(C/G)TG	25
(T/C)TTT(T/C)TCCC	14
CTT(G/T)C(T/A)G(C/A)A	14
(T/G)GAAAAA	31
CCTT(C/T)CC	13
(T/C/G)GCCT(C/G)(G/T)(G/C)C	17
(T/G)G(G/A)(T/A)GAA(G/A)	18
TGTA(T/C)GG(G/A)TG	33

[a] Number of genes containing each motif.

For the down-regulated genes, we also found that one of the motifs includes the binding sequence for the Rap1p transcription factor (Table 4.25, in Appendix). The Rap1p (repressor activator protein) transcription factor encodes an essential protein involved in many diverse, some seemingly contradictory processes, including telomere maintenance, transcriptional silencing (repression) of the silent mating *loci HML* and *HMR* (Kurtz and Shore, 1991), and high level transcriptional activation of genes encoding ribosomal proteins and enzymes of the glycolytic pathway (Shore and Nasmyth, 1987; Vignais *et al.*, 1987; Buchman *et al.*, 1988 and Lieb *et al.*, 2001; reviewed in Piña *et al.*, 2003).

These data suggest that, in addition to the Xbp1p transcription factor, Pdr1p and Pdr3p, Gcr1p, Swi5p, Uasphrp and Rap1p, may also mediate the gene expression changes observed in *isc1*Δ cells.

4.3.7. EXPRESSION OF A FUSION PROTEIN ISC1-GFP3 IN *S. cerevisiae*

A recent study revealed that during exponential growth Isc1p colocalizes with the endoplasmic reticulum, but translocates to the mitochondria during the late logarithmic and postdiauxic phases of growth, suggesting a new mechanism for the activation of Isc1p (Vaena de Avalos *et al.*, 2004). To analyse if Isc1p localization was affected by exposure to H_2O_2, a quimeric protein was produced in which GFP was fused to the C terminus of Isc1p. The *S. cerevisiae* strain with constitutive expression of the fusion protein ISC1-GFP3 was obtained by the short flanking homology method, using the protocol described in 4.2.6. The integration in the genome of the construction of interest was confirmed by PCR analysis, using ISC1-FUSION primer from *ISC1* gene and GFP-DO primer from yGFP3 sequence. A 1535 bp fragment was expected for the transformant strain (according to the integration of the yGFP3 and KanMX4 sequences in frame with *ISC1* gene), and no fragments were expected for the wild type strain. The analysis of the PCR products revealed a fragment with the expected size for the transformant strain (Figure 4.9).

Yeast cells carrying the fusion construction were grown to early exponential phase ($OD_{600} \approx 0.6$). Cells were observed using a BioRad MRC 600 confocal laser microscope (Biorad, UK) and images were captured with an AxioCam camera (Zeiss, Germany). As no fluorescence was detected under normal physiological conditions, yeast cells were submitted to several conditions known to induce *ISC1* gene transcription (Causton *et al.,* 2001; Travers *et al.,* 2000). Yeast cells were heat-shocked for 60 minutes (25ºC to 37ºC) or exposed to 2 mM DTT for 60 minutes, but no fluorescence was detected after either of these treatments.

The absence of fluorescence could be due to low expression levels of the ISC1-GFP fusion protein. For this reason, this approach did not allow the analysis of Isc1p localization.

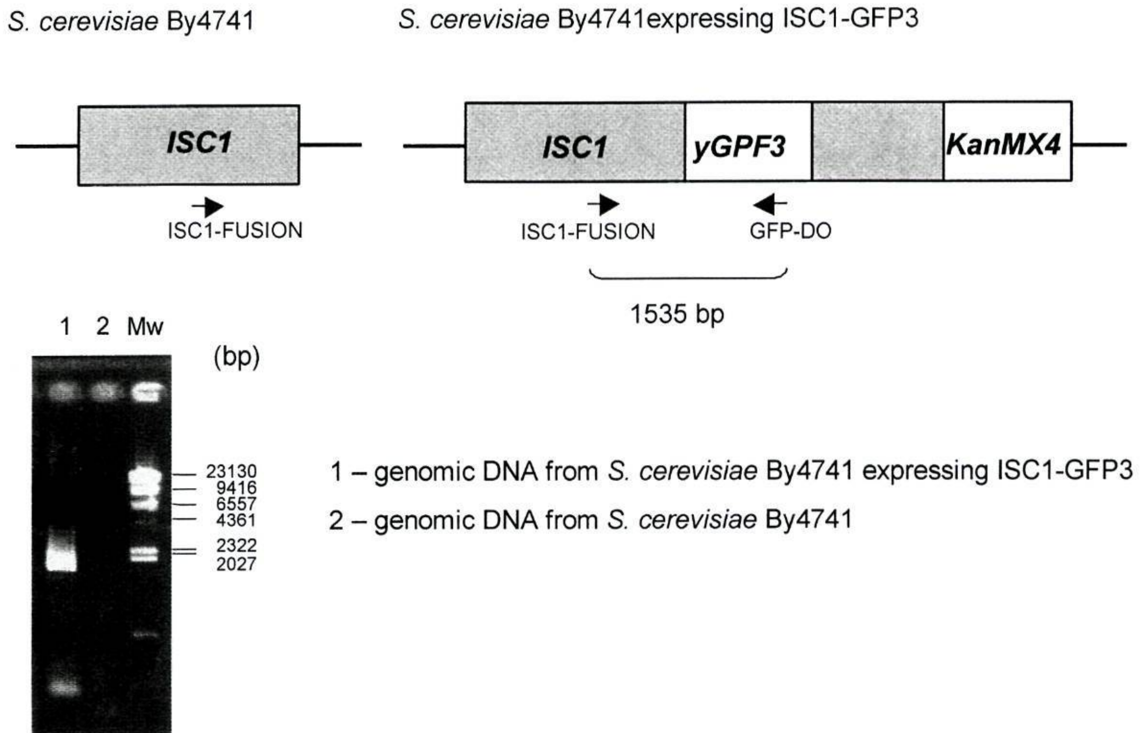


Figure 4.9.

Confirmation of *ISC1-GFP3* integration by PCR. PCR products using genomic DNA from *S. cerevisiae* By4741 or *S. cerevisiae* By4741 expressing ISC1-GFP3, and the primers ISC1-FUSION and GFP-DO, were analysed in a 0.8% agarose gel. The expected fragment sizes for each strain are indicated. The molecular weight marker (Mw) was phage λ digested with *Hin*dIII.

DISCUSSION

Sphingolipids have been referred as important signalling molecules, with roles in differentiation, senescence, cell cycle arrest, apoptosis and stress responses, including oxidative stress (Hannun and Luberto, 2000; Ohanian and Ohanian, 2001). Indeed, in mammalian cells, an increase of ceramide levels resulting from sphingomyelinase activity has been observed in response to DNA damage and oxidants (Hannun and Luberto, 2000),

S. cerevisiae ISC1 gene encodes an inositol phoshosphingolipid phospholipase C with 30% identity to the mammalian neutral sphingomyelinase. In this study, we used *isc1Δ* mutant cells to analyse the role of Isc1p in oxidative stress resistance.

It is clear from the results that Isc1p is required for optimal respiratory growth, and cells deficient in Isc1p display an increased sensitivity to H_2O_2. These mutants were still able to acquire H_2O_2 stress resistance either by pre-exposure to a sublethal stress or by growth from the logarithmic to the post diauxic phase, indicating that ceramide generated by Isc1p is not required for these adaptive responses. In agreement, *isc1Δ* cells were able to induce the expression of antioxidant defences and heat shock proteins, as part of the adaptive response to H_2O_2 (Figure 4.4). The constitutive levels of major antioxidant defences, namely superoxide dismutase and catalase, were also similar in wild type and *isc1Δ* mutants, and therefore a reduced antioxidant capacity cannot account for the sensitivity of *isc1Δ* cells.

Oxidative stress induced cell death has also been associated with the depletion of ATP (Osorio *et al.*, 2003), which may affect cell viability and integrity (Cabiscol *et al.*, 2000; Cabiscol and Levine, 1995; Huang and Philbert, 1996). The constitutive ATP content and its decrease during exposure to H_2O_2 were similar in wild type and *isc1Δ* cells, indicating that the higher sensitivity of *isc1Δ* cells is not associated to decreased energy levels. However, the oxidative stress sensitivity of *isc1Δ* cells was associated with an increased accumulation of oxidised proteins and lipids. Notably, the protein carbonyl content was constitutively higher in *isc1Δ* cells. These results suggest that ceramide generated by Isc1p may regulate some cellular functions or structures important for protection from oxidative damages.

Aiming to identify the functions important for oxidative stress resistance, we analysed the transcriptome of *isc1Δ* mutant cells. Most of the genes constitutively down-regulated in *isc1Δ* mutant cells are ribosomal protein genes. The silencing of ribosomal protein genes is observed in response to a variety of stimuli and therefore *isc1Δ* mutants may be subjected to a constitutive stressful event. The analysis of the localization of the proteins encoded by the genes upregulated in *isc1Δ* mutant cells revealed that 67% were associated to the secretory pathway, plasma membrane and the cell wall. Vesicle trafficking is responsible for the delivery of proteins to organelles as well as membrane recycling, both of which are crucial

processes for normal cell functioning. Membrane recycling is particularly important during oxidative stress, which causes damage to membrane components by lipid peroxidation and protein oxidation. Failure to repair the membranes will result in the loss of membrane integrity and ion leakage, leading to cell death. The upregulation of genes associated with the secretory pathway may therefore be associated with the constitutive higher levels of carbonylated proteins of *isc1*Δ mutant cells, since these irreversibly oxidised proteins must be targeted for vacuolar degradation. Consistently, the expression of the vacuolar protease Pep4, which has a key role in the degradation of carbonylated proteins (Chapter 2), was up-regulated in *isc1*Δ mutant cells.

An additional factor that may play a key role is iron. Indeed, iron is an essential nutrient and the yeast *S. cerevisiae* respond to iron deprivation by increasing the expression levels of genes involved in iron uptake. This response is transcriptionally mediated by the major iron-dependent transcription factor in yeast, Aft1p (Yamaguchi-Iwai *et al.*, 1995; 1996). The disruption of *ISC1* gene induced the expression of two genes encoding components of the non-reductive iron uptake system (*ENB1/ARN4* and *ARN1*) (Heyman *et al.*, 2000a,b; Lesuisse *et al.*, 1998; Yun *et al.*, 2000a,b), and two genes encoding cell wall mannoproteins involved in the retention of siderophore-iron in the cell wall (*FIT2* and *FIT3*) (Protchenko *et al.*, 2001). As most of the Aft1p targets were not up-regulated, it is unlikely that *isc1*Δ mutants suffer from iron deprivation. The up-regulation of the 4 genes associated with iron uptake may result in iron overload. Since iron can react with H_2O_2 to generate hydroxyl radicals, the lower resistance to H_2O_2 and the higher constitutive levels of carbonyls in *isc1*Δ cells may be associated to iron toxicity (Halliwell and Gutteridge, 1999).

The disruption of *ISC1* gene also increased the expression of *PDR5* and *PDR15* genes, which encode plasma membrane drug efflux pumps involved in cellular detoxification during exponential growth, and during metabolic stress or in non-growing cells, respectively (Mamnun *et al.*, 2004; Wolfger *et al.*, 2004). The transcription of these genes is regulated by the Pdr1p/Pdr3p transcription factors, through the PDRE's present in their promoters. The disruption of *ISC1* gene also induced the transcription of other 6 genes containing at least one PDRE on their promoter. The lack of up-regulation of other known targets of Pdre1p/Pdr3p is not surprising, as Pdr1p/Pdr3p dependent gene regulation varies considerably between different promoters, probably due to other factors present in the respective context (Wolfger *et al.*, 1997).

The transcriptome analysis also revealed the induction of several genes associated with cell wall biogenesis, namely *MTL1*, *ROM1* and FKS2. *MTL1* is involved in the transduction of cell wall stress signals, leading to the activation of the Rho1 GTPase (Ketela *et al.*, 1999; Sekiya-kawasaki *et al.*, 2002), and *ROM1* is the GDP/GTP exchange protein for

Rho1p (Ozaki *et al.*, 1996). The Rho1p serves as regulatory subunit of the 1,3β-glucan synthase complex, acting in the alternative catalytic subunits Fks1p and Fks2p. In addition, Rho1p regulates the cell integrity signalling pathway by binding and activating the protein kinase C (Pkc1p) (Nonaka *et al.*, 1995; Kamada *et al.*, 1996).

The cell wall of yeast cells is essential for maintaining cell morphology and to protect cells from the external environmental, and according to these functions its architecture has a highly dynamic nature (Molina *et al.*, 2000; Cid *et al.*, 1995; Smits *et al.*, 1999). Indeed, the cell wall can change in composition and structure in response to environmental stress (Klis *et al.*, 2002; Popolo *et al.*, 2001), as part of the so-called "compensatory mechanism" (Delley and Hall, 1999), and this may be essential for cell survival under stress conditions. Curiously, the expression of *YGP1*, *TSL1*, *SIM1* and *TIR4* genes, which are known to be up-regulated in response to changes in the cell wall (Kapteyn *et al.*, 2001; Garcia *et al.*, 2004; Abramova *et al.*, 2001), was induced by *ISC1* disruption. Ygp1p is a highly glycosylated extracellular protein that has been suggested as a useful marker protein for monitoring early events associated with the stress response (Destruelle *et al.*, 1994), and Sim1p is a cell wall protein involved in cell cycle regulation and cell ageing (Camougrand *et al.*, 2000). In addition, the *RSB1* gene, which encodes a putative transporter or flippase that translocates LCBs from the cytoplasmic side toward the extra-cytoplasmic side of the membrane (Kihara and Igarashi, 2002), was induced in *isc1Δ* mutant cells. These results indicate that *ISC1* disruption led to a remodelling of the cell wall, and suggest that this process may be directed through the accumulation of IPC or $M(IP)_2C$ known to occur in *isc1Δ* mutant cells (Sawai *et al.*, 2000).

The disruption of *ISC1* gene specifically increased oxidative stress sensitivity, as ethanol, acetic acid and heat stress resistance even increased in these mutants. The molecular mechanisms induced by yeast cells to cope with the deleterious effects of these stress conditions have been characterised (Alexandre *et al.*, 2001; Estruch, 2000; Piper, 1997; Decotignies *et al.*, 1998; Piper *et al.*, 1998; Holyoak *et al.*, 1999; Tenreiro *et al.*, 2000). Accordingly, the higher resistance of *isc1Δ* cells to ethanol may be associated with the constitutively higher expression of genes known to be up-regulated by exposure to ethanol (Alexandre *et al.*, 2001), namely *TSL1*, *GLK1*, *TDH1*, and *YGP1*. Similarly, the higher resistance of *isc1Δ* cells to heat-shock may also be associated with the higher constitutive expression of genes induced by heat-shock: *TSL1*, *XBP1*, *TDH1*, *FKS2* and *PDR15* (Gross and Watson, 1998; Mai and Breeden, 1997; Boucherie *et al.*, 1995; Zhao *et al.*, 1998; Wolfger *et al.*, 2004). Furthermore, the higher resistance of these cells to acetic acid may be associated with the constitutive higher expression of *PDR15* and *PDR5* genes.

The results obtained in the *in silico* analysis revealed several motifs that are highly conserved in the promoters of the up- or down-regulated genes. Some of these motifs

include the DNA binding site for previously characterized transcription factors, such as Pdr1/3p, Swi5p, Gcr1p, Uasphrp and Rap1p. The identification of other conserved sequences suggest the existence of other putative transcription factors that may be involved in the regulation of changes in gene expression triggered by Isc1p deficiency. In addition, the consensus binding sequence for Xbp1p, is present both in the up- and in the down-regulated genes, suggesting also a role for Xbp1p in this regulation. Although uncommon, the existence of transcription factors that may act both as a transcriptional activator and as a repressor, depending on the DNA sequence binding context was already described in *S. cerevisiae* (*e.g.* Rap1p) (Piña *et al.*, 2003).

As the activity of Isc1p may depend on its localization, a quimeric protein was engineered in which GFP was fused to the C terminus of Isc1p, to analyse if the localization of Isc1p was effected by exposure to H_2O_2. To clarify if the absence of fluorescence in cells carrying the quimeric protein could be due to low expression levels or if there was some problem in the construction (DNA sequence), several approaches can be followed in the future: Western Blot analysis for detection of the fusion protein (using an antibody against Gfp3p), RT-PCR (reverse transcription-polymerase chain reaction) or Northern-Blot for mRNA detection, insertion of the DNA construct in a plasmid with a strong promoter, or sequencing of the DNA construct.

In summary, the yeast Isc1p is essential for constitutive, but not induced, H_2O_2 resistance. Isc1p deficiency did not affect the level of antioxidant defences and some heat shock proteins or ATP content. However, the increased sensitivity to H_2O_2 in the *isc1Δ* mutants was correlated to a higher accumulation of protein carbonylation and lipid peroxidation, suggesting that ceramide generated by Isc1p may regulate cellular functions or structures important for oxidative stress resistance. The major changes in the transcriptome of *isc1Δ* mutant cells are related with the secretory pathway, proteins involved in iron transport, multidrug or long chain base resistance, cell wall biogenesis and cell signalling. The disruption of *ISC1* gene did not result in a general stress sensitivity. Curiously, *ISC1* disruption increased the cellular resistance to heat shock, ethanol and acetic acid, what may be associated to the constitutive upregulation of genes known to be involved in cellular response to these stress conditions. The overall results suggest that *ISC1* disruption led to a remodelling of the cell wall, and that Isc1p may mediate cell signalling in response to H_2O_2.

APPENDIX

Table 4.8. Genes constitutively up-regulated in *isc1*Δ mutant cells that contain the C(C/T)C(T/C)(T/C)T(C/T)T(T/C) sequence.

Position [a]	*Sequence*	*Gene*
-658	CTCTCTCTC	*YOR152c*
-72	CTCTTTTTT	*YOR152c*
-319	CTCTTTTTT	*YOR152c*
-276	CTCTCTCTC	*PGK1*
-575	CTCCTTCTT	*PGK1*
-59	CTCTCTCTT	*YGR069w*
-99	CTCTCTCTT	*TDH1*
-232	CCCCTTCTT#	*TDH1*
-319	CTCTCTCTT	*XBP1*
-328	CCCCTTCTC	*XBP1*
-467	CTCTTTCTT	*YHR214w-a*
-302	CTCTTTCTT	*MTL1*
-455	CCCCCTCTC	*MTL1*
-528	CCCTCTCTC	*RNR2*
-114	CCCTTTTTC	*RNR2*
-145	CTCTCTTTT	*PRY1*
-209	CTCTCTTTT	*GSC2*
-392	CTCTTTTTC#	*GLK1*
-415	CTCTTTTTC#	*GLK1*
-89	CTCTCTTTT	*ARN1*
-240	CTCCCTTTC#	*PDR15*
-391	CCCCCTCTC#	*SRP40*
-530	CTCTTTTTT#	*YFR039c*
-517	CCCTTTTTT#	*YFR039c*
-188	CTCCCTTTT#	*TIR4*
-182	CCCCTTCTC#	*TSL1*
-406	CTCCTTTTC	*DRS1*
-212	CCCTTTTTC#	*YGP1*
-437	CCCCTTCTT#	*YGP1*
-412	CTCCTTTTT	*FIT3*

[a] Position relative to the ATG. # Means the Crick strand.

Table 4.9. Genes constitutively up-regulated in *isc1Δ* mutant cells that contain the (G/A)CAA(C/G)A(A/C)(C/A)A sequence.

Position [a]	*Sequence*	*Gene*
-641	GCAACACCA	*YDL023c*
-335	GCAACAACA#	*MTL1*
-442	ACAACACCA	*MTL1*
-670	ACAACAACA	*MTL1*
-323	GCAACAACA#	*RNR2*
-667	GCAAGACCA	*SIM1*
-574	GCAAGAAAA	*SIM1*
-565	GCAAGAACA#	*YHR214w-a*
-268	ACAACACCA#	*YHR214w-a*
-483	GCAAGAACA	*PDR15*
-36	ACAACACCA	*GLK1*
-447	ACAACAAAA	*GLK1*
-468	ACAACAAAA#	*GLK1*
-239	ACAACAACA	*PGK1*
-342	ACAAGAACA#	*DRS1*
-42	GCAACAAAA	*TIR4*
-268	GCAACAAAA#	*ROM1*
-507	GCAAGACAA	*ROM1*
-464	ACAAaAACA#	*ROM1*
-209	ACAACACAA	*PEX8*
-213	ACAACAAAA#	*YFR039c*
-570	GCAAaACCA#	*FIT3*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides.

Table 4.10. Genes constitutively up-regulated in *isc1Δ* mutant cells that contain the TTTCTTC sequence.

Position [a]	*Sequence*	*Gene*
-198	TTTCTTC	*YGR069w*
-341	TTTCTTC#	*YGR069w*
-503	TTTCTTC	*YGR069w*
-613	TTTCTTC	*YDL023c*
-414	TTTCTTC	*YFR039c*
-139	TTTCTTC	*PEX8*
-432	TTTCTTC#	*PEX8*
-34	TTTCTTC	*PRY1*
-257	TTTCTTC	*MTL1*
-429	TTTCTTC#	*TIR4*
-580	TTTCTTC#	*ROM1*
-56	TTTCTTC#	*GSC2*
-477	TTTCTTC	*GSC2*
-148	TTTCTTC#	*HXT3*
-211	TTTCTTC	*PGK1*

[a] Position relative to the ATG. # Means the Crick strand.

Table 4.11. Genes constitutively up-regulated in *isc1Δ* mutant cells that contain the A(G/A)(A/G)G(G/C)AAG(G/C/A) sequence.

Position [a]	*Sequence*	*Gene*
-547	AGGGGAAGG#	*PDR15*
-277	AGGGGAAGC	*RNR2*
-321	AG**AGGAAG**G	*YFR039c*
-117	AA**AGGAAG**C#	*YFR039c*
-121	AAGGGAAGG	*TIR4*
-519	AAAGCAAGG	*TIR4*
-601	AA**AGGAAG**G#	*SIM1*
-503	AA**AGGAAG**C#	*YOR152c*
-575	AG**AGGAAG**A	*YOR152c*
-429	AA**AGGAAG**C#	*TDH1*
-298	AAGGCAAGC	*TDH1*
-376	AGGGCAAGG#	*YDL023C*
-144	AAGGGAAGA	*PEP4*
-406	AGGGGAgGG	*PRY1*
-672	AGAGGAgGC	*GLK1*
-480	AGGcGAAGC#	*HXT3*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides. The consensus binding site for the Uasphr transcription factor, AGGAAG, is in bold.

Table. 4.12. Genes constitutively up-regulated in *isc1Δ* mutant cells that contain the (A/T)(A/G)GA(G/A)AA(G/A)A(A/G) sequence.

Position [a]	*Sequence*	*Gene*
-60	AAGAGAAGAA	*GCS2*
-152	AAGAGAAGAA	*HXT3*
-55	AAGAGAAAAA	*SIM1*
-63	AAGAGAAAAA#	*PGK1*
-201	AAGAGAAAAA	*ARN1*
-643	AAGAAAAGAA#	*YOR152c*
-571	AAGAAAAGAA	*PDR15*
-589	AAGAAAAGAA#	*FIT3*
-134	AGGAAAAGAA#	*RNR2*
-612	TAGAGAAGAA#	*YDL023c*
-166	AAGAAAAAAA#	*PEX8*
-312	TGGAGAAGAA#	*XBP1*
-532	AGGAAAAAAA#	*PRY1*
-452	TGGAGAAAAA	*YFR039c*
-522	AAGAAAAAAG#	*SRP40*
-184	AGGAGAAGgG	*TSL1*
-402	AAGAAAAAtA#	*RSB1*
-221	AAAAGAAGAA#	*PEP4*
-487	AGGAGAAtAG	*ENB1*
-420	TGGAAAAAgA	*GLK1*
-229	TAGAAAAGtG	*MTL1*
-347	AAGAGcAGgG	*FIT2*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides.

Table 4.13. Genes constitutively up-regulated in *isc1Δ* mutant cells that contain the C(T/A)A(T/C)(C/T)TT(T/C)(T/G)C sequence.

Position [a]	*Sequence*	*Gene*
-57	CTCTCTTTCC	*YGR069W*
-497	CTCTCTTTCC	*PDR5*
-145	CTCTCTTTTC	*PRY1*
-393	CTCTTTTTCC#	*GLK1*
-241	CTCCCTTTCC#	*PDR15*
-319	CTCTTTTTTC	*YOR152C*
-691	CACTCTTTCC#	*PGK1*
-261	CACTTTTTCC	*SIM1*
-188	CACCCTTTCC#	*FIT3*
-209	CTCTCTTTTG	*GSC2*
-30	CACCTTTTTC#	*YFR039C*
-189	CTCCCTTTTG#	*TIR4*
-215	CTCTCaTTTC#	*RNR2*
-30	CACTTTTTTG#	*ENB1*
-530	CTCCTaTTCC	*YHR214w-a*
-510	CACTCaTTTC#	*FIT2*
-87	CTCTTTTTgC	*ARN1*
-99	CTCTCTcTTG	*TDH1*
-12	CACCTTcTCC	*XBP1*
-211	CTCCCTTTTt#	*YGP1*
-302	CTCTTTcTTG	*MTL1*
-190	CACTTTTTCt#	*SRP40*
-581	CTtCTTTTTC#	*PEP4*
-674	tTCTTTTTTC#	*HXT3*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides.

Table 4.14. Genes constitutively up-regulated in *isc1Δ* mutant cells that contain the CTT(C/G)CT(C/T)(G/T)(T/G)T sequence.

Position [a]	*Sequence*	*Gene*
-364	**CTTCC**TCGTT#	*FIT3*
-83	CTTGCTCGTT	*XBP1*
-577	**CTTCC**TCTTT#	*YOR152c*
-488	CTTGCTCTTT#	*PDR15*
-649	CCTGCTCGTT#	*ARN1*
-170	CTTGCTTGTT#	*PRY1*
-674	CCTCCTCTGT#	*GLK1*
-579	CTTGCTCGcT#	*SIM1*
-592	**CTTCC**TTTTT#	*PGK1*
-390	CgTCCTCGTT#	*FIT2*
-429	**CTTCC**ACGTT#	*YDL023c*
-392	CgTCCTCGGT#	*PEX8*
-631	CTaGCTCGTT	*HXT3*
-573	CTTCgGTCGGT#	*MTL1*
-554	CTTCgTCTTT#	*ROM1*
-48	CTTGCTTTGT#	*SRP40*
-418	CcTGCTCTCT#	*GSC2*
-367	**CTTCC**TgTTT#	*TDH1*
-505	CcTCgTCTGT	*TSL1*
-635	CTTGCaTGTT#	*ENB1*
-553	CcTGCTTGAT	*PEP4*

[a] Position relative to the ATG. " Means the Crick strand. Small letters mean non-conserved nucleotides. The consensus binding site for the Gcr1p transcription factor, C(A/T)TCC, is in bold.

Table 4.15. Genes constitutively up-regulated in *isc1*Δ mutant cells that contain the (T/C)T(C/G)(C/G)N(C/T)CCT sequence.

Position [a]	*Sequence*	*Gene*
-110	TTCCTCCCCT#	*ARN1*
-228	CTCCCCCCCT#	*TDH1*
-352	CTCCGCCCCT#	*PEP4*
-511	CTCCTCCCCT	*SRP40*
-433	TTCGGCCCCT#	*YGP1*
-280	TTCCCCTCCT#	*RNR2*
-634	TTGCTCCCCT	*SIM1*
-137	TTGCACCCCT	*ENB1*
-406	TCCCTCCCCT#	*PRY1*
-330	CCCCGCCCCT	*TSL1*
-175	TTGGGCCCCT#	*ROM1*
-525	TTCCTCCtCT	*YFR039c*
-457	CTCCCCCtCT	*MTL1*
-414	TcCCGCTCCT	*DRS1*
-338	TTGCCCCtCT	*XBP1*
-37	TTCCGCTtTCT	*YHR214w-a*
-549	gTCGCCCCCT	*RSB1*
-246	CaACCCCCCCT	*FIT2*
-580	TTtCCCTCCT	*PGK1*
-477	TTCGAgCCCT#	*YGR069w*
-74	CTCGTtCCCT	*TIR4*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides.

Table 4.16. Genes constitutively up-regulated in *isc1Δ* mutant cells that contain the (G/A)A(A/C)A(C/G)(C/A)AGCA sequence.

Position [a]	*Sequence*	*Gene*
-632	GACA**CCAGCA**	*MTL1*
-49	GAAA**CCAGCA**	*TIR4*
-639	AACA**CCAGCA**	*YDL023c*
-573	AAAA**CCAGCA**#	*FIT3*
-436	GACACCAGCG#	*YGR069w*
-371	GACACCcGCA#	*PEP4*
-425	AACACCcGCA#	*SRP40*
-636	GgCA**CCAGCA**	*GLK1*
-35	cACACAAGCA	*PDR15*
-587	GACACAAGCg#	*YFR039c*
-583	CAAACAAGCA	*TSL1*
-182	AAAACCAtCA	*YGP1*
-543	GAAACCAaCg	*YHR214w-a*
-64	CAAACCAtCA#	*ENB1*
-577	GAtACCcGCA#	*FIT2*
-207	AACACAAtCA	*PEX8*
-37	AgAAGCAGCA	*RSB1*
-650	GAAAGAAaCA	*SIM1*
-301	AAtACCAaCA#	*HXT3*
-381	GtAACAAGCA	*PGK1*
-46	AACACAcaCA	*TDH1*
-501	cAAAGAAaCA	*ROM1*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides. The consensus binding site for the Swi5p transcription factor, (T/C)CAGC(C/A), is in bold.

Table 4.17. Genes constitutively up-regulated in *isc1Δ* mutant cells that contain the T(C/G)(C/A)(G/T)CG(G/C)(A/C) sequence.

Position [a]	*Sequence*	*Gene*
-585	**TCCGCGGA**	*YGP1*
-389	**TCCGCGGA#**	*HXT3*
-644	TtCGCGGA	*HXT3*
-312	**TCCGCGGA**	*PDR5*
-492	**TCCGCGGA#**	*PDR5*
-374	**TCCGtGGA**	*PDR5*
-588	TCgGCGGC#	*PDR5*
-380	**TCCGCGGA#**	*PDR15*
-443	cCCGCGGA#	*PDR15*
-449	**TCCGCGGA#**	*XBP1*
-567	**TCCGCGCA**	*TIR4*
-173	TCCGaGGA	*SRP40*
-558	TCAGCGGA#	*ARN1*
-539	**TCCGtGGA#**	*PEX8*
-32	TGCGCGCA	*PEX8*
-136	TCCGaGGA	*MTL1*
-287	TCCGCcGC#	*SIM1*
-208	TCAGCGaA	*YGR069w*
-696	TCAGCcCA#	*TDH1*
-352	TCCTCGCA	*ENB1*
-484	TCAGCGGt#	*YHR214w-a*
-290	TGCTCGaA	*TSL1*
-122	TCATCGCA#	*DRS1*
-684	TtCGCcCA	*GLK1*
-55	aGAGCGGA#	*PRY1*
-642	gGCGCGaA	*PGK1*
-441	**TCCGCGGA**	*XBP1*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides. The consensus binding sequence for Pdr1/3p, TCCG(C/T)GGA, TCCACGGA, TCCGCGGGA and TCCGCGCA (Nourani *et al.*, 1997; Katzmann *et al.*, 1995, 1996; Wolfger *et al.*, 1997; DeRisi *et al.*, 2000), are in bold.

Table 4.18. Genes down-regulated in *isc1*Δ mutant cells that contain the (T/A)(C/G)TGC(C/T)(T/G)(C/G)TG sequence.

Position [a]	*Sequence*	*Gene*
-214	TCTGCCTCTG#	*RPL20B*
-279	ACTGCCGCTG	*RPL42B*
-668	TCTGCTGCTG	*RPL35B*
-396	ACTGCCGCTG	*RPL38B*
-298	TCTGCCTCGgG#	*RPL27A*
-636	gCTGCTGCTG	*RPS10A*
-223	TtTGCCTCTG#	*RPS18B*
-132	TGcGCCTCTG	*RPL21A*
-632	TGcGCCTCTG	*RPL24B*
-261	TCTcCCTCTG	*RPL16A*
-221	gGTGCCGGTG	*RPS1B*
-331	TCTGCCTCTc#	*RPL37B*
-42	TCTGCTGtTG	*RPL25*
-134	AtTGCCTCTG#	*RPL9A*
-26	TCTGCTTtTG#	*RPS28A*
-254	TtTGCCTCgG#	*RPL39*
-137	TCTGCCaCTG#	*RPL32*
-95	gCTGCTTtTG#	*RPL27B*
-645	AGTGCgTCTG#	*RPS31*
-609	gGTGCCTGgG	*RPS1A*
-665	ACTcCCTGTG#	*RPS26A*
-407	TtTGCCGtgG#	*BUD19*
-515	TGcGCTGTTG	*RPL24A*
-240	TtccCCGCTG	*RPS9B*
-17	AtTcCTGCTG#	*RPL13A*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides.

Table 4.19. Genes consitutively down-regulated in *isc1Δ* mutant cells that contain the (T/C)TTT(T/C)TCCC sequence.

Position [a]	*Sequence*	*Gene*
-446	TTTTTTCCC#	*RPS20*
-492	TTTTTTCCC	*RPS1B*
-396	TTTTTTCCC	*RPS10A*
-489	TTTTTTCCC#	*URA1*
-286	CTTTTTCCC	*RPL21A*
-42	CTTTTTCCC	*RPL40B*
-114	TTTTCTCCC	*RPL24B*
-535	TTTTCTCCC#	*RPS1A*
-316	CTTTCTCCC#	*RPS26A*
-77	CTcTTTCCC	*RPL17B*
-440	CTgTTTCCC	*RPL13A*
-391	TTTTTTCCa	*RPL27B*
-381	TTTTTTCtC#	*RPL9A*
-289	TTTTCTaCC	*RPL21B*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides.

Table 4.20. Genes consitutively down-regulated in *isc1Δ* mutant cells that contain the CTT(G/T)C(T/A)G(C/A)A sequence.

Position [a]	*Sequence*	*Gene*
-175	CTTGCTGCA	*RPL27A*
-64	CTTGCAGCA	*RPL13A*
-657	CTTGCAGCA#	*RPL21B*
-490	CTTGCTGAA	*RPL16A*
-32	CTTGCTGAA#	*RPL40B*
-294	CTTTCAGCA#	*RPL17A*
-194	gTTGCTGCA	*RPS7A*
-193	CTTcCAGCA#	*RPL21A*
-596	CTTGCAGAA	*URA1*
-594	CTgGCTGCA#	*RPL32*
-254	CTTTCTGAA#	*RPS9B*
-158	CTTTCTGAA#	*BUD28*
-76	CTTTCTaCA	*RPL39*
-32	CTTGCTaAA#	*RPS10A*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides.

Table 4.21. Genes consitutively down-regulated in *isc1Δ* mutant cells that contain the (T/G)GAAAAA sequence.

Position [a]	*Sequence*	*Gene*
-575	TGAAAAA#	*RPL42B*
-697	TGAAAAA	*RPL42B*
-290	TGAAAAA#	*RPL13A*
-307	TGAAAAA#	*RPL13A*
-321	TGAAAAA#	*RPL13A*
-285	TGAAAAA#	*RPL27B*
-390	GGAAAAA#	*RPL27B*
-272	TGAAAAA	*RPL17A*
-317	TGAAAAA	*RPL17A*
-517	GGAAAAA	*RPL17A*
-161	TGAAAAA	*RPS27A*
-427	TGAAAAA#	*RPL39*
-29	TGAAAAA#	*RPL24B*
-287	TGAAAAA#	*RPL17B*
-566	GGAAAAA	*RPL17B*
-275	TGAAAAA#	*RPL9A*
-217	TGAAAAA	*RPL21B*
-312	TGAAAAA	*RPL35B*
-160	TGAAAAA#	*RPL35B*
-216	TGAAAAA	*RPL38*
-581	GGAAAAA#	*RPL38*
-28	TGAAAAA	*RPL24A*
-237	TGAAAAA	*RPL24A*
-458	TGAAAAA#	*RPL27A*
-600	TGAAAAA#	*RPL27A*
-443	TGAAAAA	*RPS7A*
-566	TGAAAAA#	*RPS7A*
-186	TGAAAAA#	*RPS28A*
-46	TGAAAAA	*RPS18B*
-338	TGAAAAA	*RPS18B*
-631	TGAAAAA	*RPS18A*
-166	TGAAAAA	*RPS9B*
-195	TGAAAAA#	*RPS9B*
-200	TGAAAAA	*RPS1A*
-442	TGAAAAA#	*RPS1B*
-491	GGAAAAA#	*RPS1B*
-505	TGAAAAA	*RPS10A*
-368	GGAAAAA#	*RPS10A*
-163	TGAAAAA	*RPS31*
-191	TGAAAAA#	*RPS31*
-191	TGAAAAA#	*RPL40B*
-41	GGAAAAA#	*RPL40B*
-164	TGAAAAA#	*URA1*
-446	GGAAAAA#	*URA1*

Table 4.21. (Cont)

Position [a]	*Sequence*	*Gene*
-488	GGAAAAA	*URA1*
-341	GGAAAAA#	*RPL13A*
-285	GGAAAAA#	*RPL21A*
-276	GGAAAAA#	*RPS17B*
-414	GGAAAAA	*RPS20*
-445	GGAAAAA	*RPS20*
-573	GGAAAAA	*RPS26A*
-255	GGAAAAA#	*BUD19*

[a] Position relative to the ATG. # Means the Crick strand.

Table 4.22. Genes consitutively down-regulated in *isc1Δ* mutant cells that contain the CCTT(C/T)CC sequence.

Position [a]	*Sequence*	*Gene*
-152	CCTTCCC#	*RPL42B*
-121	CCTTCCC#	*RPL37B*
-112	CCTTCCC	*RPS26A*
-257	CCTTCCC#	*RPS1B*
-17	CCTTCCC#	*BUD19*
-150	CCTTTCC#	*RPL21A*
-119	CCTTTCC	*RPL27B*
-515	CCTTTCC#	*RPL20B*
-442	CCTTTCC#	*RPL27A*
-196	CCTTTCC	*RPS18A*
-639	CCcTCCC	*RPL25*
-555	CCcTCCC#	*RPS26A*
-585	CCcTCCC	*RPS31*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides.

Table 4.23. Genes consitutively down-regulated in *isc1Δ* mutant cells that contain the (T/C/G)GCCT(C/G)(G/T)(G/C)C sequence.

Position [a]	*Sequence*	*Gene*
-205	CGCCTCGGC	*RPS1B*
-255	TGCCTCGGC#	*RPL39*
-239	CGCCTtGGC	*RPL39*
-224	CGCtTCGGC#	*RPL39*
-185	TGCCTGGGC#	*RPL21A*
-150	TGCCTGGGC	*RPS18B*
-597	TGCCTCGCC	*RPS28A*
-270	GGCCTCGCC#	*RPS7A*
-332	TGCCTCTCC#	*RPL37B*
-469	GGCCTGGCC	*RPL13A*
-230	GGCCTGGCC#	*RPL20B*
-640	GGCCTGTGC	*URA1*
-203	CGCCcGGGC#	*RPS31*
-609	CGCCcCGCC#	*RPL32*
-586	TGCCTCcGC#	*RPL32*
-520	TGtCTCGGC	*RPL25*
-121	CGtCTCTGC#	*RPL9A*
-638	CGtCTCTGC#	*RPL24A*
-277	TGCCgCTGC	*RPL42B*
-456	CGCCTGaGC	*RPL40B*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides.

Table 4.24. Genes consitutively down-regulated in *isc1Δ* mutant cells that contain the (T/G)G(G/A)(T/A)GAA(G/A) sequence.

Position [a]	*Sequence*	*Gene*
-681	TGGTGAAG	*RPL37B*
-341	TGGTGAAG#	*RPL21A*
-633	TGGTGAAG	*RPL21B*
-674	TGGTGAAG	*RPS1A*
-342	TGGTGAAG	*RPS31*
-496	TGGAGAAG	*RPL32*
-247	TGGAGAAG	*RPL24A*
-308	GGGTGAAG	*RPL27B*
-169	GGGTGAAG	*RPS17B*
-222	GGGAGAAG#	*RPS10A*
-291	TGGTGAAA#	*RPS7A*
-569	TGGAGAAA	*RPS20*
-632	TGAAGAAG	*RPL35A*
-597	TGAAGAAG#	*RPL9A*
-240	TGAAGAAG	*BUD28*
-149	TGATGAAA	*RPL24B*
-406	TGGgGAAG#	*RPL20B*
-220	TGAAGAAA#	*URA1*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides.

Table 4.25. Genes consitutively down-regulated in *isc1Δ* mutant cells that contain the TGTA(T/C)GG(G/A)TG sequence.

Position[a]	*Sequence*	*Gene*
-369	TGTACGGGTG#	*RPL20B*
-397	TGTACGGGTG	*RPL25*
-360	TGTA**TGGGTG**#	*RPL42B*
-280	TGTA**TGGGTG**	*RPL13A*
-218	TGTATGGGTC#	*RPL13A*
-523	TGTATGGGTG	*RPL17B*
-296	TGTA**TGGGTG**#	*RPS17B*
-320	TGTATGGATG#	*RPS17B*
-339	TGTA**TGGGTG**	*RPS10A*
-358	TGCtTGGATG#	*RPS10A*
-346	TGTACGGATG#	*RPL17A*
-364	TGTtTGGATG#	*RPL17A*
-416	TGTACGGATG#	*RPL27A*
-325	TGTACGGATG#	*RPS18A*
-310	TGcA**TGGGTG**#	*RPS18A*
-372	TGTATGGATG#	*RPS18B*
-397	TGTACGGATt#	*RPS18B*
-396	TGTATGGATG#	*RPS1B*
-366	TGTACtGGTC#	*RPS1B*
-179	TGTt**TGGGTG**#	*RPL35B*
-284	TGTACGGGTt#	*RPL35B*
-473	TGTt**TGGGTG**#	*RPS7A*
-498	TGTA**TGGGTt**#	*RPS7A*
-287	TGcACGGATG"	*RPL38*
-377	TGTtCGGATG#	*RPL42B*
-240	TGTACaGGTG#	*RPL37B*
-352	TGTACaGGTG#	*RPL21A*
-257	TGTACaGGTG#	*RPS20*
-292	TGTATGGGTC#	*RPL35A*
-404	TGTACtGGTG#	*RPL16A*
-221	TGTACGGATt#	*RPS9B*
-205	TGTACaGATG#	*RPS9B*
-263	gGTACGGATG#	*RPL21B*
-143	gGTATGGATG#	*RPL21B*
-335	TGTgTGGGTG#	*RPL27B*
-242	TGTc**TGGGTG**	*RPL40B*
-370	TGTATGGGcG	*RPL39*
-371	TGTACtGATG	*RPL9A*
-309	TGTt**TGGGTT**	*RPL9A*
-211	TGcACGGGTT#	*RPS27A*
-652	TGcATaGGTG#	*BUD28*
-177	TGTACaGAcG	*RPS28A*

Table 4.25. (Cont.)

Position [a]	*Sequence*	*Gene*
-581	TGCATGGGgG#	*RPS31*
-558	TcTATGGATG#	*URA1*
-245	TGTtTGGGTt#	*RPS1A*

[a] Position relative to the ATG. # Means the Crick strand. Small letters mean non-conserved nucleotides. The consensus binding site for the Rap1p transcription factor, TGGGT(T/G)(T/C), is in bold.

CHAPTER 5

Conclusions and perspectives

5.1 – The molecular mechanisms associated to the recovery from hydrogen peroxide stress

Unicellular organisms are continuously exposed to different types of environmental and physiological stress conditions. To cope with the deleterious effects of stress, cells have developed rapid molecular responses to survive and acquire the ability to resist to higher doses of the same or other forms of stress. Yeast cells display common molecular mechanisms of defence that are activated when cells are challenged with different stresses, such as heat-shock, ethanol, metal ions, high osmolarity or oxidants. This response is usually referred as the environmental stress response and requires a complex network of sensing and signal transduction pathways, which are however condition-specific. Indeed, transcription factors that independently regulate the expression of subsets of the environmental stress response genes in response to heat shock, osmotic shock or oxidative stress, are not involved in regulating their expression under other conditions. The activation of the signal transduction pathways leads to adjustments of the gene expression programme and metabolic activities that are essential for the adaptation of yeast cells to the new conditions.

In yeast cells, reactive oxygen species are produced as normal by-products of cellular metabolism. Under physiological conditions, the cell defence mechanisms are able to avoid molecular damages by neutralising the ROS (primary defences). This balance is disturbed when cells are exposed to stress conditions and the levels of ROS exceed the antioxidant capacity of the cells. When cellular damages occur, the repairing of molecular damages or degradation of oxidised molecules by the secondary defences are key events for cellular recovery. Hydrogen peroxide *per se* is a weak oxidant, but through its conversion into the highly reactive hydroxyl radicals by the Fenton reaction, it is able to induce oxidative damages to lipids, carbohydrates, proteins and nucleic acids (Halliwell and Gutteridge, 1999).

This work aimed at understanding the molecular mechanisms involved in the recovery from oxidative damage. We have searched for cellular functions associated with cellular recovery from hydrogen peroxide stress, and analysed the role of the ubiquitin-26S-proteasome system and vacuolar proteolysis in the degradation of carbonylated proteins during recovery.

Our results showed that H_2O_2 led to a growth arrest and decreased protein synthesis rate, but the cells were able to resume the growth and protein synthesis rates when transfered to non-stress conditions. The expression of genes associated to aminoacid and protein biosynthesis, aminoacid uptake, transcription and ribosomal proteins was down-regulated. The combined effects of the decrease in transcription and protein synthesis may

represent a mechanism for the cell to preserve mass and energy, necessary for cell adaptation to its new conditions. Supporting this idea, and since that H_2O_2 cause depletion of the ATP pool in the cell (Osorio *et al.*, 2003), genes related to ATP generation were up-regulated during cellular recovery.

To recover, cells must remove additional ROS that may be formed by reduction of H_2O_2 through the Fenton reaction, restablish the redox balance, and repair or degrade oxidatively damaged molecules. In agreement, genes encoding proteins with scavenging properties, or involved in ionic homeostasis or in control of redox potential were up-regulated. Since H_2O_2 induces oxidative damages to nucleic acids, it is not surprising the up-regulation of genes encoding enzymes involved in DNA repair, nucleotide metabolism, and DNA processing during recovery from H_2O_2 stress.

Molecular damages to lipids involve oxidation of polyunsaturated fatty acids to fatty acids hydroperoxides. The activity of the glutathione / glutathione peroxidase and thioredoxin / thioredoxin peroxidase systems has been shown to be involved in the reduction of lipid hydroperoxides generated by H_2O_2 (Moradas-Ferreira and Costa, 2000) and require NADPH as the reducing power source. The existence of such protecting mechanisms prevents lipid fragmentation. However, oxidised lipids must be replaced and synthesis *de novo* of lipid molecules should be activated. Accordingly, our results showed that genes related with ergosterol, GPI anchors, phosphatidylcholine and sphingolipid biosynthesis were up-regulated. It is conceivable that NADPH may also be required for these biosynthetic processes. The supply of NADPH is mainly provided by the pentose phosphate pathway (Godon *et al.*, 1998; Juhnke *et al.*, 1996). In aggreement, glucose-6-phosphate dehydrogenase (Zwf1p) was not oxidatively inactivated by exposure to H_2O_2 (in agreement with Shenton and Grant, 2003). Besides, Zwf1p activity increased during recovery from H_2O_2 stress, associated with an induction of *ZWF1* gene expression. In addition, the increased synthesis of glucose-6-phosphate (by up-regulation of hexocinase 1), and the down-regulation of genes encoding glycolytic enzymes and regulators of glycolytic activity during cellular recovery, supports a slowdown of the glycolytic flux favouring the pentose phosphate pathway. The rate of glucose consumption decreased during the first hours of recovery, which is consistent with the oxidative inactivation of glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase and enolase, during H_2O_2 stress (Grant *et al.*, 1999; Cabiscol *et al.*, 2000; Costa *et al.*, 2002; Shenton and Grant, 2003). The analysis of the recovery of *zwf1*Δ cells will be an important challenge for a future work, as it will contribute to our understanding the role of the molecular mechanisms involving NADPH during cellular recovery.

Protein damages induced by H_2O_2 are related to oxidation of methionine and cysteine residues. Although cysteine residues are among the most easily oxidised residues in

proteins, oxidation of cysteine to sulphenic (Cys-SOH) derivatives can be reversed by S-thiolation with reduced glutathione, thus preventing the irreversible oxidation to higher oxidation states that may lead to cell death (Coan *et al.*, 1992; Grant *et al.*, 1998; Demasi *et al.*, 2003; Shenton and Grant, 2003). It has been shown that protein S-thiolation is important for survival during exposure to oxidative stress conditions. Indeed, under physiological conditions, S-thiolation levels are low, but increased after H_2O_2 stress (Grant *et al.*, 1998). H_2O_2 also induces specific protein carbonylation, leading to an irreversible protein inactivation as there is no repair mechanism of carbonyls.

Once the proteins become carbonylated and loose their biological function, they are targeted for proteolysis. As refered above, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase is a major target oxidatively inactivated during exposure to H_2O_2, and the decrease of Tdh activity has been related both to S-thiolation and carbonylation (Grant et al., 1999; Cabiscol *et al.*, 2000; Costa *et al.*, 2002). The glyceraldehyde-3-phosphate dehydrogenase Tdh3p isozyme is the major target S-thiolated following exposure to H_2O_2, but the activity is restored after removal of H_2O_2, by dethiolation mediated by the glutaredoxin Grx5p (Shenton *et al.*, 2002). In contrast with Tdh3p, the Tdh2p isoform is not S-thiolated and its activity in only partially restored during recovery from H_2O_2 stress (Shenton *et al.*, 2002). As Grx5p activity requires NADPH as reducing power, the increase of NADPH production resulting from the increased Zwf1p activity that we observed during cellular recovery, may also be important for Tdh3p dethiolation. As both Tdh3p and Tdh2p isozymes are irreversibly carbonylated after exposure to H_2O_2 (Costa *et al.*, 2002), we raised the hypothesis that the proteins have to be targeted for degradation. Consequently, Tdh dethiolation would be insufficient to fully restore Tdh activity after H_2O_2 removal. In agreement, our results show that in addition to dethiolation, protein *de novo* synthesis partially contributed for the recovery of Tdh activity. Besides, total Tdh protein remained unchanged during cellular recovery, indicating that Tdh synthesis *de novo* and Tdh degradation occurred at similar rate. In addition, Tdh degradation was associated with a decrease of carbonylated Tdh. As Tdh2p, in contrast to Tdh3p, is not S-thiolated, the turnover of Tdh2p protein could be somehow affected by the lack of protection from oxidation to higher oxidation states. However, using *tdh3Δ* and *tdh2Δ* mutants, we showed that the rate of protein turnover during cellular recovery were similar for Tdh2p and Tdh3p. In addition, the restoration of Tdh2p and Tdh3p activities was correlated with the decrease of carbonylated Tdh2p and Tdh3p, respectively, although Tdh2p activity recovered at slower rate. Taken together, these results indicate that S-thiolation does not affect protein turnover. Our results also demonstrate that the increased sensitivity to H_2O_2 previously described for *tdh3Δ* mutants (Grant *et al.*, 1999) is not associated with higher basal or H_2O_2-induced protein

carbonylation, or with a decreased capacity to turnover carbonylated proteins during cellular recovery.

It is possible that both the proteasome pathway and the vacuolar proteases mediate the degradation of irreversibly oxidised proteins. Indeed, in *S. cerevisiae*, enzymes of the proteasome pathway and vacuolar proteases are induced by H_2O_2 (Godon *et al.*, 1998; Lee *et al.*, 1999a; Gash *et al.*, 2000). The proteasome in cells is present in two major forms, the 20S and the 26S proteasome. The 26S proteasome is a complex consisting of two 19S regulatory subunits and one 20S catalytic core. Several studies suggest that the 20S proteasome rather than the ubiquitin-26S proteasome pathway, is responsible for the degradation of oxidised proteins: i) no multiubiquitination is observed in H_2O_2 treated cells and ubiquitin-conjugating and –activating enzymes are inhibited by oxidative stress; ii) H_2O_2 treatment increases the activity of the 20S proteasome, which degrades oxidised proteins independently of ATP and ubiquitination, and concomitantly represses the activity of 26S proteasome (Shringarpure *et al.*, 2003); iii) yeast cells deficient in the 19S regulatory particle exhibits higher 20S proteasome activity and degrade carbonylated proteins more efficiently that wild type cells (Inai and Nishikimi, 2002). The upregulation of genes encoding subunits of the 20S core proteosome during cellular recovery is in agreement with the role of the 20S proteosome in the turnover of oxidised proteins.

Since cellular recovery after H_2O_2 stress was correlated with the turnover of carbonylated proteins, it would be of interest to clarify the involvement of the ubiquitin-proteasome system and vacuolar proteolysis in this process. The role of the ubiquitin-proteasome system was assessed using *doa4Δ* cells, which contain reduced levels of free ubiquitin (Swaminathan *et al.*, 1999). We show that the turnover of carbonylated proteins is not affected in cells deficient in Doa4p. It has been suggested that lysosomal proteases may operate sequentially or in parallel with the proteasomal system in the degradation of oxidised molecules (Dunlop, 2002). Supporting this hypothesis, we show that the constitutive levels of oxidised proteins is higher is *pep4Δ* mutants, deficient in the vacuolar protease Pep4p, and that the turnover of carbonylated proteins occurred at a lower rate. These results indicate that Pep4p plays a major role in the turnover of proteins oxidised under physiological conditions and during recovery from oxidative stress. In agreement, microarray analysis showed that the *PEP4* and *LAP4* vacuolar protease genes, and genes associated with protein traffic to the vacuole were induced after H_2O_2 stress and during recovery from oxidative stress, in contrast to *DOA4*, which was not induced. Taken together, these results suggest that the 26S-proteasome is not involved in the degradation of oxidised proteins, and point to a role of the vacuolar system in the degradation of oxidised proteins. Consequently, the up-regulation of genes encoding one component of the proteasome regulatory particle and ubiquitin activating- and conjugating enzymes, does not seem to be associated with the

turnover of oxidised proteins, but instead to another cellular function important for cellular recovery. The analysis of Tdh turnover using *pep4Δ* mutant cells, and the analysis of Pep4p activity during recovery of wild type cells from H_2O_2 stress will be an important future experiment to support the role of Pep4p in degradation of carbonylated proteins indicated by our results.

Several studies have provided evidence that the oxidation of proteins is a denaturing process similar to heat denaturation that exposes hydrophobic aminoacid residues from the protein's interior (Davies, 1987). Under stress conditions, these damaged proteins can be stabilized by molecular chaperones, which facilitate their proper refolding or in some cases, may help to target them for proteolytic degradation (Hayes and Dice, 1996). In agreement, genes encoding molecular chaperones were also specifically induced during cellular recovery from H_2O_2 stress. The up-regulation of 5 genes encoding enzymes of the aminoacid catabolic pathway is also consistent with this enhanced proteolytic activity during cellular recovery, as protein degradation leads to an increased pool of aminoacids.

5.2 – The key role of Isc1p sphingomyelinase in cell resistance

Sphingolipid metabolism plays a role in signal transduction from yeast to mammalian cells. In mammals, ceramide is linked to the activation of signal transduction pathways that control a variety of cellular processes, including the cell cycle, apoptosis and senescence (Ohanian and Ohanian, 2001). Ceramide is synthesised either by *de novo* synthesis or through the metabolism of sphingomyelin. In mammals, the accumulation of ceramides by activation of sphingomyelinases is induced by different stress conditions, including UV light, heat shock, DNA damage, and oxidative stress (Hannun and Luberto, 2000). How ceramide activates signal transduction pathways is not well understood, but it is reported that it inhibits protein kinase C and activates ceramide-activated protein phosphatases (Chalfant *et al.*, 2001; van Blitterswijk *et al.*, 2003).

The yeast *S. cerevisiae* contains an inositol phosphosphingolipid phospholipase C, Isc1p, which has a phosphatidylserine-dependent neutral sphingomyelinase (nSMase) activity and 30% identity to mammalian nSMase2. Isc1p hydrolyses inositol phosphosphingolipids (IPS), releasing ceramide and the polar head groups. We showed that cells deficient in the Isc1p are specifically sensitive to hydrogen peroxide. Indeed, the disruption of *ISC1* did not result in a general stress sensitivity. The resistance to acetic acid, ethanol and heat shock, in contrast to H_2O_2, increased by *ISC1* disruption. This enhanced sensitivity to hydrogen peroxide is likely to be associated with alterations on sphingolipid

signalling mechanisms. Therefore, one of the aims of this work was to address the role of Isc1p in the regulation of oxidative stress resistance.

The results revealed that Isc1p is essential for constitutive but not induced H_2O_2 resistance. The levels of antioxidant defences and heat shock proteins are key factors for cell resistance. However, we provide evidences that the higher sensitivity of *isc1*Δ cells is not associated with a decrease of the antioxidant capacity or ATP levels. In addition, these cells are able to induce the major antioxidant defences and heat shock proteins as part of the adaptive response to H_2O_2.

Oxidative stress-induced cell death has been associated with the oxidative inactivation of specific proteins. Indeed, the accumulation of oxidised proteins is correlated with stress sensitivity, as protein carbonylation was enhanced in yeast cells deficient in the stress response regulators Yap1p and Skn7p, which are hypersensitive to H_2O_2 (Costa *et al.*, 2002). Our results showed that the oxidative-stress sensitivity of *isc1*Δ cells is associated to an increase in the constitutive protein carbonyl content. In addition, the H_2O_2 induced protein carbonylation and lipid peroxidation also increased in *isc1*Δ cells. The overall results suggest that ceramide generated by Isc1p is not important for cell adaptation, but may regulate some cellular functions or structures important for oxidative stress resistance. The analysis of the changes in the transcriptome of *isc1*Δcells was performed to clarify the mechanisms of regulation.

The results show an increased expression in *isc1*Δ cells of genes associated with the secretory pathway, as well as the vacuolar protease Pep4p. Pep4p plays an key role in the vacuolar degradation of carbonylated proteins (Chapter 2) and vesicle trafficking is important for protein delivery to organelles, as well as for membrane recycling. According with the increased constitutive carbonyl content of *isc1*Δ cells, it is conceivable that the up-regulation of those genes may be associated with membrane recycling and degradation of carbonylated proteins, which are key events for the survival of *isc1*Δ cells. However, the constitutive up-regulation of *PEP4* gene expression in *isc1*Δ cells seems not to be enough to counteract the formation protein carbonyls, as constitutive carbonyl levels remain higher than in wild type cells.

The increased sensitivity to H_2O_2 and the higher constitutive levels of carbonylated proteins of *isc1*Δ cells may be associated to the increased iron uptake in *isc1*Δ cells. Indeed, the expression of four Aft1p (major iron dependent transcription factor) target genes related to iron transport is up-regulated in *isc1*Δ cells. This can result in iron overload that may lead to the generation of hydroxyl radicals by the Fenton reaction and to an increased production of protein carbonyls. Hydroxyl radicals are also able to cause damages to DNA. Consistently, two genes normally induced by DNA damages (*RNR2* and *XBP1*) are up-regulated in *isc1*Δ

cells. As a future challenge, it would be interesting to quantify the levels of iron and the levels ROS (assessed by epifluorescence microscopy and flow cytometry) in *isc1Δ* cells.

Our results suggest that the changes in the transcriptome of *isc1Δ* cells may be indirectly mediated by several transcription factors, namely Xbp1p, Pdr1/3p, Swi5p, Gcr1p, Uasphp, and Rap1p. The lack of up-regulation of other known targets of Pdre1p/Pdr3p is not surprising, as Pdr1p/Pdr3p dependent gene regulation varies considerably between different promoters, probably due to other factors present in the respective context (Wolfger *et al.*, 1997). Besides, the existence of binding sequences for Xbp1p both in the up- and down-regulated genes is not surprising. In fact, the transcription factor Rap1p may act both as a transcriptional activator and as a repressor, depending on the DNA sequence binding context (Piña *et al.*, 2003). In a future work, the use of double mutants (*isc1Δpdr1Δ*, *isc1Δpdr3Δ*, *isc1Δxbp1Δ*, *isc1Δswi5Δ*, *isc1Δgcr1Δ*, *isc1Δuasphr*Δ, and *isc1Δrap1Δ*) would be very useful to clarify the involvement of Pdr1p, Pdr3p, Xbp1p, Swi5p, Gcr1p, Uasphrp and Rap1p transcription factors in mediating the changes observed in the transcriptome of *isc1Δ* cells. Northern Blot analysis should be performed for the genes that contain binding sequences for these transcription factors in their promoters. The purification and identification of proteins that bind to the other motifs retrieved in this study is also a major goal for a future work.

Several evidences seem to point to a constitutive stressful event in *isc1Δ* cells. In addition to the constitutive higher levels of carbonylated proteins, most of the genes that are constitutively down-regulated in *isc1Δ* cells are ribosomal protein genes. The silencing of RP genes is frequent in response to a variety of stimuli and may help to preserve mass and energy under stress conditions. In addition, it is known that changes in the composition and structure of the cell wall usually occur in response to stress conditions, and that are essential for cell survival (Klis *et al.*, 2002; Popolo *et al.*, 2001; Delley and Hall, 1999. Curiously, the transcriptome analysis also revealed the up-regulation in *isc1Δ* cells of several genes involved in cell wall biogenesis, transduction of cell wall stress signals or genes known to be up-regulated in response to changes in the cell wall.

The overall results suggest that *ISC1* disruption may lead to a remodelling of the cell wall that may be related to the accumulation of IPC or $M(IP)_2C$, and as a consequence, the lipid signalling pathway is negatively affected.

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