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Abstract

Programmed cell death (PCD) has been documented not only in higher organisms but also bacteria and yeast. Proteins of the Bcl-2 family are important regulators of mammalian cell life and death, with some functioning to prevent (Bcl-2) and others to promote (Bax) apoptosis. Old yellow enzymes (OYE) group of FMN- oxidoreductases has been extensively characterized with regard to biochemical catalysis but little information exists on the biological functions of these proteins. In a genetic screen to identify modifiers of Bax dependent lethality in yeast, the C-terminus of OYE2 was isolated based on its capacity to restore sensitivity to a Bax-resistant yeast mutant strains. Overexpression of full length OYE2 suppresses Bax lethality in yeast, lowers endogenous reactive oxygen species (ROS), increases resistance to H_2O_2-induced programmed cell death (PCD) and significantly lowers ROS levels generated by organic prooxidants. Reciprocally, *Δoye2* yeast strains are sensitive to prooxidant-induced PCD. Overexpression and knockout analysis indicates these OYE2 antioxidant activities are opposed by OYE3, a highly homologous heterodimerizing protein, which functions as a prooxidant promoting H_2O_2-induced PCD in wild type yeast. OYE3 requires the presence of OYE2 to promote cell death, since the deletion of the 12 C-terminal amino acids and catalytic inactivation of OYE2 by a Y197F mutation enhance significantly survival upon H_2O_2-induced PCD in wild type cells, but accelerate PCD in *Δoye3* cells, implicating the oye2p-oye3p heterodimer for promoting cell death upon oxidative stress. Unexpectedly, a strain with a double knockout of these genes (*Δoye2 oye3)* is highly resistant to H_2O_2-induced PCD, exhibits increased respiratory capacity, and undergoes less cell death during the adaptive response in chronological aging. Simultaneous deletion of OYE2 and other antioxidant genes hyperinduces endogenous levels of ROS, promoting H_2O_2-induced cell death and sensitize towards αβ-unsaturated aldehydes (acrolein and methylvinyl ketone: in *Δoye2 glr1* yeast high levels of oxidized glutathione (6 fold than wild type) elicited gross morphological aberrations involving the actin cytoskeleton, and defects in organelle partitioning. OYE cooperate in enhancing the survival with knockouts of mitochondrial apoptoic remarks regulators (Δaif1, Δcyc1) but not cytoplasmic and nuclear (Δyca1, Δnma111). Based on this work, OYE proteins are firmly placed in the signaling network connecting ROS generation, PCD modulation and cytoskeletal dynamics in yeast.

Introduction

1. Introduction

Yeast is a collective term for ascomycetous and basidiomycetous fungi. S*accharomyces cerevisiae*, an ascomycetous eukaryotic unicellular organism proliferates by budding, with a small "daughter cell" growing form the "mother cell" which gradually increase in size. Yeasts are amenable fast growing facultative anaerobe organisms as a whole, easy to cultivate and offer a wide spectrum of genetic (small genome of 16 chromosomes, homologous recombination) and cytological tools. All these characters make the *s. cerevisiae* a favorite tool for genome wide exploration (Froehlich *et al*., 2007).

The lives of many cells in multicellular organisms seem to follow a Samurai principle: it is better to die than to be wrong. Complex biological systems are equipped with programs that eliminate portions of the system that become dangerous and unnecessary for the system as a whole. One mechanism of programmed cell death is apoptosis (Longo *et al*., 2005). The term was coined in 1972, referring to this morphologically defined form of cell death (Kerr *et al*., 1972). Apoptosis is an evolutionarily conserved mechanism by which the multicellular organisms regulate cell number. The proper regulation of this process is critical during cell accumulation and development, as well as during tissue homeostasis in adult organisms. The onset of apoptosis in an individual cell can be triggered by environmental cues, including specific signaling cascades or an insufficiency of survival factors (Harris and Thompson, 2000).

1.1 Apoptosis in mammalian system

Apoptosis takes place in a variety of biologically significant situations including embryogenesis, organogenesis and the maintenance of homeostasis, as well as normal function of the immune system (Cohen *et al.* 1992; Wyllie 1997a; and Cummings *et al*., 1997). Abnormal regulation of apoptosis has been implicated in the onset and progression of diseases both in the form of inhibited and excessive apoptosis (Thompson, 1995; Antonsson, 2001).

Apoptosis is associated with a set of characteristic changes called "apoptotic remarks" such as the shrinking of cells, fragmentation of cells, fragmentation of nuclei,

condensation and fragmentation of chromatin, fragmentation of DNA, reduction of the mitochondrial membrane potential, release of cytochrome c, increased caspase activity, and focal phagocytic elimination of the cells by surrounding cells and macrophages (Yamaki *et al.,* 2001)

During apoptosis, the dying cell separates from its neighbors and undergoes a period of membrane blebbing, condensation of cytoplasm and increase in cell density. Simultaneously the nuclear chromatin becomes compact, segregates and forms sharply delineated masses along the nuclear envelope. The nucleus splits into discrete fragments and finally, the cell splits into a cluster of membrane bound apoptotic bodies, each containing a variety of organelles. Apoptotic bodies are ingested by nearby cells and macrophages before they cause an inflammatory reaction (Kerr *et al*., 1972, Kerr *et al*., 1994). The morphologically visible process of apoptosis takes a few hours, and the majority of the time is spent on the degradation within the phagocytic cells (Wyllie *et al.,* 1997a).

1.2 Activation Pathways of Apoptosis

Apoptosis can be induced by a variety of physiologic, damage-related and therapy-associated agents (Thompson, 1995; Wyllie, 1997b). Two major apoptotic pathways have been identified: the death receptor pathway (extrinsic), and the mitochondrial pathway (intrinsic). The death receptor pathway involves at least five transmembrane receptors belonging to the TNF (tumor necrosis factor)/NGF (nerve growth factor) receptor superfamily **(**Timmer *et al*., 2002**;** Itoh *et al*., 1991). The mitochondrial pathway is mediated by mitochondrial membrane permeabilization and the release of cytochrome c (Antonsson *et al.,* 2001). In both of these pathways, the final result is the activation of the so-called caspase cascade, which leads to proteolysis of structural and regulatory proteins and cell death. (Antonsson, 2001; Timmer *et al*., 2002).

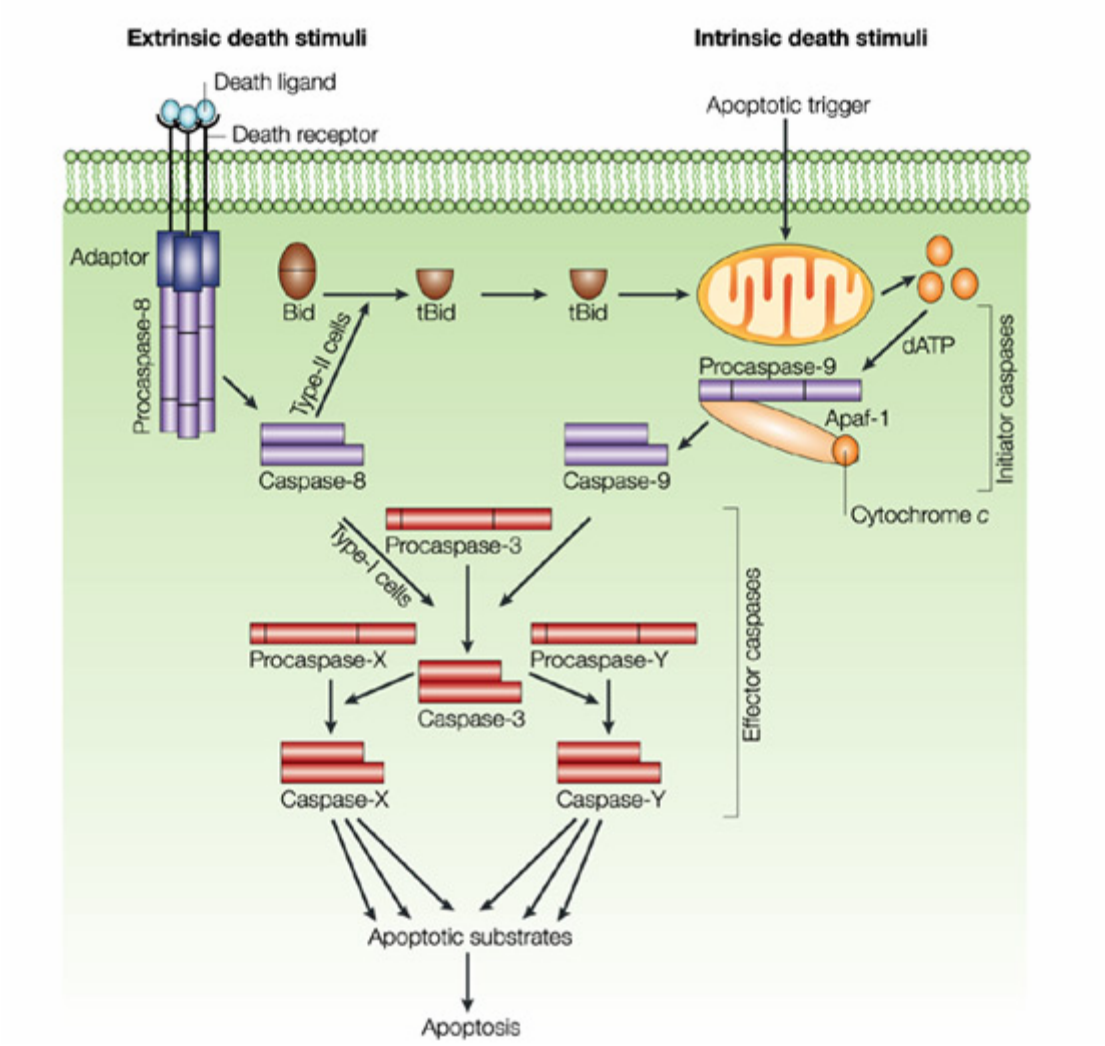


Figure 1.1. Intrinsic (mitochondrial) pathway and extrinsic (receptor) pathway of apoptosis, each of which involves the activation of specific initiator caspases such as caspase-8 and caspase-9. Both routes eventually converge at the level of caspase-3 activation. Downstream of the effector caspase-3, several signals serve to mediate apoptosis. Although the two routes operate largely independently of each other under most conditions, crosstalk can be provided by BH3-interacting-domain death agonist (Bid), the known pro-apoptotic Bcl-2 family member (Jesenberger and Jentsch 2002).

1.2.1 Death receptor or extrinsic pathway

Receptors such as tumournecrosis factor (TNF)-receptor 1 or Fas/CD95 interact with their cognate ligands (death ligands), which induces the formation of a death-inducing signaling complex (DISC) (Figure 1). This complex recruits several procaspase-8 molecules through an adaptor molecule such as Fas-associating death domain (FADD)/Mediator of receptor-induced toxicity (MORT1), which results in cross-activation of caspase-8 and the release of active caspase-8 into the cytosol. In ‘type-I cells’, sufficient levels of caspase-8 are

generated to directly initiate the effector caspase cascade through caspase-3. In 'type-II cells', low levels of caspase-8 cleave the pro-apoptotic Bcl-2 family member Bid, which results in the formation of a truncated protein, tBid, which works with other Bcl-2 family members to trigger the mitochondrial pathway. FasL is a type II membrane protein that belongs to the TNF family and is expressed predominantly in activated T lymphocytes and in tissues including small intestines, kidney, testis, and lung (Suda *et al*., 1993).

1.2.2. Mitochondrial or intrinsic pathway

Stress-induced signals such as DNA damage initiate the release of cytochrome *c* and other apoptogenic factors, such as the apoptosis-inducing factor (AIF), endonuclease G, and second mitochondria-derived activator of caspase (Smac)/DIABLO from the mitochondrial intermembrane space. AIF and endonuclease G probably function in apoptosis by exerting effects on nuclei, whereas Smac/DIABLO sequesters inhibitor of apoptosis (IAP) proteins away from caspases. Cytochrome *c* binds to the adaptor molecule apoptotic protease activating factor 1 (Apaf-1) and, in the presence of ATP or dATP, this complex recruits and activates the initiator caspase-9. Activated caspase-9 can, in turn, activate caspase-3, which is responsible for the execution of the cell (Jesenberger and Jentsch 2002).

1.3 Yeast Programmed Cell Death or Phenoptosis

Accumulating studies indicate that yeast undergo programmed cell death for several good reasons (Fabrizio *et al.,* 2004; Herker *et al.,* 2004; Knorre *et al.,* 2005; Reiter *et al.,* 2005; Allen *et al.,* 2006). Since this is a unicellular organism, the phenomenon is rather characterized as phenoptosis, or programmed cell death of an organism (Skulachev, 2006**)**. Unicellular organisms manifest genetically regulated cell death; many recent results support that yeast might have a mechanism for apoptosis. Firstly, the expression of human Bax or Bak led to cell death in S. *cerevisiae* and S. *pombe,* (Sato *et al.,* 1994; Zha *et al.,* 1996; Ink *et al.*1997; Ligr *et al*., 1998) and was inhibited by the co-expression of Bcl-2, (Ligr *et al.,* 1998; Xu and Reed 1998); likewise the introduction of *ced-4* induced cell death in both S. *cerevisiae* (James *et al.,* 1997) and S. *pombe* (Tao *et al.,* 1999). Secondly, the mutation in the cell division cycle gene CDC48 prompted the appearance of several apoptotic remarks in S. *cerevisiae* (Madeo *et al.,* 1997). Thirdly, the identification of a homologous gene of the

mammalian inhibitor of apoptosis (IAP), in both S. *cerevisiae* and S. *pombe* (Deveraux and Reed, 1999). Like mammalian cells, yeast cells (*Saccharomyces cerevisiae*) undergoing apoptosis show characteristic markers such as DNA cleavage, apoptosis-typical chromatin condensation, externalization of phosphatidylserine and cytochrome C release from mitochondria (Madeo *et al.,* 1997; Manon *et al.,* 1997; Ludovico *et al*., 2002).

1.4 Mitoptosis

The term mitoptosis was coined to describe suicide of the mitochondrion. Such a process is suggested to operate as a mechanism, ridding the mitochondrial population in the cell from malfunctioning organelles; for example those which overproduce reactive oxygen species (ROS). Its noteworthy that the mitoptosis elimination of a mitochondrion does not require any extra-mitochondrial components (Skulachev, 2006).

1.5 Necrosis

Cell death in multicellular organisms proceeds through two distinct pathways, apoptosis and necrosis. Apoptosis, or programmed cell death is an "active" cell death. Necrosis is an accidental "passive" cell death that is caused by chemical and physical stresses. It appears that the translocation of AIF from a mitochondrial to an extramitochondrial localization occurs both in apoptosis and necrosis and thus may participate in the common death pathway. Table 1 shows a comparison between apoptosis and necrosis. Necrosis presents remarkable changes such as swelling of the cells, disintegration of nuclei, loss of chromatin, swelling and breakage of mitochondria, leakage of cytoplasm, and inflammation of neighboring cells (Yamaki *et al*., 2001). The paradigmatic opposition between apoptosis and necrosis is less clear than this; it has been generally thought that necrosis can be prevented in some cases by Bcl-2. Thus, necrosis can be prevented in some cases by Bcl-2 and may involve an early mitochondrial permeabilization event (Shimizu, *et al.,* 1996; Kroemer *et al.,* 1998).

Table 1. Characteristics distinguishing apoptosis from necrosis

Defining features	Apoptosis	Necrosis
Physiologic/pathologic features		
Cellular role	Usually normal	Abnormal, accidental
Process	Active, energy dependent	Passive, results from lack/loss of energy
Distribution	Dispersed affects individual cells	Contiguous, simultaneous, and massive affects in damaged tissue areas
Triggers	100s of physiologic and noxious stimuli	Sudden transfer of energy, specific toxins, or ATP depletion
Induction	Slow (hours),	Rapid (seconds, minutes)
Tissue inflammation	Absent	Present
Cell removal	Rapid and discrete	slow
Morphologic features		
Cellular membranes	Integrity preserved, blebbing of intact plasma membrane	Loss of integrity, with spilling of cell constituents
Cell volume	Decreased, as well as the formation of small, fragmented "apoptotic bodies" or inclusions	Increased
Organelle structure	Late preservation, with exception of nuclear condensation and fragmentation	Swelling of nucleus and other organelles
Chromatin	Discrete, organized condensation, margination and fragmentation	Pattern conserved
Biochemical and molecular features		
Mitochondrial permeability transition	Moderate	Severe
Mitochondrial membrane potential (delta psi-m)	Transient loss	Permanent loss
Requirement for ATP	Yes	No
Membrane phospholipid asymmetry	Exteriorization of phosphatidylserine from inner to outer leaflet of plasma membrane	Unchanged
Cell pH	Acidification	Unchanged
DNA cleavage	Initial specific large cleavage products of 300, then 50, kbp, followed by Inter nucleosomal cleavage leading to DNA ladder pattern of 180 bp unit repeats	Random DNA cleavage
Caspase dependence	Yes	No

2. Molecular machinery of yeast apoptosis

Reactive oxygen species (ROS), the central regulator in the metazoan apoptosis, has also been identified as essential in at least some yeast apoptosis, indicating that the similarity

between the two processes is not restricted to the appearance (Madeo *et al.,* 1999). The past several years have seen the discovery of several yeast orthologues of crucial apoptotic regulators. These findings have firmly established that yeast and metazoan apoptosis are, in essence, the same cellular program, and lay the foundation for using yeast as a tool for apoptosis research. The observation that old yeast cells, as well as dying yeast cells in stationary phase, produce oxygen radicals and die apoptotically provide possible clues to a similar sequence of events in mammalian ageing (Laun *et al.,* 2001; Herker *er al.,* 2004). Mitochondria, both as an essential organelle targeted for destruction and as a perpetrator of the death pathway, play a central role in mammalian apoptosis**.** Crucial proteins of the basic molecular machinery executing cell death are conserved in yeast, including the yeast caspase YCA1, the mitochondrially located proteins apoptosis-inducing factor1 (AIF1), AMID (NDI1), HtrA2/Omi (NMA111), and the antiapoptotic proteins CDC48 and BIR1 (Buttner *et al.,* 2007).

2.1 Caspases

The cell death process is characterized by a distinct set of morphological and biochemical features, mediated by a class of specific Cys proteases called **c**ysteinyl **asp**artate specific proteinases (caspases). Iterative homology research has recently revealed two new groups of caspase related Cys proteases, designated paracaspases in humans and *C. elegans* and metacaspases in fungi, yeast and plants. The tertiary structure of caspases is conserved to the so-called "caspase hemoglobinase fold". The metacaspase present in *S. cerevisiae* YCA1 have a caspase-like fold (Uren *et al.,* 2000) and dispose caspase-like proteolytic activity which is activated when yeast is stimulated by H_2O_2 and acetic acid (Madeo et al., 2002).

Caspases are specific proteases that show high a degree of specificity with an absolute requirement for a cleavage adjacent an Asp residue and a recognition sequence of at least four amino acids N-terminal to this cleavage site. Caspases are synthesized as inactive pro-enzymes and are activated by directed proteolysis that removes the N-terminal peptide and cleaves the proteolytic domain at a specific recognition site. Caspases are divided into groups based on their substrate preferences and the extent of sequence and structural similarities (Woltering *et al.,* 2002).

In general, apoptotic cell death involves a sequence of caspase activation events in which initiator caspases activate downstream executioner caspases which process a variety of target proteins, eventually leading to apoptotic phenotype. Caspase-8 is the key initiator caspase in the death-receptor pathway. Upon ligand binding, receptors aggregate from membrane–bound complexes, which then recruit through adaptor proteins several procaspase-8 molecules which, under these conditions, cleave and activate each other. Auto processing and activation of initiator caspase-9 is mediated through association with specific activators such as Apaf1 and cytochrome c. Together, these proteins form a large protein complex (the apoptosome) that may also contain additional proteins. The initiator caspases may directly processs and activate executioner caspases 3, 6 and 7 (Woltering *et al.,* 2002). Deletion of the yeast caspase gene *YCA1* enhances the resistance against oxidative stress and also delays age-induced cell death (Madeo *et al.,* 2002), although caspase-independent apoptosis also occurs in yeast as well (Guscetti *et al.,* 2005).

2.2 NMA111

High temperature requirement A (HtrA) and its homologues constitute the HtrA familiy proteins, a group of heat shock-induced serine proteases. A defining feature of the HtrA protein family is the combination of a catalytic serine-protease domain with at least one PDZ domain (Pallen and Wren, 1997; Harris and Lim, 2001; Clausen *et al.,* 2002), and a temperature-dependent switch mechanism, which effects the change in its function from molecular chaperone to protease. This mechanism is also related to substrate recognition and the fine control of its function (Kim, 2005).

PDZ domains are small globular domains that act as protein-protein recognition modules and are involved in many signalling cascades (Clausen *et al.,* 2002). Bacterial HtrA/DegP is a heat-inducible serine protease with two C-terminal PDZ domains that is essential for viability of bacteria after heat shock (Skorko-Glonek *et al.,* 1995). Moreover, it has a dual function because it degrades misfolded proteins at higher temperatures but acts as molecular chaperone at low temperatures (Spiess *et al.,* 1999). The yeast HtrA-like protein Nma111p was identified by yeast two-hybrid screens. Nma111p is a nuclear protein and, like

its mammalian homologue HtrA2/Omi, plays a crucial role in yeast apoptosis. The pro-apoptotic activity of Nma111p is dependent on its serine-protease activity. An *nma111* deletion yeast strain has an enhanced thermotolerance at elevated temperatures and shows increasing survival rates after the induction of apoptosis by H_2O_2. By contrast, over expression of Nma111p leads to a decrease in the survival rate of yeast cells treated with H_2O_2 (Fahrenkrog *et al.,* 2004). Nma111p is evenly distributed in the nucleus at 25°C but the protein aggregates in the nucleus at 42°C and at 50°C. In addition, at 50°C, some cells exhibited translocation of Nma111p into the cytoplasm, but not mitochondria. Similarly, under apoptotic conditions, i.e. after treating the cells with (3 mM H_2O_2), these observations show that the localization of Nma111p is distinctly altered inside the nucleus, both at elevated temperatures and under apoptotic conditions (i.e. by oxygen stress), and that this redistribution within the nucleus might be directly related to the apoptosis promoting effect of Nma111p (Fahrenkrog *et al.,* 2004).

2.3 Glutathione in defense against oxidative stress

All aerobic organisms are exposed to reactive oxygen species (ROS), such as peroxide H_2O_2, the superoxide anion O^-_2 and the hydroxyl radical, during the course of normal aerobic metabolism or after exposure to radical-generating compounds. Such ROS can cause wide-ranging damage to macromolecules and have been recognized for some time as being associated with various disease processes including cancer, ageing and neurodegenerative disorders (Halliwell and Gutteridge, 1989; Gutteridge, 1993). To protect against oxidant damage, cells contain effective defence mechanisms including enzymes such as catalase, superoxide dismutase and glutathione peroxidase and antioxidants, such as **glutathione (GSH)** and vitamins C and E (Yu, 1994).

Cysteine residues are among the most easily oxidized residues in proteins, resulting in intermolecular protein cross-linking and enzyme inactivation. Such irreversible oxidation events can be prevented by protein S-thiolation, in which protein -SH groups form mixed disulphides with low-molecular-weight thiols such as GSH (Thomas *et al.,* 1995a). Protein S-thiolation is reversible, and dethiolation can occur via direct reduction by GSH. In addition,

protein dethiolation can be catalysed in vitro by glutaredoxins and thioredoxins. A recent study has indicated a correlation between protein ± SSG reduction and glutaredoxin activity in mammalian cells (Chrestensen *et al.,* 2000). Protein S-thiolation may, therefore, represent a novel form of post-translational modification which can regulate protein activity in response to growth or other cellular signals and, for example, has been implicated in the regulation of the HIV-1 protease under the control of glutaredoxin (Davis *et al.,* 1997) and in regulating ubiquitin-conjugating enzymes in bovine retina cells (Obin *et al.,* 1998).

GSH is an important antioxidant, as yeast strains lacking GSH or altered in their GSH redox state are sensitive to oxidative stress induced by peroxides and the superoxide anion, as well as the toxic products of lipid peroxidation (Grant *et al.,* 1996a). Exponential phase cells under normal aerobic conditions have a high redox ratio (GSH±GSSG) of almost <11:1 (Grant *et al.,* 1996b; Muller, 1996), indicating that most of the intracellular glutathione is maintained in a reduced (GSH) form. Exposure of yeast cells to H_2O_2 causes a reduction in GSH levels, as well as a shift in the redox balance towards the more oxidized form (Grant *et al.,* 1998). Of the total glutathione detected, most is present in a free (unbound), form either intracellularly (58%) or extracellularly (39%). In response to a challenge with H_2O_2, oxidized GSSG, protein-bound (GSSP) and extracellular glutathione are all elevated in parallel with the reduction in intracellular GSH levels. This increase in oxidized GSSG is consistent with the role of GSH as both a free radical scavenger and a cofactor for various antioxidant enzymes, including glutathione peroxidases, glutathione S-transferases and glutaredoxins (Grant *et al.,* 1998).

2.3.1 Biosynthesis of GSH

The levels of reduced glutathione (g-glutamylcysteinylglycine, GSH) are maintained by a complex series of reactions that balance its rate of synthesis and reduction (Fig. 2). The enzymes involved in the biosynthesis (Gsh1, g glutamylcysteine synthetase; and Gsh2, glutathione synthetase) and redox regulation (Glr1, glutathione reductase) of GSH have been well characterized (Grant and Dawes, 1996). A homologue of the mammalian enzyme that initiates the breakdown of GSH (g-glutamyltranspeptidase) has been identified by the yeast genome sequencing project (YLR301w) (Manning et al., 1997). g-Glutamyltranspeptidase

activity has also been detected in yeast and appears be required for supplying growth requirements such as sulphur and nitrogen from intracellular GSH pools (Penninckx and Elskens, 1993).

2.3.2 The glutaredoxin system

Yeast contains two glutaredoxin genes, designated GRX1 and GRX2 (Fig. 3), which share 61±76% similarity with those from bacterial and mammalian species (Luikenhuis *et al.,* 1997). Strains deleted for both GRX1 and GRX2 are viable but lack heat-stable oxidoreductase activity. The *grx1* mutant is sensitive to oxidative stress induced by the superoxide anion, whereas a strain that lacks GRX2 is sensitive to hydrogen peroxide, GRX2 accounts for the majority of this activity in vivo. This difference in oxidant sensitivity may reflect differences in the substrate proteins regulated by Grx1 and Grx2 or in their ability to detoxify ROS-mediated damage and is consistent with the idea that the two glutaredoxin isoforms play distinct roles during normal growth and stress conditions (Grant, 2001).

Glutaredoxins and thioredoxins are small heat-stable oxidoreductases which contain two conserved cysteine residues in their active sites (Holmgren, 1989). They were originally identified as hydrogen donors for ribonucleotide reductase, but are also required for a number of metabolic enzymes that form a disulphide as part of their catalytic cycle. Proposed roles include many cellular processes, such as protein folding and regulation, reduction of dehydroascorbate, repair of oxidatively damaged proteins and sulphur metabolism. Despite considerable functional overlap, they are differentially regulated. The oxidized disulphide form of thioredoxin is reduced directly by NADPH and thioredoxin reductase, whereas glutaredoxin is reduced by glutathione (GSH) using electrons donated by NADPH (Holmgren, 1989).

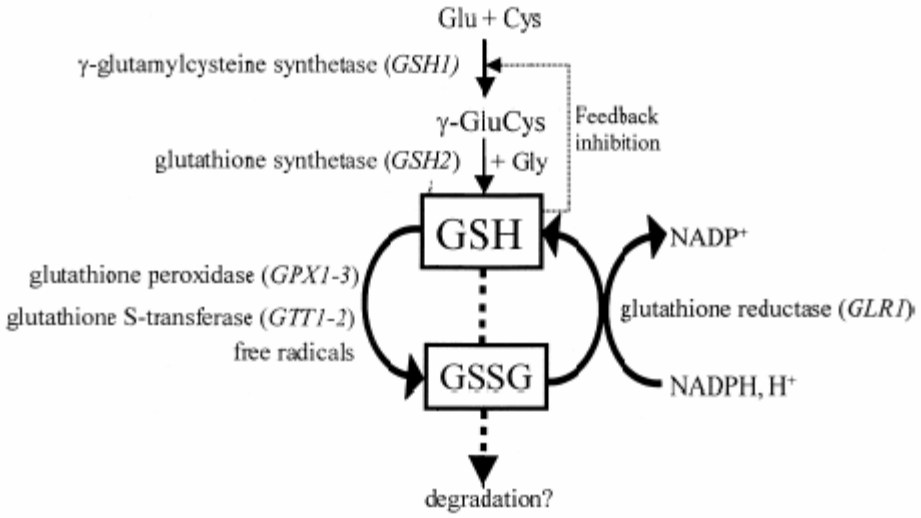


Figure 1.2. The yeast glutathione cycle. GSH is synthesized from its constituent amino acids via two ATP-dependent steps. In the first step, g-glutamylcysteine synthetase, encoded by GSH1, catalyses the formation of the dipeptide g-glutamylcysteine (g-GluCys) from glutamic acid and cysteine and, in the second step, glutathione synthetase (GSH2) catalyses the ligation of the dipeptide with glycine. The first step in GSH synthesis is the rate-limiting step and is feedback inhibited by the endproduct GSH. GSH is oxidized to GSSG by reaction with free radicals or in reactions catalysed by glutathione peroxidase or glutathione S-transferase. GSH is regenerated in an NADPH-dependent reaction catalysed by glutathione reductase (encoded by GLR1). The factors that control the degradation of GSH/GSSG are unknown in yeast (Grant, 2001).

2.3.3 The thioredoxin system

Yeast contains two genes encoding cytoplasmic thioredoxins (TRX1 and TRX2; Figure 2) which are dispensable under normal growth conditions (Muller, 1991). Deletion of both TRX1 and TRX2 affects the cell cycle, resulting in a prolonged S phase and a shortened G1 interval, which do not result from alterations in the levels of deoxyribonucleotides (Muller, 1991). Thioredoxin mutants (trx1 trx2) are defective in sulphate assimilation like glutaredoxins, TRX2 functions in protection against ROS, as trx2 mutants are sensitive to H_2O_2 and resistant to the thiol oxidant diamide (Muller, 1996).

A mitochondrial thioredoxin system has also been identified in yeast, including a thioredoxin (TRX3) and thioredoxin reductase (TRR2), which function in protection against oxidative stress generated during respiratory metabolism. However, given the lethality of strains deleted for GRX1, GRX2, TRX1 and TRX2, it does not appear that this mitochondrial system can substitute for the cytoplasmic thioredoxin or glutaredoxin systems (Draculic *et al.,* 2000).

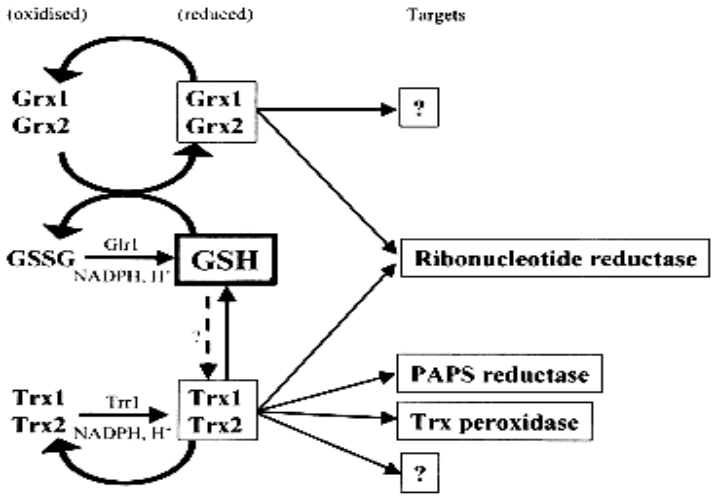


Figure1.3. The GSH/glutaredoxin and thioredoxin systems. Yeast contains two gene pairs encoding cytoplasmic glutaredoxins (GRX1, GRX2) and thioredoxins (TRX1, TRX2). The oxidized disulphide form of thioredoxin is reduced directly by NADPH and thioredoxin reductase. In contrast, oxidized glutaredoxin is reduced by glutathione (GSH), and oxidized GSSG is in turn reduced in an NADPH-dependent reaction catalysed by glutathione reductase. The redox status of GSH may provide a functional link between the GSH/glutaredoxin and thioredoxin systems, as thioredoxins function along with Glr1 to maintain the high intracellular GSH±GSSG ratio (Grant, 2001).

2.3.4 Glutathione reductase

A single glutaredoxin or thioredoxin is essential for viability in yeast. The first indication of a functional link between the thioredoxin and GSH/glutaredoxin systems in yeast came from the identification of a glutathione reductase (GLR1) null mutant in a genetic screen for mutations that confer a requirement for thioredoxins (Muller, 1996). Mutants lacking both TRX1 and TRX2 are inviable in the absence of GLR1, indicating that thioredoxin mutants require the ability to reduce GSH for survival. Limited growth of the triple trx1 trx2 glr1 mutant could be restored using anaerobic conditions, indicating that oxidative stress may play a role in the lethality of this strain (Grant, 2001).

A further overlap between the two systems is evident from the finding that loss of both TRX1 and TRX2 results in elevated levels of GSSG, indicating a link between the thioredoxin system and the redox status of GSH in the cell (Muller, 1996). Deletion of GLR1 in the trx1 or trx2 mutant further exacerbates this elevation in GSSG levels, indicating that thioredoxins may normally function along with Glr1 to maintain the high intracellular

GSH±GSSG ratio. Thus, the redox status of GSH may provide a functional link between the GSH/glutaredoxin and thioredoxin systems (Fig. 3) (Draculic *et al.,* 2000).

2.3.5 Glutathione peroxidase

Both selenium-dependent and -independent glutathione peroxidases have been described that catalyse the breakdown of hydroperoxides using GSH as a reductant. Recent studies have confirmed the presence of glutathione peroxidases in yeast. Glutathione peroxidase activity towards both H_2O_2 and organic peroxides has been detected, and three gene products (GPX1, GPX2 and GPX3) with significant homology to mammalian glutathione peroxidases have been identified from the yeast genome. The greatest sequence homology with mammalian glutathione peroxidases is found in the region around their active sites (Inoue *et al.,* 1999).

2.4 Apoptosis inducing factor

Apoptosis-inducing factor (AIF 57 kDa) is a key regulator of cell death, and is also essential for normal mammalian development and participates in pathological apoptosis. AIF, as a novel mitochondrial intermembrane flavoprotein, has a significant homology to bacterial and plant oxidoreductases (Susin *et al.,* 1999)**.**

Human AIF family members include AIF (with mitochondrial localization sequence) AMID or (PRG3), and AIFL (supposedly characterized with internal mitochondrial localization sequences instead of the N-terminal mitochondrial localization sequences) (Wu *et al.,* 2002), yeast AIF homologue Ynr074cp which controls yeast apoptosis. Similar to mammalian AIF, ynr074cp is located in mitochondria and translocates to the nucleus of yeast cells in response to apoptotic stimuli. A Purified ynr074cp degrades yeast nuclei and plasmid DNA. *YNR074C* disruption rescues yeast cells from oxygen stress and delays age-induced apoptosis. Conversely, overexpression of Ynr074cp strongly stimulates apoptotic cell death induced by hydrogen peroxide. Yeast Ynr074cp in is named AIF-1 (Aif1p, gene *AIF1*) (Wissing *et al.,* 2004).

Overexpression of Bcl-2 has been suggested to inhibit apoptosis mainly by preventing the release of cytochrome *c* (Kluck *et al*., 1997; Yang *et al.,* 1997) Mitochondrion-targeted Bcl-2 also prevents the release of AIF. Several apoptosis inducers induce the mitochondrio-nuclear translocation of AIF, suggesting that the redistribution of AIF is a general feature of the apoptotic process (Fig. 4) (Daugas *et al.,* 2000).

Apoptosis inducing factor (AIF) is an apoptotic effector protein that induces chromatin condensation and large-scale (50 kbp) DNA fragmentation. This protein is synthesized as a nonapoptogenic precursor in the cytoplasm and is efficiently imported into the mitochondrial intermembrane space. Upon proteolytic removal of the amino-terminal mitochondrial targeting sequence, attachment of a flavin adenine dinucletide group, and refolding of the protein, it becomes potentially apoptogenic (Susin *et al.,* 1999).

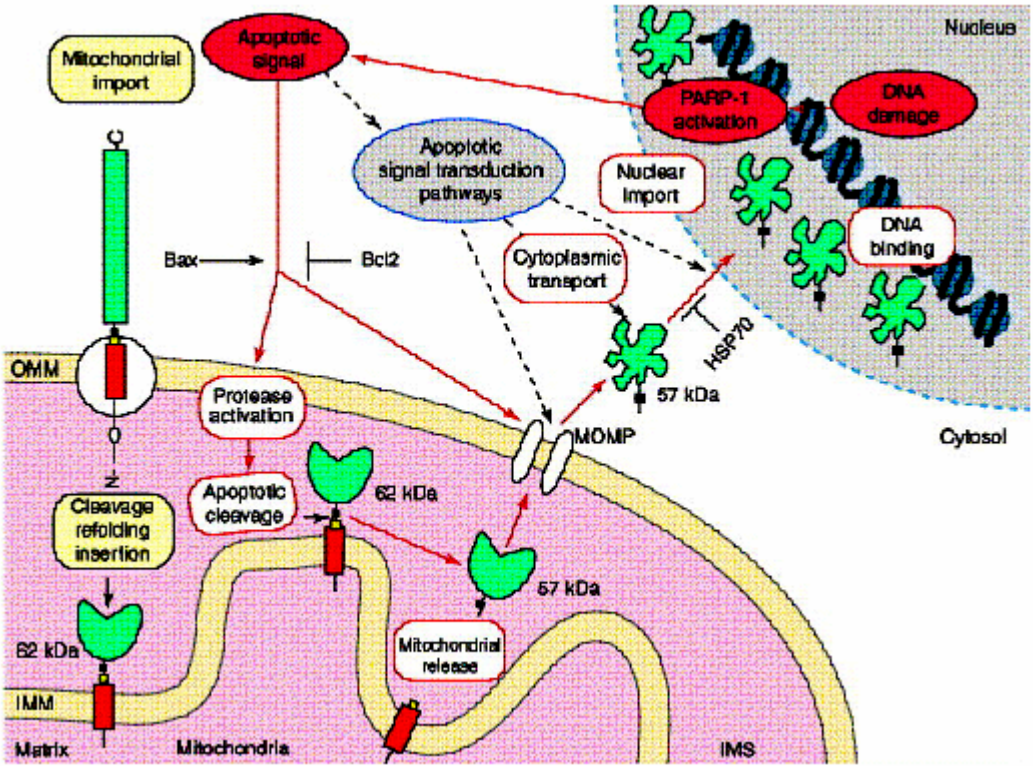


Figure 1.4. Subcellular localization of AIf. The import of unprocessed full length AIf into the mitochondria followed by the cleavage of its N–terminal LMS by a mitochondrial peptidase. AIF is inserted in the inner mitochondrial membrane (IMM), via its N-terminal transmembrane region (orange). The rest of the protein faces the intermembrane space (IMS), where it participates in the regulation of the activity of the respiratory chain. In cells undergoing genotoxic stress, the activation of AIF's nuclear lethal function might be triggered by the PARP1 signaling pathway. The apoptotic release of AIF from mitochondria requires the inducible activity of unknown protease and mitochondrial outer membrane permeabilization. Proteins from the Bcl-2 family regulate these processes. The cytoplamic transport of AIF is inhibited by the anti-apoptotic Hsp70. Upon translocation to the nucleus, AIF bind to chromatin and induces its condensation (Daugas *et al.,* 2000).

In normal cells, AIF is strictly confined to mitochondria, and thus colocalizes with heat shock protein 60 (hsp60); In mitochondria, it has an essential role in the maintenance of

the respiratory chain complexes I and III (Modjtahedi *et al.,* 2006). On induction of apoptosis, AIF (but not hsp60) translocates to the nucleus, which suggests that only the outer mitochondrial membrane (which retains AIF in the intermembrane space) and not the inner membrane becomes protein permeable. ATP depletion is sufficient to cause AIF translocation to the nucleus (Wissing *et al.,* 2004). An inducible heat shock protein HSP70 regulates the translocation by binding with amino acids (150-228) (Ravagnan *et al.,* 2001). When added to purified nuclei in a cell-free system, recombinant AIF has two major effects: It causes peripheral chromatin condensation and large-scale DNA fragmentation, yet fails to generate nuclear bodies or oligonucleosomal DNA fragments. These latter changes have been attributed to the caspase-dependent activation (Wissing *et al.,* 2004).

Moreover, the mitochondrio-nuclear translocation of AIF appears to be a constant feature of cell death, independent of the death inducing conditions Thus, the redistribution of AIF to the nucleus, which strongly correlates with large-scale DNA fragmentation, has been found to be caspase independent and ATP independent, which leads to apoptotic cytolysis (necrosis) (Wissing *et al.,* 2004).

Mitochondria release AIF relatively early; before they completely release cytochrome *c*. Kinetic analyses suggest that AIF translocation to the nucleus coincides with initial chromatin condensation (wrinkled pattern of peripheral condensation, stage I), whereas cytochrome *c* release correlates with a more advanced pattern of chromatin condensation (stage II). The differential release kinetics of AIF and cytochrome *c* might be explained by the preferential localization of cytochrome *c* to the inner mitochondrial membrane which would retard its release, (Salamon and Tollin, 1997, Jutila *et al.,* 1998).

2.5 AMID/NDI1

Phylogenetic analysis has indicated that AMID possesses homologous counterparts in eubacteria, but not in archaebacteria, in contrast to AIF, which possesses significantly homologous genes in both eubacteria and archaebacteria. AIF (apoptosis-inducing factor) and AMID (AIF-homologous mitochondrion-associated inducer of death) are both mitochondrial flavoproteins that trigger caspase-independent apoptosis. AIF orthologue (YNR074C) identified in yeast, displays sequence similarity to AIF/AMID and regulates

apoptosis in a similar way to that of AIF in mammalian cells (Wu *et al.,* 2002; Wissing *et al.,* 2004). Compared to AIF, the proapoptotic nature of AMID and its mode of action are much less clarified. However, the closest homologue of AMID in yeast is Ndi1p, instead of Aif1p. Recent results have showed that the overexpression of yeast AMID homologue, *NDI1* (internal NADH dehydrogenase), but not *NDE1* (external NADH dehydrogenase), can cause apoptosis-like cell death, and this effect can be repressed by increased respiration on glucose-limited media. This result indicates that the regulatory network of energy metabolism, in particular the cross-talk between mitochondria and the rest of the cell, is involved in Ndi1p-induced yeast cell apoptosis (Li *et al.,* 2006).

NDI1 encodes NADH:ubiquinone oxidoreductase, which is localized on the mitochondrial inner membrane and is involved in the transfer of electrons from NADH to ubiquinone in the respiratory chain. *NDE1,* a close homologue of *NDI1*, also encodes NADH:ubiquinone oxidoreductase, and is localized on the mitochondrial external membrane. In contrast to the multisubunit respiratory complex I in higher eukaryotic cells, Ndi1p and Nde1p do not pump protons across mitochondrial inner membrane during respiration (Li *et al.,* 2006).

2.5.1 Expression of NDI1 and NDE1

The apoptotic effect of *NDI1* overexpression is associated with an increased production of reactive oxygen species (ROS) in mitochondria. Interruption of certain components in the electron-transport chain can suppress the growth inhibition from Ndi1p overexpression. Electron microscopic investigation of *NDI1*-overexpression cells has also revealed extensive chromatin condensation and aggregation along the inner part of nuclear envelope, a typical sign of apoptosis. *NDI1*-overexpression cells show discernible autophagic bodies and some other abnormal structures embedded with intact membrane in the vacuoles of starved yeast cells (Takeshige *et al.,* 1992). The presence of autophagic bodies in the vacuoles of *NDI1*-overexpression cells is therefore another indicator of apoptosis-like cell death.

In glucose-rich media, *S. cerevisiae* preferentially utilizes glucose through fermentation, and turns down a large number of genes involved primarily in respiration or the utilization of other carbon sources. This is thought to be an energy-saving response, a phenomenon name glucose repression (Schuller, 2003). When glucose becomes limited, the energy generation of cells becomes more dependent on respiration and those glucose-repressed genes are induced. *NDI1* and *NDE1* are two of the genes that are repressed when cultured on glucose-rich media (Li *et al.,* 2006).

Both *NDE1* and *NDI1* encode NADH dehydrogenases, overexpression of *NDI1* accelerates apoptosis and ageing, but not *NDE1*, the difference of the two gene products is that Ndi1p catalyzes the intramitochondrial NADH, while Nde1p oxidizes NADH at the cytosolic side of the inner membrane (Luttik *et al.* 1998). Because overexpression of either of them increases ROS production, one explanation for this observed difference is that ROS produces in *NDE1*-overexpression cells is possibly more in the cytosol and ROS produced from *NDI1*-overexpression is more localized in mitochondria. It is possible that intra-mitochondrial ROS is a more potent apoptosis inducer. In addition, cytosolic ROS may well be scavenged by Sod1p, but intramitochondrial ROS may not be so well by Sod2p (superoxide dismutase), which is expressed at low levels (5-10 fold less) during growth in glucose medium (Maris *et al.,* 2001). Therefore, when cultured in a glucose-rich medium such as normal SD-URA, ROS produced by *NDI1*-overexpression cannot be detoxified efficiently, which in turn damages mitochondria (Li *et al.,* 2006).

2.6 Endonuclease G (EndoG)

Cleavage of chromatin into oligonucleosomal-sized fragments is a biochemical hallmark of apoptosis. Biochemical and genetic studies have identified at least two endonucleases important for mammalian DNA fragmentation during apoptosis, the DNA fragmentation factor (DFF) and endonuclease G (EndoG) (Zhang *et al.,* 2003).

DFF is a dimeric protein consisting of the DFF40 and DFF45 subunits also termed CAD (caspase-activated DNase) or CPAN (caspase-activated nuclease), and ICAD (inhibitor of caspase activated DNase). DFF40 contains an endonuclease activity, and its synthesis and

folding critically depend on DFF45. On induction of apoptosis, DFF45 is cleaved by caspase-3. DFF40 is then activated to cleave DNA into nucleosomal-sized fragments and to induce chromatin condensation. These observations, together with biochemical studies, have established that DFF is a major endonuclease responsible for DNA degradation after activation of apoptosis (Zhang and Xu, 2002).

EndoG is an evolutionarily conserved, nuclear-encoded endonuclease reported to be involved in mtDNA replication, recombination, and degradation. Biochemical work showed that, on activation of apoptosis, EndoG is released from the mitochondrial intermembrane space and translocates to the cell nucleus to induce DNA fragmentation (8–10). EndoG cleaves chromosomal DNA in a caspase-independent manner. DNA fragmentation can be blocked by an anti-EndoG antibody, indicating that the residual DNA fragmentation activity in these cells is at least partially attributable to EndoG. Unlike DFF, which generates double-stranded DNA breaks, EndoG degrades single-stranded DNA more rapidly than duplex DNA (Zhang and Xu, 2002). Genetic studies have showed that an EndoG mutation in *Caenorhabditis elegans* results in less effective DNA degradation and delayed apoptosis *in vivo*. These biochemical and genetic studies suggest that EndoG participates in a DNA fragmentation pathway originating in mitochondria. Despite these studies, the role of EndoG in mammalian development and apoptosis *in vivo* remains almost unknown. EndoG is required for early embryogenesis and normal apoptosis and is not required for the maintenance of normal levels of mtDNA. Cells from EndoG heterozygous mutant mice exhibit increased resistance to both the tumor necrosis factor and staurosporine-induced cell death. Moreover, spontaneous cell death of spermatogonia in EndoG heterozygous mutant mice is significantly reduced compared with wild-type mice (Zhang *et al.,* 2003).

3. Old yellow enzyme

The yeast Old Yellow Enzyme (OYE) was the first flavin-dependent enzyme identified and isolated from brewers' yeast by Warburg and Christian in 1932 during attempts to elucidate the nature of biological oxidations. Methylene blue in the presence of two components of erythrocytes (enzyme "Zwischenferment" and a small heat-stable "Coferment") were able to oxidize the glucose-6-phosphate. The (yellow enzyme) "Gelbe

ferment" identified by Warburg permitted the system to form a complete respiratory chain reacting with molecular oxygen. Later on, a second 'new' yellow enzyme which was discovered by Haas in 1938 made Warburg's enzyme termed "Old Yellow Enzyme" (Williams and Bruce 2002).

The purified OYE comprises a colorless apoprotein and a yellow dye; both are essential for enzyme activity. The yellow dye was found to be similar in nature to vitamin B_2 (riboflavin), thus OYE provided the first biochemical role for a vitamin. The yellow cofactor was in fact riboflavin 5'-phospate, which is termed flavin mono nucleotide (FMN) (Williams and Bruce, 2002).

Extensive purification and characterization of OYE was carried out for the enzymes from S. *carlsber*gensis and *S. cerevisiae*. Protein from these natural sources has been shown to be heterogeneous both in terms of chromatographic behavior and amino acid sequence. OYE isolated and sequenced from S. *carlsbergensis* was designated *oyel.* Since then, three other OYE genes have been sequenced, designated *oye2, oye3,* and *kyel.* The *oye2* gene was isolated by screening a genomic library of *S. cerevisiae* using the *oyel* gene as a probe; the *oye3* gene was isolated in a similar manner after it was discovered that an *oye2* deletion mutant still produces a protein with the properties of OYE; and the *kyel* gene was recently found in the genome of *Kluyvernmyces lactis* (Karplus *et al.,* 1995). A growing family of OYE homologs has been identified in organisms with both prokaryotic and eukaryotic origins. Recently, the cloning and sequencing of a higher plant OYE homolog from *Arabidopsis thaliana* has been described (Strassner *et al.,* 1999). Both the chromatographic and sequence heterogeneity can be explained by assuming that two OYE isozymes in each species form both homodimers and heterodimers which are in a dynamic equilibrium. In addition to the formation of heterodimers within a species, showed that mixing the oyelp and oye2p proteins (which come from different yeast species) in vitro leads to a high yield (-70%) of the "unnatural" Oyel:Oye2 heterodimer (Karplus *et al.,* 1995).

The physiological reductant of the OYE is assumed to be NADPH. Substrates capable of oxidizing include methylene blue, Fe^{3+}, quinones, cytochrome c. and ferricyanide (Fig.5) (Massey *et al.,* 1969). Reoxidation reaction is affected by molecular oxygen to yield hydrogen peroxide and superoxide, but this reaction is lower than other oxidase enzymes

which are an advantage (Schopfer *et al.,* 1991, Vaz *et al.,* 1995). Several examples of substrates as α/β- unsaturated aldehydes and ketones but not acids, ester or amides, the major inhibito is the phenolic compound *ρ*-hydroxybenzaldehyde (Fox and Karplus, 1994, Williams and Bruce, 2002).

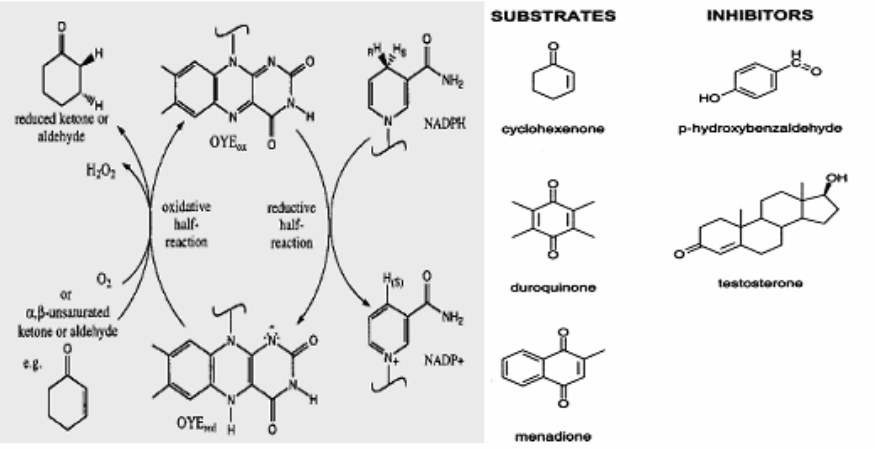


Figure 1.5. OYE from brewer's yeast. the dimer of 45 kDa subunits with one noncovalently bound FMN per subunit is rapidly reduced by NADPH and can be reoxidized by oxygen (NADPH-dependent oxidase activity). The left panel of the picture shows some subtrates and inhibitors (Kohli *et al.,* 1998).

3.1 OYE1

The open reading frame encoded a 400 residue subunit of 44,890 daltons. The structure of natural OYE1 from S. *carlisbergensis* shows that OYE folds into a single domain based on a (β/a)8 barrel (or TIM barrel) with the FMN binding within the barrel near the carboxy-terminal ends of the β-strands (Fig 6). Nine secondary structural elements exist in addition to those of the main barrel: four β-strands and five a helices.

Two strands form an anti-parallel *ββ*-supersecondary structure that covers the base (amino-terminal end) of the barrel; the other seven elements decorate the carboxy-terminal end of the barrel, forming some segments that help bind the flavin and contribute to a ligand binding pocket. All of these residues are well conserved among the known OYE sequences. The flavin itself is tilted about 45° from the barrel axis. Hydrogen bonds to FMN come from the side chains of Thr^{37} Gln^{114} Arg^{243} Arg^{348}, Gly^{72} Gly^{324}, Asn^{325}, Phe^{326}, Gly^{345}, and Gly^{347} (Fox and Karplus, 1994). The complex between the active site of OYE with the inhibitor *ρ*-hydroxybenzaldehyde shows that the phenolic ring undergoes a stacking interaction with the

flavin. Generally the OYE has an observed selectivity for NADPH over NADH (Williams and Bruce, 2002).

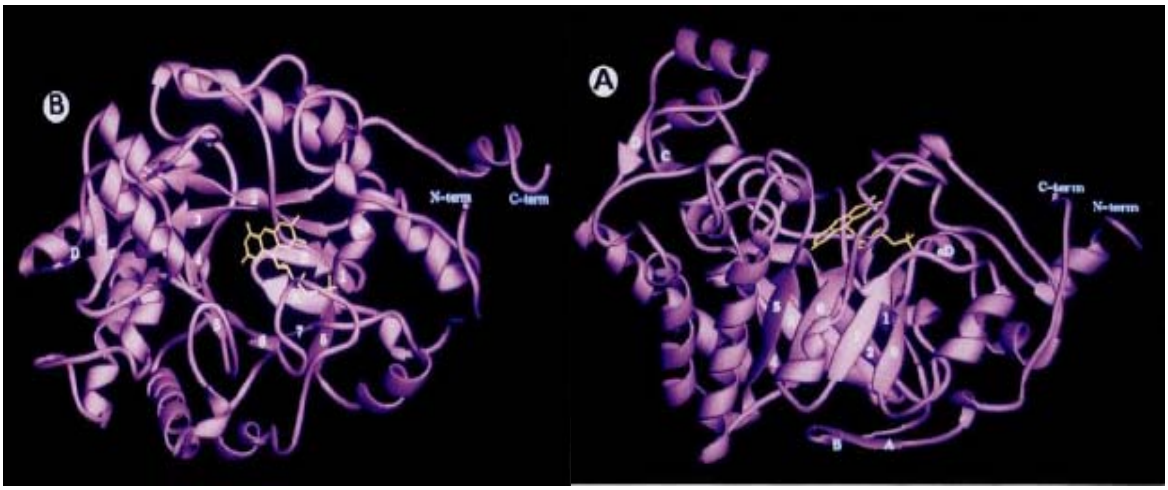


Figure 1.6. Secondary structure: four β-strands and five α-helices. Two strands form an anti-parallel ββ-supersecondary structure that covers the base (amino-terminal end) of the barrel; the other seven elements decorate the carboxy-terminal end of the barrel, forming some segments that help bind the flavin and contribute to a ligand binding pocket. This places the active sites on opposite sides of the dimer, so that in terms of function it seems that OYE can be conceptually described as a monomer. A) Perpendicular view to the barrel axis. B) A view looking clown the barrel axis (Karplus *et al.,* 1995).

3.2 OYE2

The *oye2* gene is located on chromosome VIII of *S. cererisiae*. The oye2 structural gene, like the OYE1, encodes a single polypeptide of 400 amino acids starting with the indicated methoinine which is the initiation codon and is not found on the purified protein. The calculated molecular mass of oye2 polypeptide excluding the methionine is 44.886 and is very close to that of oye1 44.890 Daltons. Of the 399 amino acids, only 33 are different (92% identity) and 95% homology including the first residue which is serine in oye1 and proline in oye2 (Stott *et al.,* 1993).

3.3 OYE3

The first gene of the OYE system in *S. cerevisiae* has been cloned and named OYE2 to distinguish it from OYE1 gene cloned from the *s. carlsbergenesis*. The analysis of an OYE2 deletion mutant has suggested that S. cerevisiae has at least two *OYE* genes, a new OYE species analyzed and named OYE3, which consists of 400 amino acid residues and has

a molecular mass of 44.788 Daltons predicted from the amino acid sequence derived from the DNA sequence. In the down stream region of the OYE3 gene which is located in chromosome XVI, the cytochrome oxidase *(COX10)* gene exists with 426 base pair intermediate sequence. Although the OYE2 and OYE3 enzymes are closely related, they show differences in ligand binding properties and in their catalytic activity with oxygen and 2-cyclohenone as acceptors. OYE2 has wavelength maxima at 380 and 462, while OYE3 has maxima at 384 and 464 nm (Niino *et al.,* 1995).

3.4 Comparison between OYE1, OYE2 and OYE3

The homology of the nucleotide sequence of the coding region between *OYE1* and *OYE2* was 85%, and between *OYE1* and *OYE3* is 74% and between *OYE2* and *OYE3 is 73%.* The amino acid sequences of the three OYE s showed that OYE1 and OYE2 have 92% identity and 95% similarity, OYE1 and OYE3 have 80% identity and 87% similarity, and OYE2 and OYE3 have 82% identity and 89% similarity. Molecular masses of OYE1,OYE2, OYE3 were 44,890, 44,856, 44,788 Daltons, respectively. At most accessible concentrations of O_2, the observed NADPH-O_2 reductase activity of OYE3 is smaller than OYE2, which id dueto a high *Km* for O_2 exhibited by OYE3. Regarding the steady state turnover OYE has been shown to employ cyclohexenone as an efficient electron acceptor in which the carbon carbon double bond is reduced to form cyclohexane. As in the case of O_2 as the acceptor, there are distinctive differences between OYE2 and OYE3 in the kinetics of the NADPH-cyclohexenone reductase activity. Cyclohexenone reoxidizes reduced enzyme very rapidly making it difficult to perform enzyme monitored turnover as the type possible with O_2 as acceptor. Thus, clearly that the OYE3 is much more poorly equipped to employ cyclohexenone as an electron acceptor than OYE2 (Niino *et al.,* 1995).

3.5 Catalytic properties of OYE

In addition to time long-known NADPH-oxygen oxidoreductase activity, OYE has been shown to catalyze the NADPH-dependent reduction of quinones , and many *a/*β-unsaturated carbonyl compounds, such as 2-cyclohexenone.

$$EFl_{red} + CHX \rightleftharpoons EFl_{red.}CHX \rightleftharpoons EFl_{ox} + CHXH2$$

The NADPH-dependent reduction of OYE thus reflects the following reaction scheme, with EFl_{ox} and EFl_{red} representing the oxidized and reduced enzyme, and the form with the asterisk (*) denoting the nicotinamide-flavin charge transfer interaction.

$$EFl_{ox} + NADPH \rightleftharpoons EFl_{ox} + NADPH \rightleftharpoons EFl^{*}_{ox} + NADPH \rightleftharpoons EFl_{red} + \mathbf{NADP}$$

$$EFl_{red} + O2 \rightleftharpoons EFl_{ox} + H_2O_2$$

$$EFl_{red} + CHX \rightleftharpoons EFl_{red.}CHX \rightleftharpoons EFl_{ox+}CHXH2 .$$

These studies provide convincing evidence for the binding of NADPH to the enzyme, even though there is no recognizable binding site involving time AMP moiety of the pyridine nucleotide (Strassner *et al.,* 1999; Niino *et al.,* 1995)

3.6 Ligand binding

Many ligands bind specifically to oxidized OYE; these include NADPH, β-NADPH, NADH), simple anions (chloride, azide), many phenols (p-hydroxybenzaldehyde, p-chlorophienol), and more recently, a number of steroids (β-estradiol, testosterone). There is strong biochemical evidence that they all bind to a common site near the flavin, as the nicotinamide dinucleotides serve to rapidly reduce the enzyme-bound FMN and the other compounds both act as competitive inhibitors of OYE reduction by NADPH and cause perturbation of the flavin spectral properties. None of the compounds bind well to reduced OYE. Many aromatic and heteroaromatic compounds that have an ionizable hydroxyl group (e.g., phenols) bind to OYE and induce marked changes in the flavin absorption spectrum, including the development of strong absorbance maxima in the 500-800 nm range. (Karplus *et al.,* 1995). The electron acceptors for reduced OYE are known including the olefinic bond of α/β-unsaturated ketones and aldehydes, suggesting that the physiological substrate may comprise such a structural feature (Kohli *et al.,* 1998).

3.6 Active site of OYE

The combination of site directed mutagenesis of OYE and crystal chromatography provided significant insight into catalytic mechanism and substrate specificity across the

OYE family as a whole, which confirms a pocket of the main residues His[191] Asn[194] and Tyr[196] (Fig. 8). In addition, a flavin which undergoes changes in the geometry and enters into a stacking interaction with the substrate. In all cases, His[191]and Asn [194] donate two hyhrogen bonds to the phenolic oxygen, which has confirmed their importance in binding the oxygen atom of substrate molecules (Kohli and Massey, 1998; Brown *et al.,* 1998).

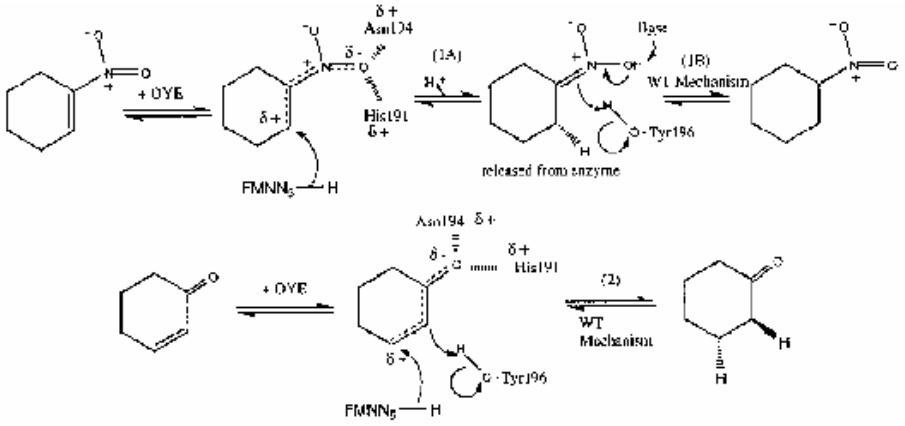


Figure 1.7. Proposed differential reactivities of 2-cyclohexenone (*CHX*) and nitrocyclohexene (*NCX*) with wild type and Y196F OYE1. The different pathways postulated to occur in wild type enzyme for two substrates. The nitrocyclohexene pathway describes the formation of an aci-nitro intermediate by transfer of hydride to the C_β followed by catalyzed tautomerization to the reduced product. The 2-cyclohexenone pathway describes a concerted type transfer where transfer of the hydride is made possible by the presence of Tyr-196 primed to protonate the C_a.

If any α/β-unsaturated carbonyl substrate binds with the carbonyl oxygen atom positioned to hydrogen bond to His-191 and Asn-194 the β carbon atom can be aligned above the flavin N-5 atom. This is consistent with earlier experiments suggesting hydride transfer from the flavin N-5 to the β position. Site directed mutagenesis has implicated Tyr-196 in the transfer of a proton to the α carbon (Kohli and Massey, 1998) completing the saturation of the double bond. Tyrosine 196 in Old Yellow Enzyme (OYE) was mutated to phenylalanine to evaluate the mechanistic role of the residue. The rate of 2-cyclohexanone reduction catalyzed by the Y196F mutant of OYE is significantly lower than the wild-type. The hydride transfer and proton transfer of 2-cyclohexanone need to be concerted, as it is not possible to form a stable carbanion intermediate.

Y196F mutant can catalyse hydride transfer to 1-nitrocyclohexene but it is unable to protonate the nitronate formed providing a strong evidence for the importance of the Tyr-196 as an active site acid (protonation reaction) (Kohli and Massey, 1998).

Y196F residue conversion demonstrates little effect on ligand binding and the reductive half-reaction, but a dramatic slowing by nearly 6 orders of magnitude of its oxidative half-reaction with 2-cyclohexenone (Kohli *et al.,* 1998). The role of Tyr-196 as an active-site acid, reductive half-reaction kinetics are expected to be unaffected in Y196F as transfer of the 4-pro-***R*** hydridic hydrogen of NADPH to oxidized flavin proceeds without a mechanistic need for a proton donor or acceptor. This is consistent with the concept that oxidation of the 2-cyclohexenone would require Tyr-196 to act as an active site base and deprotonate the non-vinyllic α-carbon to the carbonyl. An examination of the active site of OYE1 suggests a place for Tyr-196 in the mechanism of the oxidative half-reaction with reduced flavin and α/β-unsaturated compounds. While aerobic turnover with only molecular oxygen as the substrate for the oxidative half-reaction remains unaffected, the aerobic turnover reaction in the presence of 2-cyclohexenone proved to be dramatically slowed. Besides reduction of the α/β -unsaturated bond requires both hydride transfer from the flavin to the C_β and proton uptake from Tyr-196 to the C_α. The above behavior is consistent with electron-donating constituents, leading to tighter binding of substrates by increasing electron density at the carbonyl oxygen which is expected to be hydrogen- bonded to His-191 and Asn-194. Whether these components of the reaction occur in a stepwise or concerted fashion has remained an open question (Kohli *et al.,* 1998).

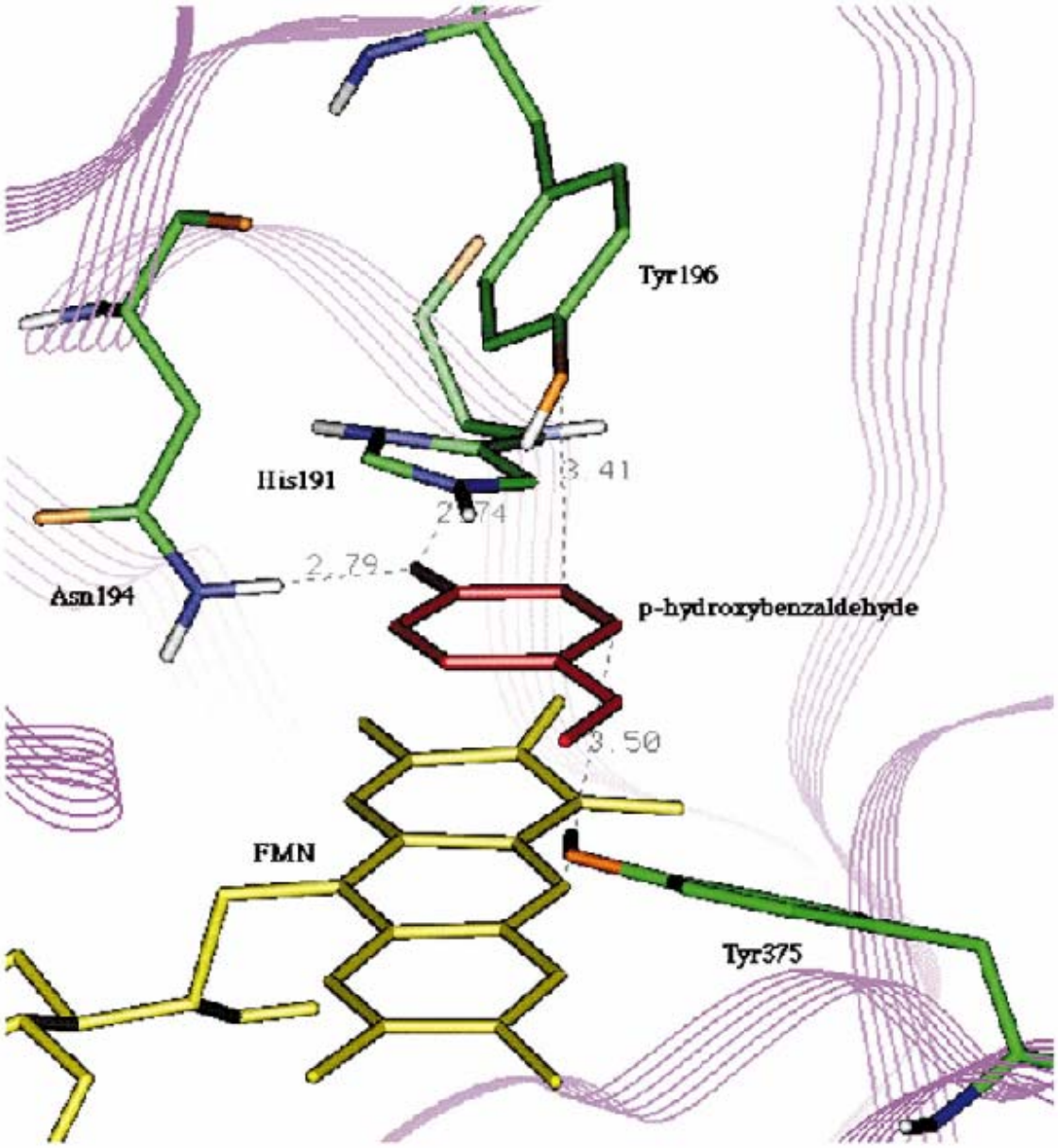


Figure 1.8. The active site of oxidized OYE1 with *p*-hydroxybenzaldehyde bound. The phenolic anion is hydrogen-bonded to the His-191 and Asn-194 residue. Hydrogen bonding distances given are between donor and acceptor atoms, ignoring the speculated location of the hydrogen atoms

3.7 Function of old yellow enzymes

OYE has served as a model for enzymologists interested in enzyme catalyzed redox reactions. Collectively, these investigations have led to a sophisticated understanding of OYE's ability to accept electrons from NADPH onto a flavin mononucleotide cofactor and transfer these electrons to diverse ligands. Unfortunately, identification of the physiologically relevant substrate(s) of OYE has been difficult and elusive (Schopfer and Massey, 1991).

3.7.1 OYE mediates resistance to unsaturated products of lipid peroxidation

All aerobic organisms are exposed to reactive oxygen species (ROS), such as H_2O_2, the superoxide anion, and the hydroxyl radical, during the course of normal aerobic metabolism or following exposure to radical-generating compounds in the environment. Unsaturated lipids are major targets of ROS attack, resulting in lipid peroxidation. During lipid peroxidation, reactive lipid and fatty acid radicals are formed, which can attack other fatty acids, leading to oxidative chain reactions. These chain reactions proceed until they come into contact with a chain-breaking antioxidant, such as vitamin E. As a result of the oxidative damage to lipids, various lipid hydroperoxides are formed. These are relatively unstable and are converted into breakdown products, which can be just as toxic as the ROS themselves. Major breakdown products include reactive aldehydes, such as malondialdehyde (MDA) and acrolein. These reactive aldehydes have a number of cytotoxic effects and inhibit a variety of enzymes. For example, these have been shown to cause membrane damage by increasing fluidity, to damage DNA, and to interact with high- and low-density lipoprotein, which contributes to atherosclerosis. In addition, they can react with nucleophiles, such as the sulfhydryl group of cysteine. Not surprisingly, therefore, reactive aldehydes are highly toxic (Trotter *et al.,* 2006).

It was shown that yeast Old yellow enzymes specifically mediate resistance to small α/ß-unsaturated compounds, but not to other aldehydes (crotonaldehyde). In vitro enzyme analysis has shown that old yellow enzymes can reduce the α/ß-unsaturated ketones methyl vinyl ketone (MVK) and 3-penten-2-one. Similar to the pattern seen with acrolein, the deletion of OYE2 caused sensitivity to MVK, and over expression of both OYE2 and OYE3 increased resistance to MVK (Trotter *et al.,* 2006).

3.7.2 Actin is a substrate of OYE

The identification of oye2p as an actin-interacting protein in a two-hybrid screen suggested that OYE might control the redox state of actin. Many studies on oxidative stress have shown that Cys^{374} of actin is highly reactive to oxidizing agents and that oxidative damage elicits changes in actin cytoskeketon organization. It has been proposed that

oxidative damage can induce intermolecular disulfides which can cross-link actin filaments (Dalle-Donne *et al*., 2001). Oxidation of actin has been directly observed in the erythrocyte cytoskeleton of patients with sickle cell anemia. The red blood cell membrane cytoskeleton consists of short (14-subunit-long) actin filaments attached at their ends to spectrin tetramers which are in turn anchored to the plasma membrane via membrane spanning glycoproteins (Haarer and Amberg, 2004). The actin filaments of this array must be dynamic for the red blood cell to undergo the cell shape changes required to pass through small capillaries. Crisis in sickle cell patients leads to severe pain, organ damage, and mortality and is correlated with the accumulation in the bloodstream of irreversibly sickled red blood cells (ISCs) that have been found to contain hyperstable membrane cytoskeletons compared with normal red blood cells (RBCs) or reversibly sickled red blood cells (RSCs). This lack of plasticity in the membrane cytoskeleton contributes to the blockage of small capillaries, in turn leading to poor peripheral circulation and organ damage (Haarer and Amberg, 2004).

Stabilization of the ISC membrane cytoskeleton is due to the accumulation of oxidized actin that contains an intramolecular Cys^{285}-Cys^{374} disulfide bond (Bencsath *et al*., 1996). Filaments formed containing this form of actin show reduced rates of disassembly in vitro (Shartava *et al*., 1995). Previously, it had been thought that the formation of a C285-C374 disulfide bond in actin was a unique result of the extremely oxidizing environment in RBCs exacerbated in sickle cell RBCs by spilling of iron from the mutant hemoglobin and decreases in cellular reductants such as reduced glutathione (Haarer and Amberg, 2004).

Blocking Cys^{285}-Cys^{374} disulfide bond formation in actin can fully suppress the sensitivity to oxidative stress caused by the loss of Old Yellow Enzyme which suggests that actin is the physiologically most significant substrate of Old Yellow Enzyme. Surprisingly, mutating these cysteines actually makes yeast cells super-resistant to oxidative stress. A strain expressing the actin mutant unable to interact with Oye2p, *act1-123* also displayed hypersensitivity to all agents tested but most dramatically to H_2O_2. The *act1-200* (C374A) and *act1-201* (C285A) alleles both suppressed the sensitivity of an Old Yellow Enzyme-deficient strain to all three compounds, indicating that the sensitivity of the Δ*oye* strain could

be entirely attributed to the formation of the actin disulfide bond. Perhaps most surprisingly, the *act1-200* and *act1-201* alleles confer super-resistance to oxidative stress, suggesting that the actin protein itself is a critically important target that determines cellular sensitivity to oxidative stress (Haarer and Amberg, 2004). This was the first demonstration that the actin cytoskeleton is such an important target of ROS that its oxidation contributes significantly to the inherent sensitivity of cells to oxidative stress. Given the connections between ROS, aging, and apoptosis, it seems likely that actin oxidation may be a contributing factor to these phenomena (Haarer and Amberg, 2004). The ROS produced by H_2O_2 or menadione will react with the sulfhydryls of free cysteine residues to initiate disulfide bond formation. This can cause oxidative stress in two ways: either indirectly by depletion of reduced glutathione or by directly inducing disulfide bonds within or between proteins. This supports that the induction of apoptosis in mammalian cells is associated with changes in the actin cytoskeleton (Kosower and Kosower, 1995).

4. The aim of the project

In a previous work the full length OYE2 suppressed Bax lethality in wild type yeast, and is a potent antioxidant protein. This activity contrasts with that of OYE3, which antagonized the protective action of OYE2 in H_2O_2-induced programmed cell death. The aim of the present work is to find out if the effect of OYE3 requires the presence of OYE2 .The absence of both OYE genes rendered cells hyper-resistant to H2O2- induced PCD, indicating that it is the oye2p-oye3p heterodimer that facilitates PCD. The respiratory competence of *Δoye2 oye3 cells* was enhanced; further examinations should be done in order to examine the increasing respiratory efficiency. Deletion of OYE2 with other antioxidant genes elevated endogenous ROS and sensitized cells further to H_2O_2-induced PCD. In the case of *Δoye2 glr1* cells, morphological aberrations, and defects in organelle partition has appeared. The cellular redox environment with high levels of oxidised glutathione may be a possible cause since the presence of high levels of ROS is not by it self the cause of cytoskeletal rearrangement.

Materials and Methods

1-Strains

1.1 Saccharomyces cerevisiae strains

BY4741 Mat a*; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*: This strain is derived from strain S288C in which commonly used selectable marker genes are deleted by design, based on the yeast genome sequence which has been constructed and analyzed. These strains minimize or eliminate the homology to the corresponding marker genes in commonly used vectors without significantly affecting adjacent gene expression.

1.2 E. coli strains

The Mach one -T1R

E. coli strain is modified from the wild-type W strain (ATCC #9637, S. A. Waksman) and has a faster doubling time compared to other standard cloning strains. It confers resistance to phage T1. Genotype is F- φ80 (lacZ) ΔM15 ΔlacX74 hsdR (rK-mK+) ΔrecA1398 endA1 tonA, (Invitrogen).

2. Nutritional media

2.1 Yeast media

2.1.1 Complete minimal (CM) dropout media

Complete dropout powder is a defined medium which contains all the amino acids necessary for growth and serves as an important selective medium. Leaving out one or more nutrients (amino acids) from the CM dropout powder (which can be complemented by plasmid acquirement) selects for yeast able to grow, only cells containing the plasmid of interest will be able to grow on such selective dropout media.

Dropout powder 0.13% (w/v),

YNB 0.67% (w/v) (yeast nitrogen base without amino acids),
Glucose 2% (w/v)

Standard autoclaving 20min, 151 lb/sq.in. on liquid cycle.

2.1.2 YPD Media (Yeast Extract/Peptone/Dextrose)

Yeast extract 1% (w/v),

Peptone 2% (w/v),

Glucose 2% (w/v).

2.1.3 YPG media (Yeast Extract/Peptone/ Glycerol)

Yeast extract 1% (w/v),

Peptone 2% (w/v),

Glycerol 3% (w/v).

2.1.4 Alternative carbon source

To induce transcription of a gene sequence fused on the GAL promoter, Galactose and Raffinose are used as an alternative carbon source as the following:

2% (w/v) Galactose,

1% (w/v) Raffinose,

0.13 % (w/v) dropout powder,

0.67 % (w/v) YNB (yeast nitrogen base without amino acids).

2.1.5 Solid media

For all the solid media, the pH is adjusted to be 5.8-6.2. Bacteriological agar was used at a concentration of 2% (w/v). Standard autoclaving (20min, 151 lb/sq.in. on liquid cycle).

2.1.6 Yeast Glycerol Stocks

For the preparation of yeast glycerol stocks, 1 volume of saturated yeast culture was added to 1 volume of a 50% sterile glycerol solution. Glycerol stocks are stored at -80ºC.

2.2 Bacterial media

2.2.1 Luria-Bertani (LB) medium

0.5 % (w/v) NaCl,

1% (w/v) bacto-tryptone,

0.5% (w/v) bacto-yeast extract,

100 μg/ml Ampicillin,

For solid LB medium, the above media was supplemented with 2% (w/v) agar after adjusting the pH to 5.8-6.2. The medium was autoclaved under standard conditions (20min, 151lb/sq.in. on liquid cycle). Antibiotics were added to the medium after being cooled down to around 55 ºC at a final concentration according to the kind of antibiotic being used. **Ampicillin** as a selectable marker for plasmids conferring resistance to antibiotics was added to a final concentration of 100 μg/ml in ddH_2O. Solidified dried plates were stored at 4ºC for up to two weeks.

2.2.2 SOC medium

This is a rich medium used to recover the transformed cells after the heat shock. It contains:

2.0 % (w/v), bacto-tryptone

0.5 % (w/v), bacto-yeast extract

10 mM $MgCl_2$

10 mM $MgSO_4$

20 mM glucose

2.2.3 Bacterial Glycerol Stocks

For the preparation of bacterial glycerol stocks, 1 volume of saturated bacterial culture in selective LB medium was added to 1 volume of a 50% sterile glycerol solution. Samples were stored at -80 ºC.

3. DNA, plasmids

The following plasmids were used:

3.1 pCRII-TOPO

Size: 3973 nucleotides. LacZα fragment: bases 1-589. M13 reverse priming site: bases 205-221. Multiple cloning sites: bases 269-383. T7 promoter/priming site: bases 406-425. M13 Forward (-20) priming site: bases 433-448. F1 origin: bases 590-1027. Kanamycin resistance ORF: bases 1361-2155. Ampicillin resistance ORF: bases 2173-3033. pUC origin: bases 3178-3851. Plasmid map and sequence in appendix

3.2 pYX143

A low copy number vector (ARS/CEN, LEU2) expressing genes under the control of the constitutive TPI promoter.

3.3 pUG72

The plasmid contains the URA3 gene from *Klyveromyces lactis* that complements the ura3 mutation of *Saccharomyces cerevisiae*. Due to the flanking loxP sites, the deletion marker can be removed by expressing cre recombinase. The plasmid is useful as a template for PCR to create gene deletions. Integration of the transformation of the respective PCR fragment can give a selection of mutants.

3.4 pYX122

A low copy number vector (ARS/CEN, HIS3) expressing genes under the control of the constitutive TPI promoter

4. Subcloning

4.1. Cloning of OYE2 into pYX122

The pYX122-mit GFP plasmid was restriction digested with EcoRI and XhoI as was the pYX143-OYE2. The digestion products were run on 1% agarose gel and the linear pYX122 (large band) and excised fragment (small band) OYE2 were gel extracted. The fragments (vector and insert) were ligated using T4 ligase (5µl vector, 10µl insert, 2µl 10x T4 ligase buffer, 1µl T4 ligase, 2µl H_2O). A control reaction which contained 5µl vector pYX122, 2µl T4 ligase buffer, 1µl ligase enzyme and 12µl H_2O, was made. The reaction tubes were incubated overnight at 4^{o}C and 3µl from each tube were used to transform Mach one *E. coli* competent cells. The plasmids from 5 randomly picked colonies were isolated and digested with HindIII and BamHI to check the correct insert and, after running in 1% agarose gel (Fig. 2.1), the plasmid with the correct insert (OYE2) was picked and stored at -20^{o}C.

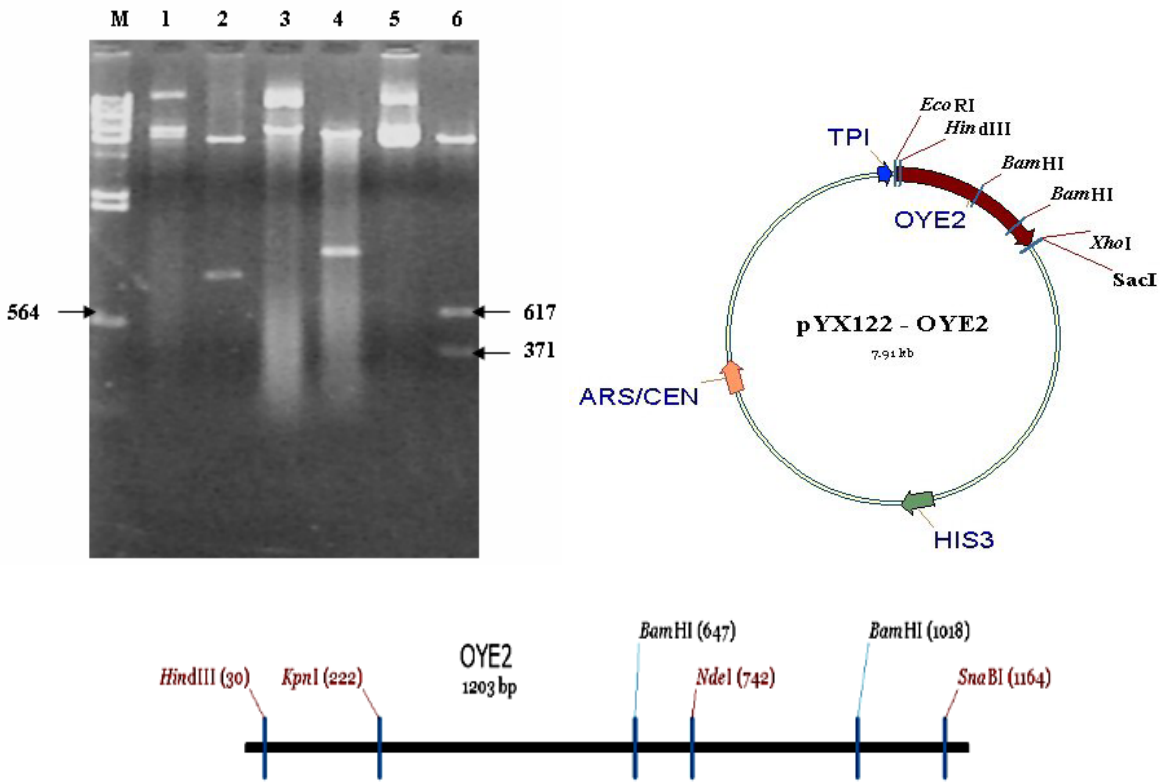


Figure 2.1. Cloning of OYE2 to pYX122. M: Gene ruler, λ DNA/HindIII marker, 1: pYX122-mitGFP undigested, 2: pYX122-mitGFP digested with EcoRI and XhoI, 3: pYX143-OYE2 undigested, 4: pYX143-OYE2 digested with EcoRI and XhoI, 5: pYX122-OYE2 undigested, 6: pYX122-OYE2 digested with HindIII and BamHI**.**

4.2. Cloning of OYE2 (3' truncated at 388bp) into pYX122

The pYX122-mitGFP plasmid was restriction digested with EcoRI and XhoI as was the pYX143-OYE2 (1-388). The fragments were run on 1% agarose gel and the linear pYX122 (large band) and excised fragment (small band) OYE2 (3' 1-388) were gel extracted, and the fragments (vector and insert) were ligated using T4 ligase (5µl vector, 10µl insert, 2µl 10x T4 ligase buffer, 1µl T4 ligase, 2µl H_2O). A control reaction which contained 5µl linear vector pYX122, 2µl T4 ligase buffer, 1µl ligase enzyme and 12µl H_2O, was made. The reaction tubes were incubated overnight at 4^{o}C and 3µl from each reaction was used to transform Mach one *E. coli* competent cells which were selected on selective LB-Ampicillin

plates. Plasmids from 5 randomly picked colonies were isolated and digested with BamHI and HindIII. After running in 1% agarose gel (Fig. 2.2), the plasmid with the correct inset (OYE2 3' truncated) was picked and stored at -20°C.

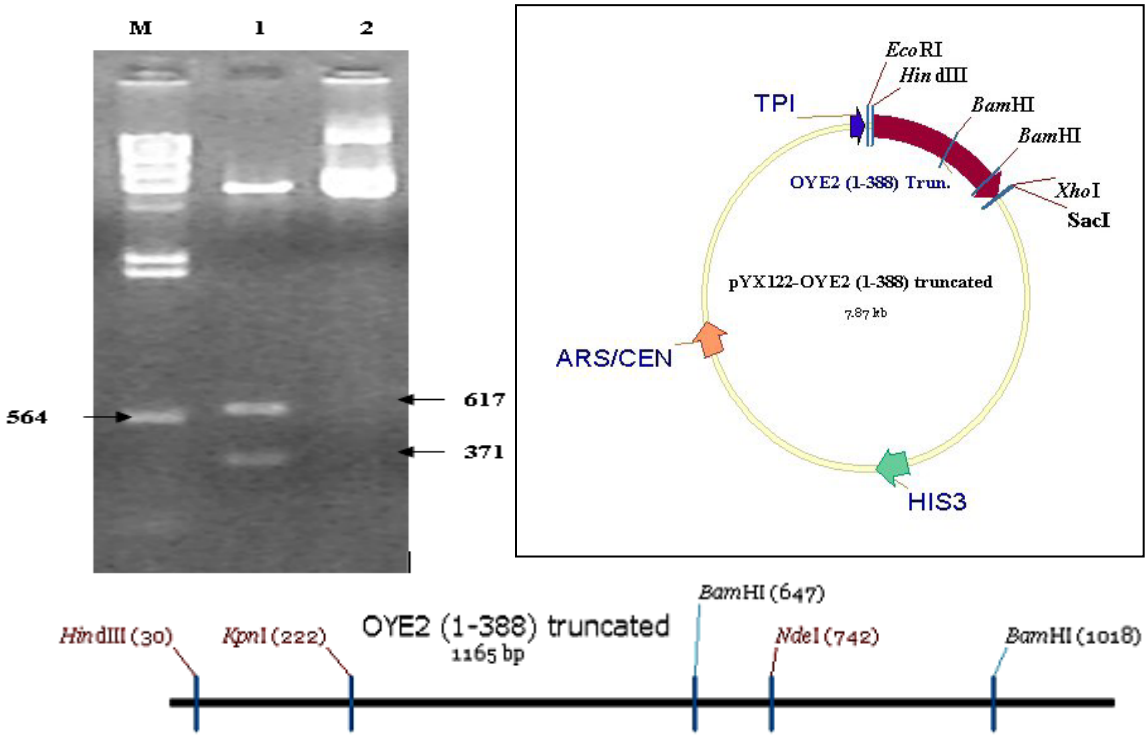


Figure 2.2. Cloning of OYE2 (1-388) to pYX122. M: Gene ruler, λ DNA/HindIII marker, 1: pYX122-OYE2 (1-388) digested with HindIII and BamHI, 2: pYX122-OYE2 (1-388) undigested.

4.3. Cloning of OYE2 (3' truncated at 388bp) into pYX143-HA

The pYX143-HA plasmid was restriction digested with EcoRI and XhoI as was the pYX143-OYE2 (1-388). The fragments were run on 1% agarose gel and the linear pYX143-HA (large band) and excised fragment (small band) OYE2 (3' 1-388) were gel extracted, and the fragments (vector and insert) were ligated using T4 ligase (5µl vector, 10µl insert, 2µl 10x T4 ligase buffer, 1µl T4 ligase and 2µl H_2O). A control reaction which contained 5µl linear vector pYX143-HA, 2µl T4 ligase buffer, 1µl ligase enzyme and 12µl H_2O, was made. The reaction tubes were incubated overnight at 4°C and 3µl from each tube were used to transform Mach one *E. coli* competent cells. Plasmids from 5 randomly colonies that appeared on the selective plates were isolated and digested with BamHI and HindIII and after running in 1% agarose gel (Fig. 2.3), the plasmid with the correct inset (OYE2 3' truncated) was picked and stored at -20°C.

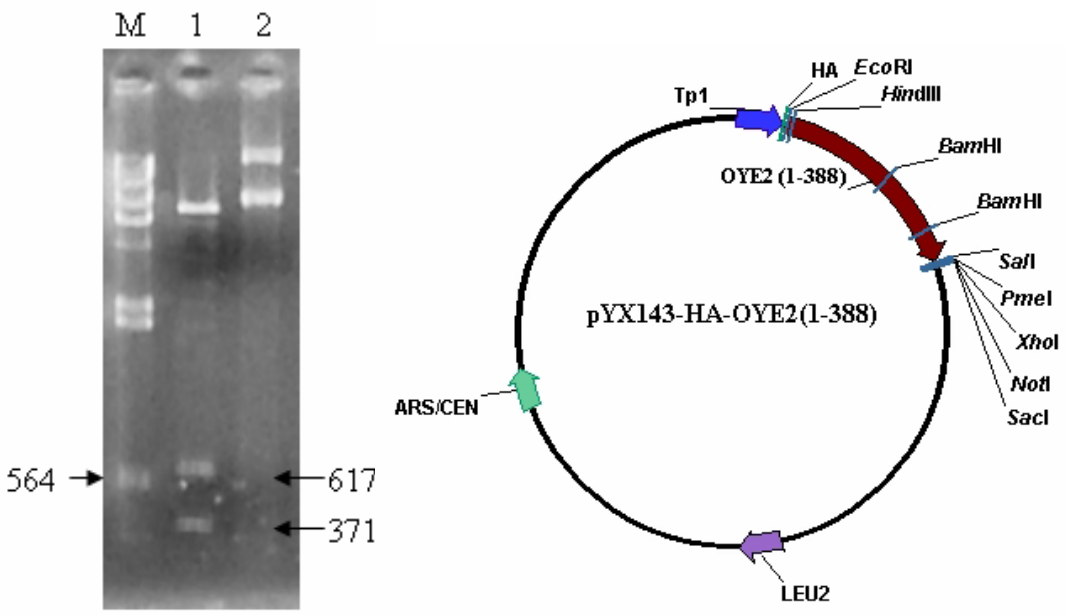


Figure 2.3. Cloning of OYE2 (1-388) to pYX143-HA. M: Gene ruler, λ DNA/HindIII marker, 1: pYX143 HA-OYE2 (1-388) digested with HindIII and BamHI, 2: pYX143 HA-OYE2 (1-388) undigested.

4.4. Cloning of OYE3 into pYX122

The pYX122 plasmid was restriction digested with EcoRI and XhoI. Also the plasmid pTOPO/OYE3 was restriction digested with XhoI and MfeI. The digestion products were run on 1% agarose gel and the linear pYX122 (large band) and excised fragment (small band) OYE3 were gel extracted, and the fragments (vector and insert) were ligated using T4 ligase (5µl vector, 10µl insert, 2µl 10x T4 ligase buffer, 1µl T4 ligase and 2µl H_2O). A control reaction was made in parallel and contained only 5µl linear vector pYX122, 2µl T4 ligase buffer, 1µl ligase enzyme, 12µl H_2O. The reaction tubes were incubated overnight at 4^{o}C and 3µl from each tube were used to transform Mach one *E. coli* competent cells. Plasmids from 5 randomly picked colonies were isolated and digested with BamHI, and after running in 1% agarose gel one band of 408bp appeared, which confirm the correct pYX122-OYE3 plasmid (Fig.2.4). The plasmid was picked and stored at -20^{o}C.

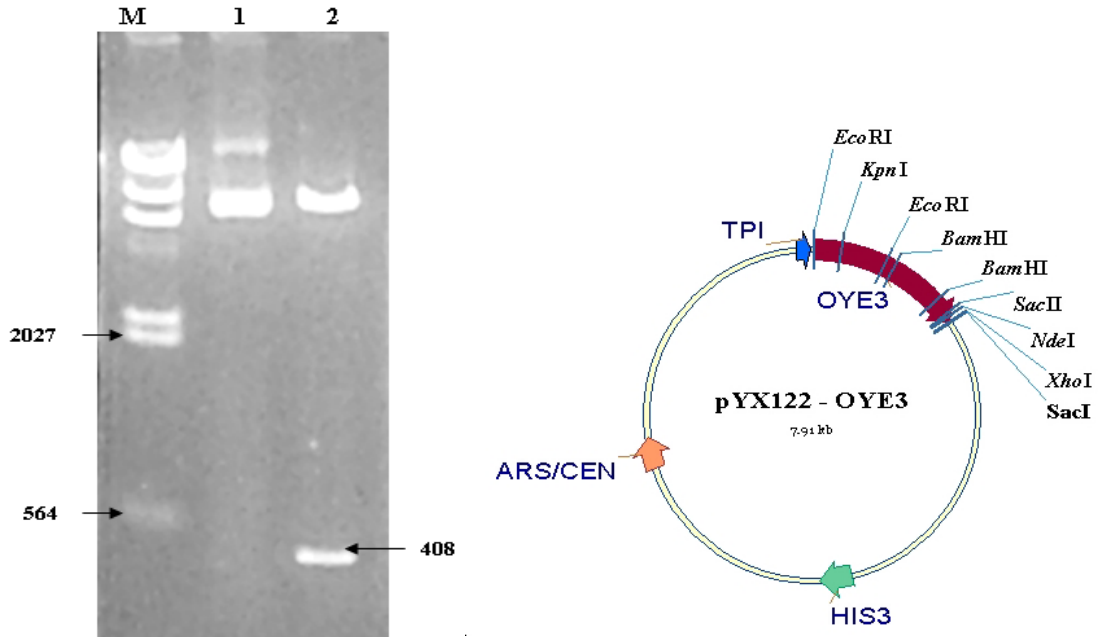


Figure 2.4. Cloning of OYE3 to pYX122. M: Gene ruler, λ DNA/HindIII marker, 1: pYX122-OYE3 undigested, 2: pYX122-OYE3 digested with BamHI.

4.5. Cloning of OYE2 (Y197F) into pYX143

The pYX143 plasmid was restriction digested with EcoRI and SalI. Also the plasmid pTOPO/OYE2 (Y197F) was restriction digested with EcoRI and XhoI. The digestion products were run on 1% agarose gel and the linear pYX143 (large band) and excised fragment (small band) OYE2 (Y197F) were gel extracted, and the fragments (vector and insert) were ligated using T4 ligase (5µl vector, 10µl insert, 2µl 10x T4 ligase buffer, 1µl T4 ligase, 2µl H_2O). A control reaction which contained only 5µl vector, 2µl T4 ligase buffer, 1µl ligase enzyme and12µl H_2O, was made. The reaction tubes were incubated overnight at 4^{o}C, 3µl from each tube were used to transform Mach one *E. coli* competent cells. The plasmids from 5 randomly picked colonies grew on LB-Ampicillin selective plates were isolated and digested with HindIII and BamHI and after running in 1% agarose gel (Fig. 2.5), the plasmid pYX143 with the correct inset OYE2 (Y197F) was picked and stored at -20^{o}C.

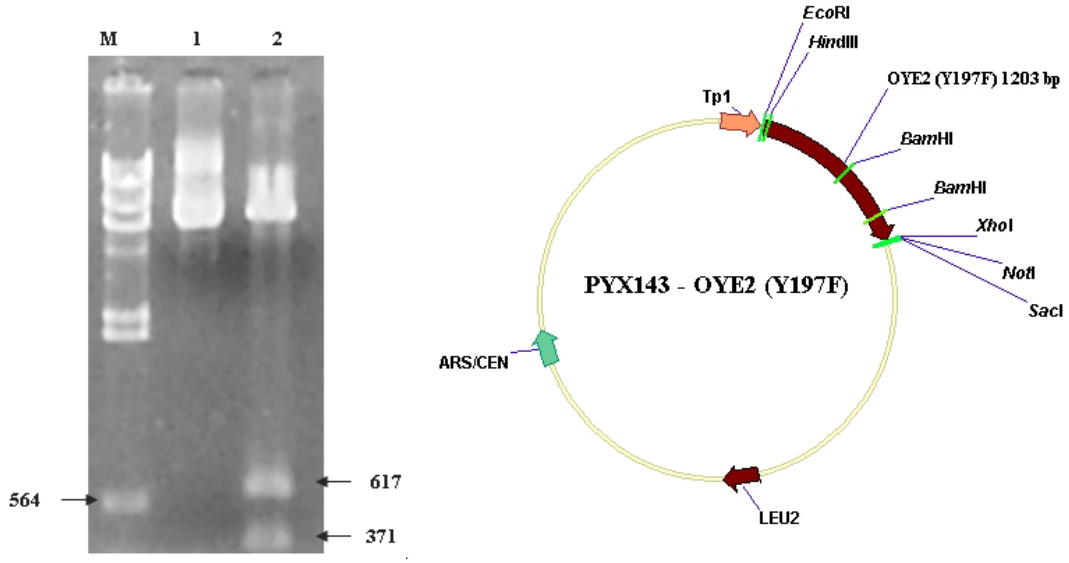


Figure 2.5. Cloning of OYE2 (Y197F) to pYX143. M: Gene ruler, λ DNA/HindIII marker, 1: pYX143-OYE2 (Y197F) undigested, 2: pYX143-OYE2 (Y197F) digested with HindIII and BamHI.

4.6. Cloning of OYE2 (Y197F) into pYX143-HA

The pYX143-HA plasmid was restriction digested with EcoRI and SalI. Also plasmid pTOPO/ (Y197F) OYE2 was restriction digested with EcoRI and XhoI. The digestion products were run on 1% agarose gel and the linear pYX143-HA (large band) and excised fragment (small band) OYE2 (Y197F) were gel extracted, and the fragments (vector and insert) were ligated using T4 ligase (5µl vector, 10µl insert, 2µl 10x T4 ligase buffer, 1µl T4 ligase, 2µl H_2O). A control reaction which contained only 5µl vector, 2µl T4 ligase buffer, 1µl ligase enzyme and 12µl H_2O, was made. The reaction tubes were incubated overnight at 4^oC and 3µl from each tube were used to transform Mach one *E. coli* competent cells. Plasmids from 5 randomly picked colonies were isolated and digested with HindIII and BamHI. After running in 1% agarose gel (Fig. 2.6), the plasmid pYX143-HA with the correct insert (Y197F) OYE2 was picked and stored at -20^oC.

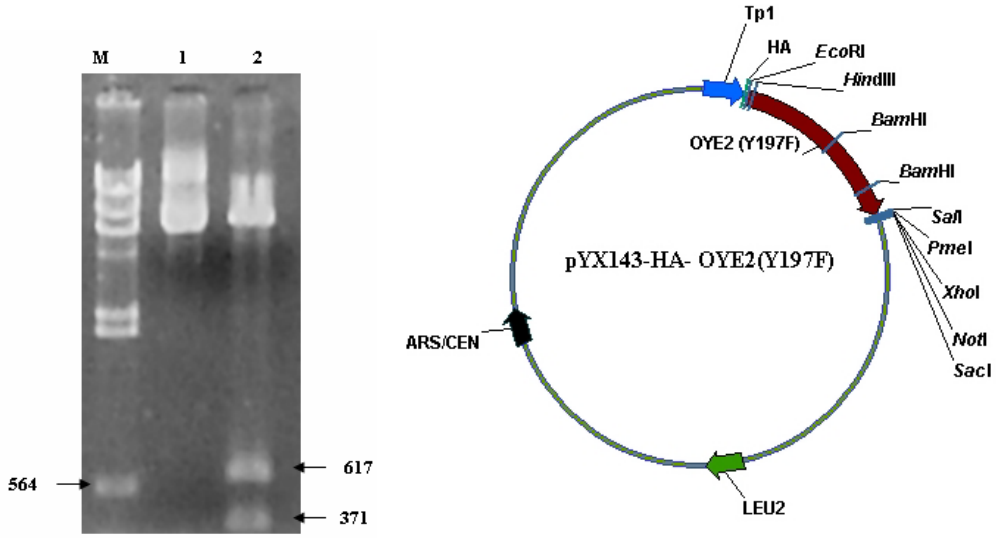


Figure 2.6. Cloning of OYE2 (Y197F) to pYX143-HA. M: Gene ruler, λ DNA/HindIII marker, 1: pYX143 HA-OYE2 (Y197F) undigested, 2: pYX143 HA-OYE2 (Y197F) digested with HindIII and BamHI.

4.7. Cloning of OYE3 into pYX143 HA

The pYX143-HA plasmid was restriction digested with EcoRI and XhoI, Also plasmid pTOPO/OYE3 was restriction digested with Mfe1 and XhoI. The digestion products were run on 1% agarose gel and the linear pYX143-HA (large band) and excised fragment (small band) OYE3 were gel extracted, and the fragments (vector and insert) were ligated using T4 ligase (5µl vector, 10µl insert, 2µl 10x T4 ligase buffer, 1µl T4 ligase, 2µl H_2O). A control reaction which contained only 5µl vector, 2µl T4 ligase buffer, 1µl ligase enzyme and 12µl H_2O, was made. The reaction tubes were incubated overnight at 4^{o}C. 3µl from each tube were used to transform Mach one *E. coli* competent cells. 5 colonies were picked randomly and colony PCR (10 µl of cells boiled for 10 minutes, 3 µl of each samples were used as DNA template for the PCR reaction) was performed using the primers 5'OYE3-CAA TTG ATG CCA TTT GTA AAA GGT TTT GAG CCG ATC-3' and 3'OYE3 5'-CTC GAG TCA GTT CTT GTT CCA ACC TAA ATC TACT GC-3' OYE3. After running in 1% agarose gel (Fig. 2.7), the plasmid pYX143-HA-OYE3 with the correct insert OYE3 was picked and stored at -20^{o}C. Further checking was done by transforming this plasmid to wild type BY4741 and western blotting for the protein utilizing the HA tag for immunodetection.

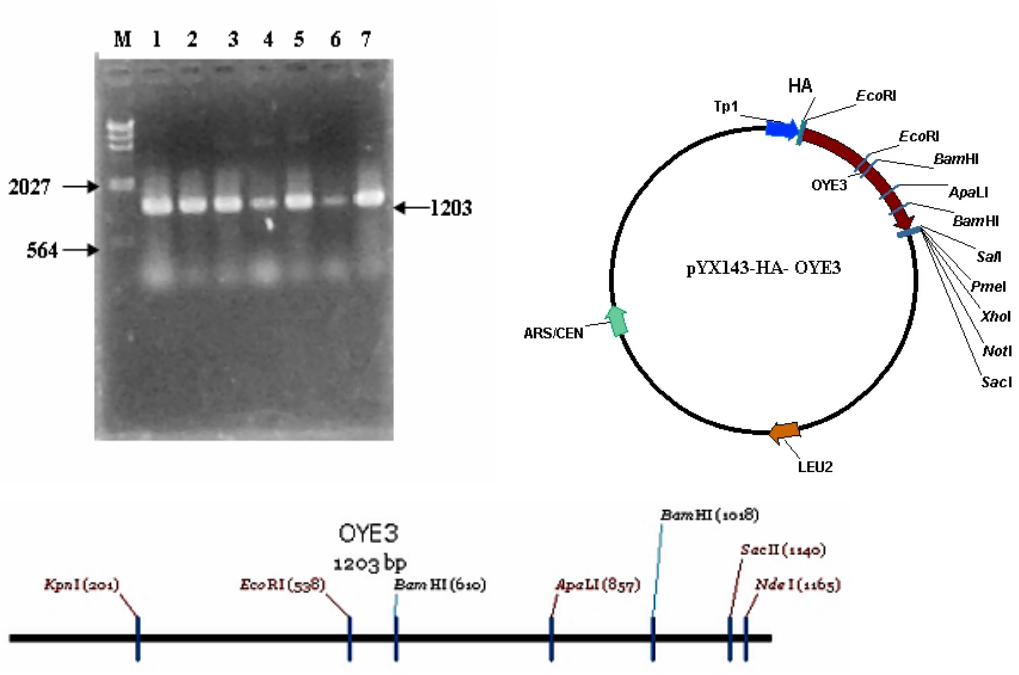


Figure 2.7. Cloning of OYE3 to pYX143-HA. M: the figure shows the PCR amplification of plasmids harboring the OYE3 gene. M: Gene ruler, λ DNA/HindIII marker, 1, 2, 3, 4: pYX143-HA-OYE3. 5: pTOPO-OYE3, 6: pyx143-OYE3, 7: pEG202-OYE3.

4.8. Cloning of OYE3 into pEG202

The pEG202 plasmid was restriction digested with EcoRI and XhoI. Also the plasmid pTOPO/OYE3 was restriction digested with Mfe1 and XhoI. The digestion products were resolved on 1% agarose gel and the linear pEG202 (large band) and excised fragment (small band) OYE3 were gel extracted, and the fragments (vector and insert) were ligated using T4 ligase (5µl vector, 10µl insert, 2µl 10x T4 ligase buffer, 1µl T4 ligase, 2µl H_2O). A control reaction which contained only 5µl vector pEG202, 2µl T4 ligase buffer, 1µl ligase enzyme and 12µl H_2O, was made. The reaction tubes were incubated overnight at 4^{o}C, 3µl from each tube were used to transform Mach one *E. coli* competent cells. Plasmid DNA from 5 randomly picked colonies were isolated and digested BamHI and after running in 1% agarose gel (Fig. 2.8), the plasmid pEG202-OYE3 with the correct insert OYE3 was picked and stored at -20^{o}C. Further checking was done by transforming this plasmid to wild type EGY48 and western blotting for the protein utilizing the LexA tag.

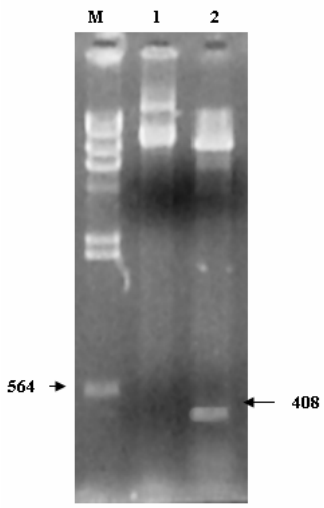


Figure 2.8. Cloning of OYE3 to pEG202. M: Gene ruler, λ DNA/HindIII marker, 1: pEG202-OYE3 undigested, 2: pEG202-OYE3 digested with BamHI.

5. Plasmid extraction

5.1 Bacterial plasmid extraction

5.1.1 Plasmid Extraction using the QIAGEN miniprep kit

Plasmid extraction was performed according to the manufacturer's instructions. (QIAprep Miniprep Handbook, 2006). Bacterial cultures were collected by centrifugation, then resuspended by resupension buffer (P1) solution, Alkaline lysed by lysis buffer (P2) and subsequently neutralized by the neutralization buffer (N3), lysates were cleared by centrifugation. The cleared lysates were then applied to the QIAprep columns where plasmid DNA adsorb to the silica-gel membrane. Impurities are washed away and pure DNA is eluted in a small volume of elution buffer or hot water.

5.1.2 QIAprep Spin Columns regeneration solution:

Recycling solution

3M NaCl,

0.15% Triton-X100,

The columns were washed with recycling solution (750 µl) at least three times.

6. Agarose gel electrophoresis

6.1 Materials

Agarose (Invitrogen)

Running Gel Buffer, TAE Buffer 50X stock

24.2% (w/v) Tris base,

3.72% (w/v) EDTA, the pH was adjusted to 8.0 with ~57 ml Glacial acetic acid and brought to 1 liter with distilled water. The working solution was 1x TAE prepared by dilution of the 50x buffer (40ml 50x TAE and the volume completed to 2 liters with distilled water).

STEB (6X Loading buffer) for DNA

100 mM Tris HCl, pH 7.5,

20% Glycerol (w/v),

1 mM EDTA,

Few grains of Bromophenol blue.

***Staining solution*:** 50 µl of 1 mg/ml Ethidium bromide stock added to 500 ml of distilled deionized water.

***Destaining solution*:** clean deionized distilled H_2O

6.2 Method

Agarose gel electrophoresis was used to separate, identify and purify DNA fragments. 1% agarose gel was prepared by adding 100 ml of 1x TAE to 1g of agarose, mixed and then heated for 2-3 min in a microwave oven until the solution became clear. The tray was assembled with the comb into the gel apparatus. After being cooled down to 55ºC, the gel was poured into the tray and left until solidified. The tank was filled with 1xTAE solution covering the top of the gel. The DNA samples were mixed with the corresponding volume of 6x loading buffer (to a final concentration of 1x) and the samples were applied to the wells. The gel was run at 150V for at least half an hour to ensure an optimal separation of the fragments, then stained with Ethedium Bromide for 15-20 min. The gel was transferred to the distaining solution for 5 minutes to remove the excess ethidium bromide before visualizing it under UV light.

7. Restriction Digestion of plasmid DNA

Materials

10x BSA stock (10 mg/ml) New England Biolabs

10x restriction enzyme buffer, NEBuffer, New England Biolabs

Restriction enzyme

Plasmid DNA (at a concentration no more than 200 ng/µl)

Method

Restriction digestion of the plasmid was performed for excision of certain inserts to be cloned in another plasmid, also used for the identification of an insert being cloned after cloning. The reaction mixture usually contained:

15µl plasmid DNA,

4µl of 10x BSA,

4µl of 10x restriction enzyme buffer,

1µl of each restriction enzyme,

Sterile water was added to a final volume of 40µl. The reaction mixture was incubated at 37ºC for 2hours and then the fragments were resolved by agarose gel electrophoresis.

8. Vector-Insert Ligation

The DNA concentration in the ligation should be of 0.2 µM to favour intramolecular interaction, thus the formation of monomeric circles. The digested samples were ligated with 1µ1 of T4 ligase in a 40µl reaction volume overnight at 4°C. For a successful ligation, after digestion of plasmid and excision of the insert, the concentrations of linear vector and insert were estimated by running on agrose gel. Approximately 1:1 or 1:2 vector: insert DNA was used.

The reaction mixture contained:

5µl vector,

10µl insert,

2µl 10x T4 ligase buffer,

1µl of T4 ligase

PCR sterile water was added to make up the final volume 20µl. The reaction mixture was incubated overnight at 4ºC and 3µl of the reaction was used to transform Mach one competent cells. Colonies grown on the selective medium were first amplified overnight and then DNA plasmid was extracted. Confirmation of the new clone was justified by digestion with restriction enzymes other than those used for the cloning purpose and by PCR.

9. PCR Amplification

Materials

Primers 100 pmol/ µl.

DNA Template (Usually the amount of template DNA is in the range of 0.01-1ng for plasmid DNA and 0.1-1µg for genomic DNA, for a total reaction mixture of 50 µl).

Taq DNA polymerase,

Platinum Taq buffer – $MgCl_2$ 50mM,

10mM, dNTPs

PCR Water.

Method

The final concentration of primers was 100pmol/µl. A master mix wass prepared, consisting of 5µl Taq buffer (10x), 1µl dNTPs (10mM) and multiplied by the number of reaction tubes needed. After adding the master mix to the PCR tubes, the other components were added: 2µl DNA template (diluted 1:10); 1µl of each primer, and then water was added to reach 49µl volume. Reactions were incubated allowed to cycle in Gene Amp PCR 2400 system (Perkim-Elmer) under the conditions needed depending on the reaction, 1µl of Taq polymerase was added to the reaction mixture when the temperature reached 94^{o}C, to have final volume of 50µl.

9.1. OYE2 Mutation Y197F, QuikChange Site-Directed Mutagenesis

The QuikChange site-directed mutagenesis kit is used to make point mutations, switch amino acids, and delete or insert single or multiple amino acids. Mutagenesis Kit, instruction manual Catalog #200518, #200519.

Materials

PfuTurbo DNA polymerase

PfuTurbo 10xbuffer

pTOPO-OYE2

Temperature cycler

H_20

PfuTurbo DNA polymerase replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) pTOPO-OYE2 vector with an insert of interest OYE2 and two complementary primers containing the desired mutation, OYE2 (Y197F)5 5'-CCA CAG CGC TAA CGG TTT CTT GTT GAA CCA GTT CTT G-3' and OYE2(Y197F)3 3'-CAA GAA CTG GTT CAA CAA GAA ACC GTT AGC GCT GTG G 3'. The oligonucleotide primers, each complementary to opposite strands of the vector, were extended 16 cycles by *PfuTurbo* DNA polymerase. At the end of amplification reaction, the products were treated with *Dpn* I. The *Dpn* I endonuclease (target sequence: 5´-Gm6ATC-3´) is specific for methylated and hemimethylated DNA and it is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. The PCR product possibly harboring the mutated insert was then transformed into competent cells, then plasmid DNA was extracted from colonies appearing on the selective plates. The mutation of OYE2 into OYE2 (Y197F) was verified by sequencing.

10. Transformation protocols

10.1 Bacterial transformation

10.1.1. Preparation of Competent Cells

Transformation Buffer –X

50 mM $CaCl_2$,

5% (v/v) Glycerol,

10 mM MOPSO pH 6.6 (adjusted with NaOH only, MOPSO= 3 [N- morpholino] propanesulfonic acids) pH 6.2-7.6, FW: 231.2.

Filter sterilized; bring up to the desired volume (300 ml) with ddH_2O.

Method

A 5 ml overnight culture of cells was grown in LB broth. 1ml of the overnight culture was inoculated into 400 ml of pre-warmed LB broth and grown to ~ 0.2 OD_{600} (2-3 hours). The cells were moved to 50 ml sterile falcon tubes and chilled on ice for 10 min. Later on cells were pelleted at 5500 rpm at 4ºC for 10 min and resuspended in 50ml of ice-cold transformation buffer-X. The resuspended cells were incubated on ice for 20 min and spun again for 10 min (5000-6000 rpm, 4ºC). The pellet was resuspended with 3ml of ice-cold transformation buffer-X (for each falcon). 50µl aliquots of the cells were prepared into 1.5-ml eppendorf tubes, fast frozen in liquid nitrogen and directly kept at -80°C.

10.1.2. Bacterial Transformation.

A 50µl aliquot of competent cells was chilled on ice for 10 min to thaw. 1-3µl of DNA was subsequently added and the cells were incubated on ice for 30 min. The cells were heat shocked for 45 seconds at 42ºC in a water bath and then incubated on ice for 2 minutes. After the end of heat-shock, 300µl of recovery LB or SOC medium was added and the tubes were further incubated shaking for 1 hour at 37ºC. Aliquots of 100µl of cells were plated on selective LB-agar plates and the plates were incubated overnight at 37ºC till distinct colonies appeared.

10.2 Yeast transformation

Reagents for LiOAc yeast transformation

10X Lithium acetate solution

Lithium acetate FW: 102.02 g/mol

The lithium acetate solution is prepared as a 1M stock in distilled water and filter sterilized. The final pH should be between 8.4-8.9 (pH 7.5).

10X TE buffer (pH 7.5)

Tris HCL

0.5 M EDTA stock

The buffer is a mixture of 100 mM Tris HCL (pH 8.0), and 10 mM EDTA,
pH is then adjusted to 7.5 and sterilized by standard autoclaving for 20 minutes.

Polyethylene glycol (PEG 50% w/v)

The polyethylene glycol (PEG), MW 3350 (Sigma) is made up to 50% (w/v) with distilled water and then filter sterilized. For optimal transformation efficiencies, care must be taken to ensure that the PEG solution is at the proper concentration. In addition, it is important to store the PEG in a tightly capped container to prevent evaporation of water and a subsequent increase in PEG concentration. 50 g of polyethylene glycol is dissolved in 35 ml of water in a 150 ml glass beaker by stirring for at least 30 minutes. The liquid is then transferred to a 100 ml graduated cylinder and the volume is then adjusted to 100 ml by adding distilled water. The solution is then filter sterilized and stored in a securely capped bottle.

Sonicated single –stranded carrier DNA (sssDNA)

sssDNA was used as a carrier which enhances the transformation efficiency, 2mg of high-molecular weight DNA (deoxyribonucleic acid sodium salt, type 3 from salmon testes, Sigma) per 1ml of sterile TE buffer was sonicated, aliquoted and stored at -20ºC. Prior to use, it should be boiled for 10 minutes and quick-cooled on ice.

***Solution 1*:** 1M Lithium Acetate, 10x TE Buffer, dd H_2O, 1:1:8 ratios respectively.
***Solution 2*:** 1M Lithium Acetate, 10x TE Buffer, 50% PEG, 1:1:8 ratios respectively.

Method

5ml of an overnight culture were diluted at OD_{600} to ~ 0.1 in 50ml fresh medium and grown till OD_{600} ~0.5. Cells were pelleted by centrifugation at 4000 rpm for 5 minutes, and then washed twice with ddH_2O water and resuspended with 300µl of solution 1. 50µl of these cells were added to new eppendorfs containing 3µl of plasmid DNA mixed with 3µl of the carrier sssDNA. 300µl of Solution 2 was added to each tube, and then incubated at 30ºC for 30-45 minutes. Cells were then heat chocked at 42ºC for 10 minutes. Cells were then plated on the desired plate at a volume depending on the excepted transformation efficiency.

11. Western blot analysis of (Y196F) oye2p expression in BY4741 cells.

In order to determine whether (Y196F)oye2p mutant protein was expressed or not, and to check the level of its expression, the total protein lysates from the wild type BY4741 cells over-expressing OYE2, (1-388) OYE2 truncated, (Y196F)OYE2 subjected to the SDS-PAGE electrophoresis followed by western blot to detect and visualize the expression of these proteins. 5 ml of cells overexpressing the desired genes were grown overnight on a Glu/CM lue liquid medium, and then washed twice with ddH2O and resuspended in 30ml new fresh for 8 hours to induce the protein expression. At the end of the incubation period, the cells were pelleted by centrifugation at 4000 rpm for 5 min. After being washed twice with distilled sterile water and protein extraction were followed.

11.1 Protein extraction

Pellets of cells overexpressing the desired protein already washed with ddH_2O after induction. Pellets were resuspended in 0.5 ml of yeast lysis buffer (protein extraction buffer) (120 mM NaCl; 0.5 % NP-40; 1 mM PMSF) and then transferred to 1.5 ml eppendorf tubes containing equal volume of glass beads. Cells were then vortexed (alternative 30 sec vortexing followed by 30 sec incubation on ice) 20 times. After being centrifuged (twice for better purity), supernatants were transferred to new tubes. The total protein concentration was estimated by the Bradford assay method, which was based on the fact that the color of Coomassie Brilliant Blue in dilute acid solution changes proportionally as the dye binds to proteins. Samples are then stored at -20ºC. Known BSA concentrations were used to generate a linear curve in order to calculate the unknown concentrations of our samples.

11.2 SDS-PAGE electrophoresis

Electrophoresis, which is used to determine the molecular mass and the level of purity of protein samples, is carried out on the sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) discontinuous buffer system in a dual slab cell gel apparatus (Hoefer; Bio-rad mini-protean II).

Materials and solutions

Mini-Protean 3 Electrophoresis Cell (BIO-RAD, cat 165-3301)

Power Pac 300 Power Supply (BIO-RAD, cat 165-5051)

Tris-HCl pH, 8.8

1M Tris-HCl dissolved in 1L of ddH_2O. The pH is then adjusted to 8.8 and the solution is sterilized by autoclaving.

Tris-HCl pH, 6.6

1 M Tris HCl dissolved in 1L of ddH_2O. The pH is then adjusted to 6.6 and the solution is sterilized by autoclaving.

Acrylamide/bisacrylamide monomer solution
Provided as 37.5:1 solution of 40% acrylamide (Fluka, Biochemika), stored at 4ºC.

Ammonium persulfate stock solution (APS)
10% w/v ammonium persulfate in ddH_2O, stored at 4ºC.

Sodium dodecyl sulfate stock solution (SDS)
10% w/v sodium dodecyl sulfate in ddH_2O, stored at 4ºC.

TEMED (Sigma) supplied ready solution for usage.

Water saturated butanol

Equal volumes of water and n-butanol were mixed, shaken vigorously, and allowed to stand. Top layer was used.

1x Tris-glycine electrophoresis buffer, pH 8.3

1.44 % (w/v) Glycine

0.3% (w/v) Tris-HCl

0.1 % (w/v) SDS

Adjust the pH to 8.3

2x sample buffer (2xSAB)

125 mM Tris-HCl, pH 6.8

20% Glycerol

4 % (w/v) SDS

1.43 M ß-Mercaptoethanol

Few grains of Bromophenol blue.

Protein marker

Prestained molecular marker (Fermentas)

Preparation of SDS resolving gel

a) Resolving gel (Final concentration 12%)

Components	**Final volume**
DD water	3.04 ml
1 M Tris-HCl, pH 8.8	3.75 ml
40% Acrylamide/bisacrylamide	3.0 ml
10% SDS	100 µl
TEMED	10 µl
10% w/v APS	100 µl
Total	**10 ml**

b) Stacking gel (final concentration 4.5%)

Components	**Final volume**
DD water	7.54 ml
1 M Tris-HCl, pH 6.8	1.25 ml
40% Acrylamide/Bisacrylamide	1 ml
10% SDS	100 µl
TEMED	10 µl
10% w/v APS	100 µl
Total	**10 ml**

Method

Gel Preparation - Electrophoresis

The mini protein slab gel apparatus was assembled according to the manufacturer's instructions. The resolving gel mixture was prepared as described above and transferred to the glass plate sandwich using a Pasteur pipette up to approximately 2/3 of the glass plate sandwich total volume. n-butanol was added immediately to remove oxygen from the surface and to make the surface of resolving gel horizontal. After polymerisation, the n-butanol was drained off and the gel surface rinsed with distilled water. Any remaining traces of water were removed with filter paper, taking care not to damage the gel surface. The stacking gel mixture was prepared in the same way as described in the material section and overlaid on the resolving gel up to the brim of the glass plate sandwich. Taking care not to trap any air

bubbles, a 10-slot teflon comb was inserted in such a way that approximately 1-1.5 cm were left between the top of the resolving gel and the bottom of the comb. The monomer was then left to polymerize. After the stacking gel had polymerized, the comb was removed carefully and the wells that had formed were washed with DD water to remove any unpolymerized acrylamide solution. The polymerized gel slab-glass plate sandwich is first removed from the casting stand and transferred to the upper buffer chamber, and then the entire unit was introduced into the larger lower buffer chamber. 1xTris-glycine electrophoresis buffer was added to the lower (approx. 2/3) and then to the upper chambers to the brim of the sandwich, so that all wells are properly covered in buffer before the combs were carefully pulled out. Samples were then boiled for 5 minutes to denature the proteins and then centrifuged for 1 min at 13,000 rpm to avoid protein streaking during electrophoresis due to cell debris. Using a Prot/Elec loading tip, the desired amounts of the protein samples or prestained SDS-PAGE molecular weight standard markers were loaded into each lane and the gel was run at a constant current (30mA, 200V) until the dye front reaches the end of the gel or the lowest band of the prestained marker is approximately 0.5 cm from the gel bottom. The gel was then removed to be used in western blotting or to be stained with commassie blue.

11.3 Western blot and immunodetection of Recombinant Proteins using anti-HA

This method provides a system for the transfer of protein from a gel to a solid support for visual detection by probing with antibodies that are specific for particular sequences or tags (epitopes) of amino acids displayed by the target protein attached to the solid support.

Materials and solutions

Tank Electrophoretic Transfer system

Power Pack 300 Power Supply (BIO-RAD, cat165-5051)

PVDF Protein Transfer (NEN™, Life Science Products, cat NEF1002)

Cellophane Nylon Wrap

HypercassetteTM18x24cm (Ameshram LIFESCIENCES, Batch240195, RPN1642)

Fluorescence Sticker

Scientific Imaging Film (Kodak, cat1651454)

Methanol 100% Methanol (Absolute Methanol)

Solutions

Methanol

100% Methanol (Absolute Methanol)

Transfer Buffer

48 mM (5.82g) Tris-HCl

39 mM (2.93g) Glycine

pH is adjusted to 8.0, then 200 ml methanol is added up to final volume 1liter.

1x Tris Buffered Saline (1x TBS)

10mM Tris-HCl

150mM NaCl

pH is adjusted to 7.5

Tris Buffered Saline/Tween 20 (TTBS)

0.1% (v/v) Tween 20 in 1x TBS.

Blocking solution

5% powder milk in 1x TBS

Primary antibody stock

In 1X TBS we add 3% milk and the antibody according to the dilution factor. The antibody is then stored at -20C.

Anti HA epitope 1:1000 dilution.

Secondary antibody stock

In 1X TBS we add, 3% milk and the antibody according to the dilution factor. The antibody is then stored at -20C.

HRP-anti mouse, 1:8000 dilution.

Western blotting detection reagent* (*SuprSignal Western Pico Chemiluminedcent substrate, PIERCE 34080)

Obtained ready to use, equal volumes used from both bottles (1 ml each)

Developer and Replenisher (Kodak GBX, cat1901859)
To 103 ml of the stock solution, DD water was added to a final volume of 473 ml and kept in a dark place.

Fixer and Replenisher (Kodak GBX, cat1901859)
To 103 ml of the stock solution, DD water was added to a final volume of 473 ml and kept in a dark place.

Method

Western blotting involves transferring of electrophoretically separated components from a gel to a solid support, such as a nitrocellulose or nylon filter, and probing with reagents that are specific for particular sequences of amino acids. The probes are usually antibodies that bind to antigenic epitopes displayed by the target protein attached on the solid support. Western blotting is extremely useful for the identification and quantitation of specific proteins in a complex mixture of proteins. A 1-5ng of an average sized protein can be detected by western blotting. The gel cassette is opened and lifted gently and the gel is cut, stacking gel is thrown away, resolving gel is immersed in transfer buffer for 15 minutes, thus allowing it to equilibrate. A piece of the PVDF membrane is cut to the dimension of the gel. One corner of the membrane is notched for later correspondence with the corner of the gel. The membrane is then wetted in 100% methanol for 15s, and then soaked in distilled water for 2 minutes and finally equilibrated into transfer buffer for at least 5 minutes. The 3MM paper, the PVDF membrane and the gel are assembled in the cassette in the following order starting from the black part: foam pad, paper, gel, PVDF membrane, paper, foam pad. The cassette is closed and placed in the tank blotting apparatus so that the side of the cassette holder with the gel is facing the cathode. Transfer buffer is then added to the blotting apparatus until the cassette is totally covered. The tank is then connected to the power output and the system is turned on for 2 hours at 30 volts. The tank is soaked in ice to prevent overheating of the buffer during the transfer process. After removing the foam pad and filter papers, the membrane is blocked in 5 % milk for one hour and then washed twice for 5 minutes in TTBS. The membrane is then transferred to a plastic bag containing the

primary antibody, and it is incubated for one hour shaking at room temperature. The membrane is washed with 1x TBS once for 15 minutes and then two times for 15 minutes each in 1x TTBS, then once with 1x TBS for 5 minutes. The membrane is transferred to a new plastic bag, and the HRP-labelled second antibody is added and incubated with the membrane for another one hour. The membrane is washed again with 1x TBS once for 15 minutes and then two times for 15 minutes each in 1x TTBS then once with 1x TBS for 5 minutes. Chemiluminescence reagent is prepared by mixing an equal volume of solution from bottle 1 and bottle 2. The membrane is then agitated with at least 0.125 ml of chemiluminescence reagent per cm^2 for 5 minutes. Excess reagent is removed by draining and the blot is enclosed in a plastic wrap, placed in the film cassette and exposed to an autoradiography film (Fuji) for 1 minutes. The film is then processed for 1 minute in developing solution, 2 minutes in fixing solution after a brief wash with water. The size of protein of interest is identified by comparison with the molecular markers.

12. Glutathione measurements

12.1 Materials and solutions:

a- 1% Oxalic acid (or 0.5% Formic acid)

b- 2-vinyl pyridine

c- Stock buffer: 125 mM Na-Phosphate, 6.3 mM Na-EDTA. Adjust pH to 7.5

d- 5 mM Triethanolamine: 1M stock is made by mixing 132 µl Triethanolamine into a total volume of 1 ml of H2O. To prepare a 5 mM stock, add 50 µl from the 1 M stock into 10 mls total volume of H_2O.

e- Three different working solutions made in stock buffer

I. 0.3 mM NADPH (24 mgs in 10 ml stock buffer)

II. 6 mM DTNB (0.115 gr in 50 ml stock buffer)

III. ~50 Units of Glutathione Reductase

f- GSH stock: 250 mM stock

250 mM stock of GSH prepared by measuring 0.15 g and dissolving it in 2 ml of sterile dH_2O. The following dilutions were prepared:

500 µM	2 µl in 1 ml of 1% oxalic acid
1 mM	4 µl in 1 ml of 1% oxalic acid
2 mM	8 µl in 1 ml of 1% oxalic acid
5 mM	20 µl in 980 µl of 1% oxalic acid
10 mM	40 µl in 960 µl of 1% oxalic acid

g- GSSG stock: 100 mM stock GSSG.

Dissolve 0.12 grams in 2 ml dH_2O. The same dilutions made as above.

12.2 Method:

5 ml cultures of the yeast strains were grown in Glu/CM overnight. The next day cells were resuspended in 50 ml medium in Erlemeyer flasks at OD_{600}~0.15 and were grown cells till saturation for about 10 hours. 50 ml falcon tubes were labeled and weighed empty and samples were centrifuged in the falcons (3,800 rpm for 8 min.) and then washed two times in sterile H_2O. At the end of the second centrifugation, falcons were dried with a paper towel to remove any excess water on the walls of the tube. The tubes were weighed with the pellets and the wet pellet weight was calculated. Cells resuspended in equal concentrations in 1% Oxalic acid, (cells with the lowest weight in 5 ml, and all other cells in proportion to their weight volume of 1% oxalic acid). Cell suspensions were sonicated to break (1 min should be enough, check turbidity), then centrifuged and the supernatants collected and divided into two different sets of eppendorfs: set A for total glutathione (GSH) and set B for oxidized glutathione (GSSG). 200 µl of supernatants were transferred to each respective tube; to all samples add 1.8 µl of 5 mM stock triethanolamine (45 µM final concentrations) were added**.** To set B (GSSG) 4 µl of 2-vinyl pyridine were added and incubated for 30 min at RT. The following mastermix is prepared in accordance to the total number of reactions:

700 µl	**Sol I** (0.3 mM NADPH) ~210 µM NADPH final
100 µl	**Sol II** (6 mM DTNB), 0.6 mM DTNB final

800 µl of mix was added per reaction, the reactions were equilibrated at 30°C for 5 min, and the contents transferred to 1 ml cuvette. **1 µl Enzyme** (~0.5 Units of Enzyme Glutathione Reductase) was added and the absorbance was monitored at 412 nm. Control reactions containing known quantities of GSH and GSSG should be prepared (250 µM, 500 µM, 1 mM, 2 mM, 5 mM, 10 mM) in 1% Oxalic acid solution.

13. Yeast Genomic DNA Isolation

Matreials

YPD growth medium (1% yeast extract, 2% peptone, 2% glucose),

Lysis buffer: 2% Triton-X100, 1% SDS, 100mM NaCl, 10mM Tris-HCl pH8.0, 1mM EDTA pH 8.0.

Absolute Ethanol

70% Ethanol

Chloroform

TE buffer: (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0)

RNase A

Method:

1.5 ml of liquid culture grown for 20-24h at 30^{o}C was transferred into a micro-centrifuge tube, and cells were pelleted by centrifugation at 13,000 rpm for 5 minutes. The pellet was resuspended in 200µl of lysis buffer. The tube was frozen at -80^{o}C by immersing in ice cold ethanol bath for 2 minutes and then transferred to 95 ^{o}C water bath for 1 minute. This process was repeated twice. The cells were subsequently vortexed for 30 seconds, and 200µl of chloroform was added to the tube and vortexed for 2 minutes. The tubes were centrifuged at 13,000 rpm for 3 minutes at RT and the upper aqueous phase was transferred to a new centrifuge tube containing 400µl ice cold 100% ethanol (which favors DNA precipitation), mixed by inversion or gentle vortexing, then incubated at -20 ^{o}C for 30 min for precipitation, and then centrifuged again at 13,000 rpm for 5 minutes at RT. The pellets were washed with 0.5 ml ice cold 70% ethanol (salts dissolve in the aqueous phase) and were dried in vacuum dryer (5 minutes at 60^{o}C). Finally, the pellets were resuspended in 40µl TE buffer and 3µl RNase was added to the tube to destroy any remaining RNA during the purification procedure.

14. Purification of the PCR product

14.1 Gel extraction

DNA fragments were purified using gel extraction kit (QIAGEN, gel extraction kit, cat.28704).

14.2 Purification of the PCR product by ethanol chloroform precipitation

Phenol is slightly water-soluble, used as water-saturated solution buffered with Tris and gives a 'fuzzy' interface that is sharpened by the presence of chloroform. Ethanol precipitation is a method used to concentrate DNA. DNA is polar, soluble in water, which is also polar. Based on the principle of "like dissolves like", it is insoluble in the relatively less polar ethanol. The DNA precipitation in this step is due to the ethanol interacting with the water so that fewer water molecules are available to dissolve the DNA molecules.

Materials:

Tris-buffered 50% phenol

Tris-buffered 50% chloroform

100% ethanol

70% ethanol

Method

To 200µl of DNA sample into a 1.5ml microcentrifuge tube, one volume of Phenol:chloroform is added which will give a sharp interface, vortexed vigorously one minute to mix the phases. Centrifugation was carried out at top speed for 3 min at 13000 rpm to separate the phases, and then the aqueous phase is removed to a new tube. The aqueous phase is usually the upper one. The loss of DNA into the organic phase can be reduced by adding a second volume of water, mixing, centrifuging, and removing again. The extraction step with (1:1) V of phenol:chloroform which is repeated once, and then followed by extraction with 1volume of chloroform, which will ensure that no phenol whatsoever remains. DNA is initially ethanol-precipitated by adding 2V of ice cold 100 % ethanol and 1/10 volume of 3M Sodium Acetate pH 5.8. The samples were incubated 30 min at -80°C or 2 hours at -20°C. This will precipitate DNA, as well as the salts that form ionic bonds with it. The suspension was centrifuged for 20 min in a microcentrifuge tube at 4°C and the supernatant was removed, leaving a pellet of the crude DNA. In the next step, 70% ethanol was added to the precipitated pellet, without vortexing or disturbing the pellets (fast), to re-suspend the DNA. This allows the 20-30 % water to access the salts present in the pellet.

This suspension was centrifuged again for 20 min at 13000 rpm, and the supernatant was removed by aspiration. The pellet was air-dried giving the purified DNA and were resuspended with the10-20µl of 1x TE or elution buffer.

15. Creating a Double-Knockout strain by mating and sporulation

Aim: Generate double knockout of *Δoye3* background with the single deletion strains to compare the effect of Δoye3 deletion compared to the double knockouts in the *Δoye2* background.

Haploid *Saccharomyces cerevisiae* strains:

BY4741: MAT**a**; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0, TRP+

All single deletion mutants (Δ*oye2*, Δ*oye3*) have the BY4741 genetic background.

Mat α control: his5, this is a test strain to determine the mating type of the generated haploids.

Mat a control: his5, this is a test strain to determine the mating type of the generated haploids.

Haploid strains form Research Genetics, with G418 cassettes listed in **Appendix**

15.1 Solutions and reagents for sporulation and random spore analysis.

Presporulation medium (PSP)

0.8% (w/v) yeast extract

0.3% (w/v) peptone

1% (w/v) glucose

The solids were dissolved in ddH_2O and the pH was adjusted to 5.8-6.2, then 2 % of agar was added to the medium to solidify, after which the medium was autoclaved (20 min, 151 lb/sq.in. on liquid cycle). After autoclaving, the media were cooled down in water bath at 50°C and plated in the Petri dishes.

15.2 Sporulation medium

10% (w/v) potassium acetate,

1% (w/v) yeast extract,

0.5% (w/v) glucose

The solids were dissolved in ddH_2O and the pH was adjusted to 5.8-6.2, then 2% of agar was added, after which the medium was autoclaved (20 min, 151 lb/sq.in. on liquid cycle).

15.3 Solutions for random spore analysis

Diethyl ether (analytical reagent M =74.12 g/mol).

50 mM Sodium Citrate pH 5.8 (Sodium citrate-2-anhydrate M=294.10 g/mol).

β-glucuronidase (SIGMA)

GENETICIN (G418 Sulfate 725µg/mg) stock solution 40 mg/ml. working stock: 500µl from the stock solution were added into each 100 ml Agar medium.

15.4 Mating

The mating was done by streaking both strains over each other on YPD media, and then the plates were placed in 30^{o}C incubator for two days, selection for mated cells was made using the suitable selective media.

15.5 Sporulation

The diploid cells were selected on Glu/CM G418^{+} ura^{-}, dropout plates, colonies formed were subcultured on new PSP plate and incubated at 30^{o}C for two days. Then the colonies were subcultured on sporulation medium plate and incubate at 30^{o}C for 4-5 days. Samples of sporulating colonies were examined under the microscope to check for the appearance of tetrad spores stage. After the visualization of the tetrad, the cells were resuspended in eppendorf tube containing 900µl sterile dd H_2O. Then 120-150µl of diethyl ether was added to the cells (the ether was added in order to destroy the vegetative cells). The culture was vigorously shaken by vortexing. The tube was left open in the laminar flow in order to let the ether evaporate. After complete evaporation of the ether, the cells were centrifuged for 2 min. at 13,000 rpm at RT, and then resuspended in 300µl Sodium Citrate (50mM) and 10µl of glucuronidase to break the cell wall.

The cells were incubated at 30^{o}C without shaking for 1 hour. After the period of incubation ended, the cells were subjected to sonication for 30 seconds to release the tetrad. Then 100µl from 10^{-3}, 10^{-4}, and 10^{-5} dilutions were plated on Glu/CM G418^{+} ura^{-} plates and the plates were incubated at 30^{o}C, until the colonies appeared.

15.6 Verification of the haploid nature and establishment of the mating type

20-50 colonies from YPD/G418 were picked to test for mating type by mating with the mating controls. Each colony was replica plated by streaking on three YPD plates (a master plate, one plate to mate with Mat **a** control strain, and one to mate with Mat **α** control strain).The next day the mated cells were transferred to a new Glucose/CM-His plates (if mating has been successful, then the diploid cells are His+ since the BY4741 background will offer the His5 gene and the mating control MAT **a** or MAT **α** cell will offer the His3 gene). A haploid strain should grow in one mating but not the other, which automatically assigns the mating type of the spores.

15.7 Creating a *ΔOYE3* strain with URA cassette

Aim: To generate a mutant *ΔOYE3* with URA3 selection marker.

This strain will enable us to generate double knockout strains with single knockout strains bearing G418 cassette, since during sporulation we can select for Diploids as URA+ and G418+. Having double knockouts, will allow us to test the effect of multiple genes involved with OYE3 in the cell-death adaptation pathway and will delineate the role of OYE3 in the programmed cell death. The approach is based on generating a URA3 cassette of 50 bp OYE3 sequence flanking the cassette on both sides. High efficiency transformation is required for successful integration by homologous recombination.

15.7 Materials:

BY4741 cells
Glu/ura plates
pUG72 plasmid
5'oye3 lox primer
3'oye3 lox primer
10mM dNTPs
Taq polymerase
Sterile PCR H_2O
10x Taq polymerase buffer with $MgCl_2$

Method:

PCR amplification of URA3 cassette flanked by 50b oye3 on both sides was generated by preparing 8 parallel reactions to ensure that there is enough insert for the transformation. The PCR reaction contained the following materials.

1µl of plasmid pUG72

5µl 10x Taq buffer with $MgCl_2$

1µl 10Mm dNTPs

1µl 5'primer

1µl 3' primer

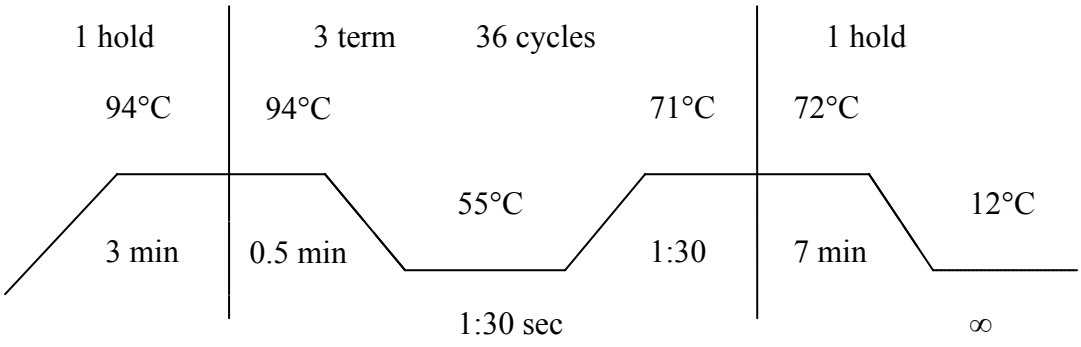
1µl Taq polymerase

40µl H_20.

The primers are 5'OYE3 lox 5'-ATA TAT ACA ACA ACT GTA GTT CAG TAT AGC GAA GTT TAA ATT TAG AAG CAG CTG AAG CTT CGT ACG -3', and 3'OYE3 lox 5'-GCG GGC ACG AGA AAG CTG CCC TAT AAC AAA GAT CGA GTC TTT TGC ATA GGC CAC TAG TGG ATC TG -3' which contain a flanking regions from 5' and 3' of OYE3 gene. From the master mix an aliquot of 49 µl was added to 8 different tubes.

Volume	Reagents	8x volume
1µl	1:10 pUG72 vector	8µl
5µl	10x PCR buffer	40µl
1µl	5' oye3 lox primer	8µl
1µl	3' oye3 lox primer	8µl
1µl	10mM dNTPs	8µl
1µl	Taq polymerase	8µl
40µl	Sterile PCR H_2O	162µl

The tubes were then loaded to the PCR machine (GeneAmp PCR system 2400, PERKIN ELMER). Hot start program was used by adding the Taq polymerase upon heating the PCR machine at 94^{o}C. The following program parameters were used:



5µl of the eluted material were run on a gel to quantify the efficiency of PCR procedure..

After being purified by Phenol / chloroform extraction and concentrated to 10µl the product was used to transform BY4741 cells (200µl of cells). Cells were selected by plating in Glu/CM-ura. Yeast cells were selected on Glu/ura plates and 50 colonies were picked, out of which 6 were tested for the integration of the URA cassette was confirmed by extracting the genomic DNA of the transformed colonies (as described below). Three parallel PCR reactions were done using the genomic DNA extracted from the positive colonies grown on the selective media (Glu/CM-ura) with different combinations of primers to confirm the deletion of OYE3. Genomic DNA from BY4741 was used as a template DNA which serves as controls for the reaction.

a) 5' promOYE3, URA-D (5'AGT GGT GGA TCA GAT ATC 3')

b) 5' promOYE3, 3' OYE3 (LOX)

c) 5'OYE3 3'OYE3

The product in the first two reactions was positive and negative in the third, which confirms the deletion of OYE3.

15.7.1 Creating a Mat α, URA3, *Δoye3* strain

The cells that were transformed with the URA3 cassette were BY4741 wild type Mat **a** mating type, and they should be Mat **α** in order to mate with the other mutants, that have the BY4741 background (Mat a) as well. To achieve this goal the cells were mated with OSO13 cells which are (mat α (BY4741 *oye2*Δ::G418X EGY48) oye2::G418, ura3, his3, trp1). The diploid cells were selected on Glu/CM $G418^{+}$ ura^{-} , then diploid cells were sporulated as before. The haploid cells (spores) which were Mat **α** URA^{+}, $G418^{-}$, trp, his, leu

were selected to be used in mating with the other single mutants in order to generate double knockouts.

15.7.2 Generating of a double knockout

The strains (*oye3*Δ::URA), $G418^-$, his3, trp1, leu2, and haploids with G418 cassette were mated by streaking both strains over each other on the YPD plate and were incubated at 30^oC. The next day the cells were subcultured in Glu/CM $G418^+$ ura^- and incubated as before. Two days later cells were transferred to new PSP media and were incubated for two days and then transferred to sporulation medium for further incubation for about 5 days. Samples of the cultures were examined under the microscope to check for the formation of the tetrad ascospores. Once the tetrad stage had been seen the spores were collected and treated as mentioned previously (sporulation). The double knockout spores were selected in Glu/CM $G418^+$ ura^- plates, and the spores were mated with the mating controls to check for the mating type of each spore.

16. Growth Curves

Overnight cultures of the different strains were grown in the appropriate glucose media. The next morning the cells were washed two times with sterile ddH_2O by centrifugation at 4000 rpm for 5 minutes at 10^oC. The pellets from the second wash were resuspended in new appropriate glucose medium in 50ml cultures at OD_{600}=0.1 and the flasks were incubated at 30^oC in an orbital shaker (150rpm). Every two hours 1ml sample of each culture was taken and the OD_{600} were measured using (HEWLETT PACKARD 8452A Diode array spectrophotometer). The growth curves showed the variation in the growth in all strains (mutants and wild type). The growth curves were repeated for the same strains, but each flask was supplemented with 1.5mM H_2O_2 final concentration after 2 hours of resuspension of the OD_{600}=0.1 dilution, then the $OD_{600}$was taken as before.

17. Determination of the viability of cells treated with pro-oxidants

The strains were grown in 5ml Glu/CM overnight. The next day cells were diluted at OD_{600}= 0.1 in 40 ml Glu/CM in 200 ml flasks and the cultures were kept shaking at 30^oC until they reached OD_{600} 0.5-0.7. For each strain, 50ml FALCON tubes containing 10ml

media (Glu/CM) were prepared, and each tube was inoculated with 200µl of the corresponding diluted cultures. 100µl sample was taken from each tube and 10^{-1}, 10^{-2} dilutions was prepared and spread on YPD plates. Prooxidant (H_2O_2) was added to each tube to a final concentration of 1mM final. The treated cultures were incubated shaking in 30^{o}C shaker for 2 hours. After a two-hour incubation period, samples were taken as before (100µl sample from each tube was taken, and 10^{-1}, 10^{-2} dilutions were made on YPD plates and the plates were incubated incubate in 30^{o}C incubator until the appearance of the colonies).

18. Generation of ρ^0 cells:

Strains used

BY4741

EGY48

Δ*oye2oye3*

Materials:

YPD medium (broth and plates)

YPG medium (broth and plates)

Glucose complete medium

10µg/ml Ethidium bromide

1M H_2O_2

Spectrophotometer

Method:

Yeast cells were grown in 5ml YPD broth culture containing ethidium bromide to a final concentration of 10µg/ml for 3 days at 30 °C. then on in order to test the strains for their growth capacity to grow, from each culture a 1:5 (40 culture: 160µl ddH_2O) serial dilution was prepared and plated to YPD and YPG plates to assess the respiration efficiency.

18.1 Determination of viability of ρ^0 cells treated with pro-oxidants:

The strains were grown in 5ml YPD overnight till saturation. The next day, cells were diluted to OD_{600}=0.1 in 50ml YPD in 300ml flasks and kept shaking at 30 °C until reaching OD_{600}=0.5-0.7. Out of the cultures 200 µl were inoculated into 10ml of fresh culture in 50ml falcon tube. 100µl of each sample was taken from each falcon and used for $10^{-1}$and 10^{-2} dilutions, which were plated on YPD and incubated for 2 days at 30 °C and enumerated when the distinct colonies appeared. The remaining 10ml samples were inoculated with H_2O_2 to a final concentration of 1.5mM and incubated for 2 hours shaking at 30 °C and later subjected to the same procedure of serial dilution mentioned above. Plated samples were incubated at 30 °C for 2 days and the colonies that appeared were enumerated.

18.2 Growth recovery curves

Fresh overnight cultures of the various yeast strains grown in glucose complete medium or glucose media lacking the aminoacid used as auxotrophic marker were washed with ddH_2O and resuspended in fresh medium at OD_{600}=0.1. Aliquots were taken serially at regular time intervals (2hours) and absorbance was measured at OD_{600}. When the OD_{600} >1, the aliquots were serially diluted (1:4, 1:10, 1:20) and new measurements were taken to examine the effect of a transient pulse of H_2O_2. Cultures were resuspended in fresh medim at OD_{600}=0.1 and 2 hours later were supplemented with 1.5 mM H_2O_2 and were incubated shaking at 30°C and the ability of cell populations to recover from H_2O_2 insult was assessed by measuring the OD_{600} at regular time intervals of 2hours.

19. Determination of the viability of cells treated with pro-oxidants

Materials

1M Diamide(dissolve 0.172g in 1ml of sterile ddH_2O)

1M Acrolein (Stock ,90% solution, MW 56.06)

1M MVK (Methyl vinyl ketone

Sterile ddH_20 Stock

YPD plates

YPD broth

ddH_2O

Preparation of media: YPD medium was prepared and was then autoclaved under standard conditions (20min, 1511b/sq.in on liquid cycle). After cooling down around 55°C, the corresponding volume of Diamide (1.5 Mm and 2mM and 2.5mM), and Acrolein (0.1mM, 0.25mM, 0.5mM, 1mM, 2mM, 3mM) and MVK (0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM, 1 mM) were added separately. In last step the media was poured into plates immediately in sterile conditions, then the plates were wrapped up and stored at 4°C up to 2 weeks at maximum. Aliquots from fresh overnight cultures diluted 1:5 in ddH2O plated on the plates and incubated for three days at 30°C.

20. Microscopy of yeast organelles

Materials

Leica DM LB microscope equipped with a 100-mercury lamp linked to a Leica DC300F digital camera, Microscope slides and cover slips.

Evans Blue

As an exclusion dye, viable cells are able to exclude the dye and do not stain. 500µl aliquots of overnight grown cultures were centrifuged for 2 minutes at 13000 rpm, washed twice with PBS, then resuspended in 25% Evans Blue stain (375µl 35% Glycerol with 125µl 4% Evans Blue dye). The cells were left for 20 min at RT shaking prior to microscopy. 15µl of each sample was mounted on a slide covered with a cover slip and observed under the microscope.

Results and Discussion

1- The FMN-oxidoreductase OYE2 suppresses Bax-induced lethality in yeast.

The Bcl-2 family of proteins is at the core of metazoan cell death machinery, Bax, a proapoptotic member of the family, is critical in the control of cell death in mitochondria and endoplasmic reticulum in mammalian cells. Yeast cells overexpressing Bax exhibited depletion of intracellular glutathione levels, transient increases in reactive oxygen species, alteration in mitochondrial membrane potential and mitochondrial lipid oxidation. In previous work carried out to identify yeast genes that participate in PCD processes, an EMS-mutant strain R13, which exhibits defects in mitochondrial protein targeting and resistance to Bax-induced lethality utilized in a reverse genetic screen to identify programmed cell death mediators. The C-terminus of OYE2 (Old Yellow Enzyme, FMN-oxidoreductase) was isolated based on its capacity to restore sensitivity to Bax in R13 strain. Overexpression of the full length of OYE2 in wild type EGY48 cells expressing Bax suppressed the Bax lethality which indicates the dominant negative effect of the C-terminus of the gene. Bax causes change in the morphology of mitochondria, and a percentage of the organelles become fragmented. Obviously the Bax fail to target and oligomerize to the mitochondria in the resistant strain R13 contrary to the normal behaviour in wild type Bax-sensitive strains. Reekmans *et al,* have recently showed that deletion of OYE3, an FMN oxidoreductase homologous to OYE2, attenuated Bax-induced growth arrest, cell death and caused a decrease in NADPH in yeast, which supports the protective role of OYE against Bax leathality. In addition the overexpression of Bax in *Δoye2* cells was more toxic relative to the wild type cells.

2- OYE2 and OYE3 have opposing functions in the regulation of oxidative stress in wild type yeast.

Bax-induced lethality in yeast is strongly linked to enhanced intracellular oxidative stress. OYE3 modulates Bax-dependent PCD, and heterodimerizes with OYE2 *in vivo* and *in vitro*. OYE2 and OYE3 share 82% identity at the amino acid level, suggesting a related activity. To explore the functional relationship of OYE2 and OYE3, they were expressed at moderate levels in yeast and assessed at basal and induced levels of oxidative stress. Yeast

overexpressing OYE2 or OYE3, were treated with prooxidants hydrogen peroxide H_2O_2, tert-butylhydroperoxide (*t*-BOOH), cumene hydroperoxide (CHP). In untreated cells, the overexpression of OYE2 modestly lowered, and of OYE3 elevated, endogenous ROS, when compared to wild type BY4741 cells with empty vector. OYE2 or OYE3 overexpression did not significantly influence ROS levels following treatment with H_2O_2; however, the treatment of cells with organic prooxidants generated more dramatic differences in ROS levels dependent on OYE status. Overexpression of OYE2 caused a substantial reduction in ROS levels in both *t*-BOOH and CHP treated cells compared to their wild type counterparts, suggesting antioxidant activity. In contrast, the overexpression of OYE3 caused a substantial increase in ROS levels, implying a prooxidant activity. Further analysis of OYE function through experiments comparing basal or induced ROS in wild type parental BY4741 yeast with ROS in strains deleted for OYE2 or OYE3 (*Δoye2* and *Δoye3*) showed that both deletion strains have similar ROS levels under basal conditions, or following prooxidant treatment. *Δoye2* cells treated with CHP exhibited a small increase in ROS; whereas *Δoye3* cells showed lower ROS. These changes in ROS again suggested antioxidant activity for OYE2, and prooxidant activity for OYE3.

3- *Double inactivation of oye2 with other antioxidant genes increases ROS and H_2O_2-induced PCD.*

Based on the data presented in the work of Osama Odat (M.Sc. thesis, 2006) which indicate that OYE2 regulates intracellular redox conditions, combined deletion of OYE2 with deletion of additional redox control genes such as *Δsod1*, *Δctt1*, *Δyca1*, *Δglr1* and their double knockout counterparts'*Δoye2 sod1*, *Δoye2 ctt1*, *Δoye2 yca1*, and *Δoye2 glr1* were checked for the ROS and were analyzed by FACS. Comparison of ROS levels between wild type BY4741 cells and the single knockouts *Δsod1*, *Δctt1, Δyca1* and *Δglr1* showed increased ROS only in the case of *Δsod1*. When their double knockout counterparts with oye2 were compared to the single knockouts, in all cases except *Δoye2 ctt1,* they exhibited substantial increases in ROS levels, confirming further the role of OYE2 as an antioxidant gene, since deletion of the gene further exacerbates the endogenous oxidative environment in cells harboring deletions in antioxidant genes.

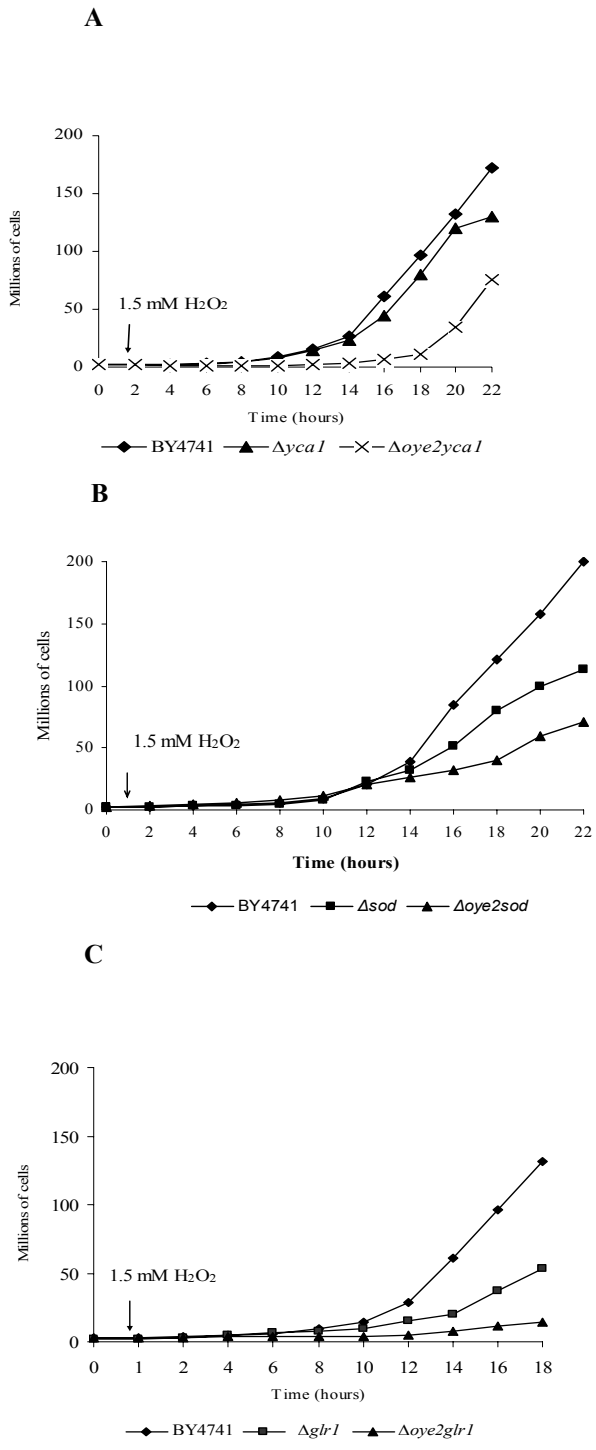


Figure 3.1 Behavior of Single knockouts and their double knock out counterparts under H_2O_2 stress. The growth of single knockout strains with their counterparts of *oye2* double deletion and the control wild type cells growth recovery was monitored at 2-hour time intervals after being subjected to a shot (1.5- 1.7 mM) of H_2O_2, (1A): BY4741, Δ*sod1,* Δ*oye2sod1*, (1B): BY4741, Δ*yca1,* Δ*oye2yca1*, (1C) BY4741, Δ*glr1,* Δ*oye2glr1*. In all the cases, the single knockouts recovered faster than their double knock out counterparts.

In order to check the ability of single and double knockout strains to recover from H_2O_2 treatment, they were examined in growth recovery assays, where the growth was monitored in regular time intervals after being subjected to a pulse of H_2O_2 which induces the programmed cell death. The sensitivity to H_2O_2-induced PCD of single and DKO, *Δyca1* and *Δoye2 yca1*, (Fig. 3.1A), *Δsod1* and *Δoye2 sod1* (Fig. 3.1B), *Δglr1* and *Δoye2 glr1* (Fig. 3.1C*)* confirmed increased sensitivity to H_2O_2-induced PCD in the double knockouts, as compared to the single knockouts or wild type cells.

4. Double knockout Δoye2oye3 mutants are resistant to H_2O_2 induced cell death

The cell death process is characterized by a distinct set of morphological and biochemical features, mediated by the Cys proteases caspases. The metacaspase present in *S. cerevisiae* YCA1 have a caspase-like fold (Uren *et al.,* 2000) and dispose caspase like proteolytic activity which is activated when yeast is stimulated by H_2O_2 and acetic acid (Madeo *et al.,* 2002). In general, apoptotic cell death involves a sequence of caspase activation events in which initiator caspases activate downstream executioner caspases that process a variety of target proteins eventually leading to apoptotic phenotype. Deletion of the yeast caspase gene *YCA1* enhances the resistance against oxidative stress and also delays age-induced cell death (Madeo et al., 2002). Starting from the premise which implied that oye2p-oye3p heterodimers promote the cell apoptotic death and basically deletion of both OYE2 and OYE3 render the cells more resistant to H_2O_2 insult and cell death, the growth of *Δyca1*, *Δoye2 oye3*, and wild type cells we monitored after being subjected to different concentrations of H_2O. Fresh cells were grown overnight till saturation and on the next day washed with distilled water twice, and resuspended at 0.1 OD_{600nm} and incubated at 30˚C shaking. After 2 hours of incubation, a shut (0.8-2 mM) of H_2O_2 was inoculated, and the growth was monitored regularly at 2-hour intervals. These experiments showed that the double deletion of OYE2 and OYE3 offers more protection for the cell than the deletion of YCA1, as the *Δoye2oye3* were able to recover faster than *Δyca1* cells (Fig. 3.2).

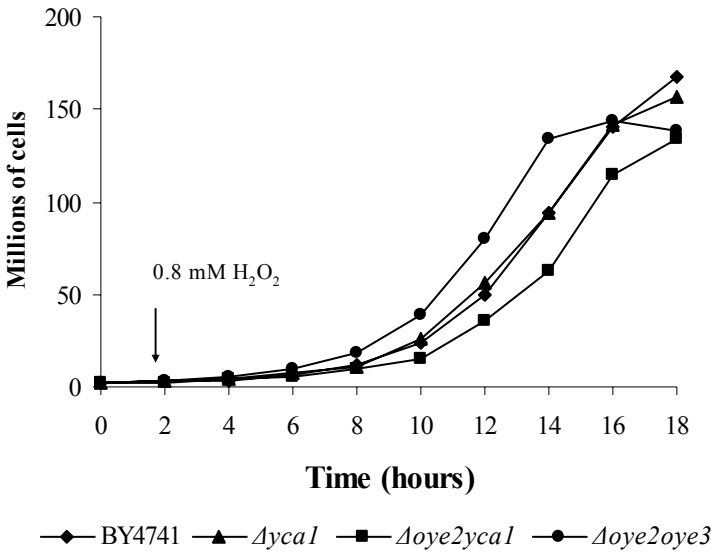


Figure 3.2. *Δoye2 oye3* recover faster than *Δyca1*. *Δyca1* cells, *Δoye2 oye3, Δoye2 yca1* and wt cells were resuspended at the same OD600 in fresh medium. Equal volume aliquots were used to inoculate fresh media at very low density, and were subsequently treated with o.8 mM H2O2 for 2 hours and their capacity to recover was monitored. *Δoye2 oye3* cells recovered faster compared to *Δyca1*cells and obviously faster than *Δoye2 yca1*.

5. Generation of OYE2 (Y197F)

Tyrosine 196 in Old Yellow Enzyme (OYE) of *S. carlsbergensis* was mutated to phenylalanine by Kohli and Massey, and the resulting mutant enzyme was characterized to evaluate the mechanistic role of the enzyme. They demonstrated little effect on ligand binding and the reductive part of half-reaction, but a dramatic slowing by nearly 6 orders of magnitude of its oxidative half-reaction with the used substrate (2-cyclohexenone) (Kohli and Massay,1998). The enzyme exists as a dimer of 49-kDa subunits containing one non-covalently bound FMN per subunit, which indicates the significance of small organic molecules in catalysis. The crystal structure of OYE1 at a resolution of 2.0 Å, identified several residues likely involved in catalysis and ligand binding. The structure supported the long established fact that NADPH serves as the physiological reductant for the enzyme and provides insight into the nature of potential oxidants. Refereing to the previous work, which implied that the OYE system participates in the oxidative stress and programmed cell death; namely indicating OYE2 as an antioxidant and OYE3 as a prooxidant; a choice was made to

mutate the OYE2 corresponding Tyrosine197 into phenyl alanine. The mutant OYE2 (Y197F) was generated by the method described before (Materials and Methods, 9.1) and was cloned into the plasmids pYX143 and pYX143-HA where the protein expression was detected by immunoblotting utilizing the 3-HA tag (Fig. 3.3).

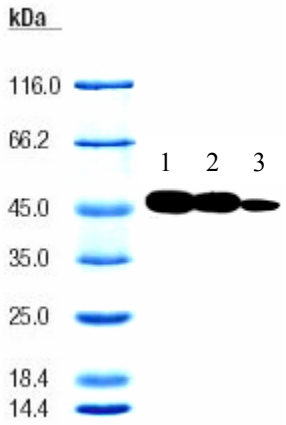


Figure 3.3. Immunodetection of OYE proteins by HA tag. Total protein lysates from the wild type BY4741 cells over-expressing the proteins OYE2, (1-388) OYE2 truncated, OYE2 (Y197F) subjected to SDS-PAGE electrophoresis followed by western blot (utilizing the HA tag) to detect and visualize the expression of these proteins. (1) oye2p, (2) oye2p (Y197F) (3) (1-388) oye2p.

5.1 OYE2 (Y197F) exerts a protective effect in wild type cells but is lethal in *Δoye3 cells*

Although the OYE2 and OYE3 enzymes are closely related, they show differences in ligand binding properties and in their catalytic activity with oxygen and 2-cyclohenone as acceptors. (Niino *et al.,* 1995). Homology of nucleotide sequence of the coding region between OYE2 and OYE3 was 73%. The amino acid sequences showed that OYE2 and OYE3 have 82% identity and 89% similarity. It is also known that deletion of OYE3 attenuates Bax lethatlity (Reekmans *et al.,* 2005) and heterodimerizes with OYE2 *in vivo* and *in vitro* (Stott *et al.,* 1993). To elucidate the functional relationship of OYE2, and OYE3, we expressed them at moderate levels in yeast and assessed basal and induced levels of oxidative stress. To assess whether the death promoting capacity of OYE3 requires the presence of OYE2 and possibly formation of heterodimers, OYE3 was overexpressed in *Δoye2* cells harbouring a deletion for the OYE2 gene. OYE3 homodimers in *Δoye2* cells exerted a protective function (Fig. 3.4A). Inversely over-expression of OYE2 in *Δoye3* cells maintained its protective function. However, overexpression of (1-388) OYE2 offered no

protection in *Δoye3* cells, indicating that the protective effect of the C-terminal truncation is exerted upon heterodimerization to OYE3 (Fig. 3.4B). In addition over expression of Y197F OYE2 in BY4741 wild type cells caused an important increase in cell viability upon H_2O_2-induced PCD as in the case of C-terminal truncation OYE2 (1-388) (Fig. 3.4C). Moreover, to assess whether the effect of Y197F OYE2 is exerted on the heterodimer with OYE3, we over-expressed the mutant protein in *Δoye3* cells (Fig 3.4D). Whereas over expression of OYE2, that is OYE2 homodimers, were protective in H_2O_2-induced PCD, expression of the Y197F OYE2 in *Δoye3* cells was lethal and cells were unable to recover after 30 hours incubation. The data indicate that formation of oye2p-oye3p heterodimers contributes to the induction of PCD upon oxidative stress, and that obstruction of heterodimer formation in wild type cells by co-expressing (1-388) OYE2 or OYE2 (Y197F) leads to elevated survival.

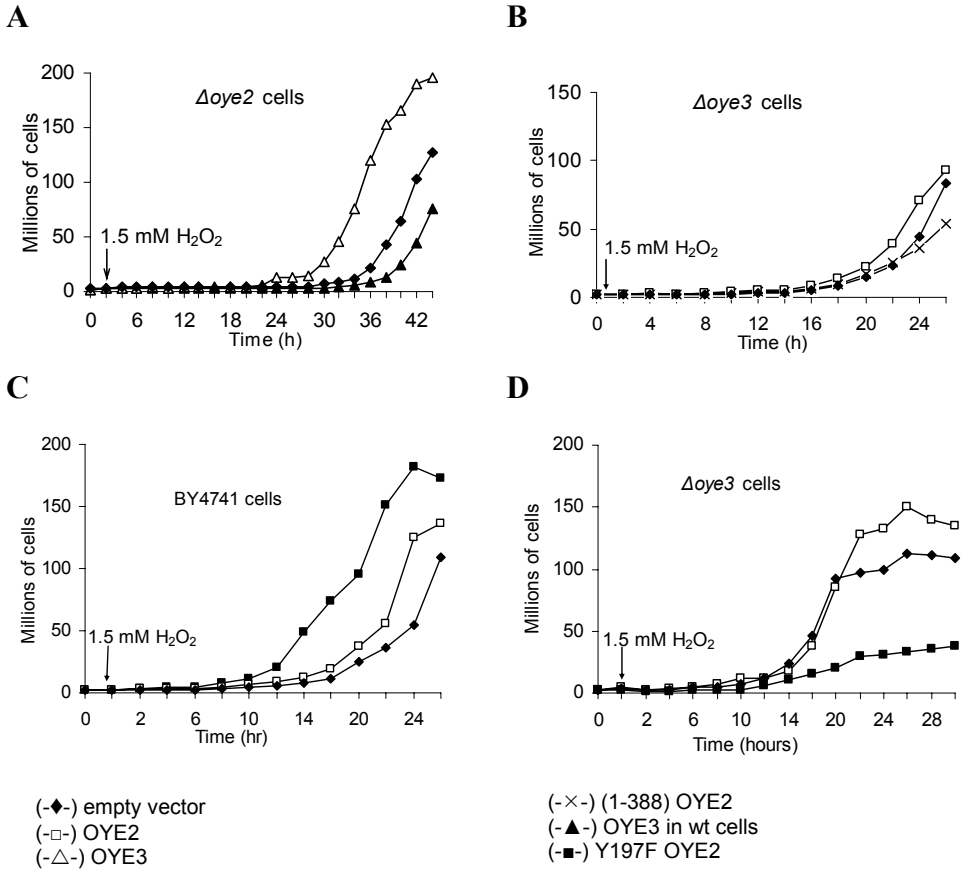


Figure 3.4. (OYE) overexpression in wild type, *Δoye2, and Δoye3* cells. (A) overexpression of OYE3 in *Δoye2* cells is compared to *Δoye2* cells harboring empty vector and wild type cells overexpressing OYE3 in the growth recovery assay described above; (B) *Δoye3* cells overexpressing OYE2, (1-388) OYE2 or empty vector were monitored for growth recovery subsequent to H_2O_2 insult. The (1-388) OYE2p did not exert any protection in the absence of OYE3. (C) BY4741 wt cells overexpressing OYE2, Y197F OYE2 or empty vector were monitored for growth recovery subsequent to H_2O_2 insult. (D) *Δoye3* cells overexpressing OYE2, Y197F OYE2 or empty vector were monitored for growth recovery as above.

6. Generation of Rho^0 cells

In order to examine whether active respiration in the double knockout strain Δ*oye2oye3* is important for cell death and resistance to oxidative stress, the ρ^0 strains (lacking the functional mitochondria) from the isogenic Δ*oye2oye3* strain were generated with control strain EGY48, known for its resistance (as a positive control), and the wild type BY4741 (as a negative control). Yeast cells were grown in 5ml YPD broth culture containing ethidium bromide (to eliminate the mitochondrial DNA) to a concentration of 10µg/ml for 3 days at 30 °C. After the end of the incubation period, serial dilution from the culture was made and plated on YPD medium to get single colonies. Five grown colonies were tested in parallel to isogenic Δ*oye2oye3,* and the wild type cells EGY48 and BY4741 untreated cells. To confirm the respiratory competence the cells were grown in 5ml cultures overnight till saturation. From of each culture, 1:5 (40 culture: 160µl ddH_2O) serial dilutions were prepared and plated to YPD and YPG plates in parallel to assess the respiration efficiency and to verify the absence of functional respiration. All tested strains were confirmed to be ρ^0. As a control the wild type cells BY4741 were not able to grow, while EGY48 grew well on YPG medium (Figure 5A).The growth of cells was monitored under normal conditions; wild type BY4741 cells, the isogenic Δ*oye2oye3* cells and three different samples of ρ^0 Δ*oye2oye3* cells in the YPD medium, ρ^0 cells behaved almost similar to the wild type cells (Fig. 3.5B).

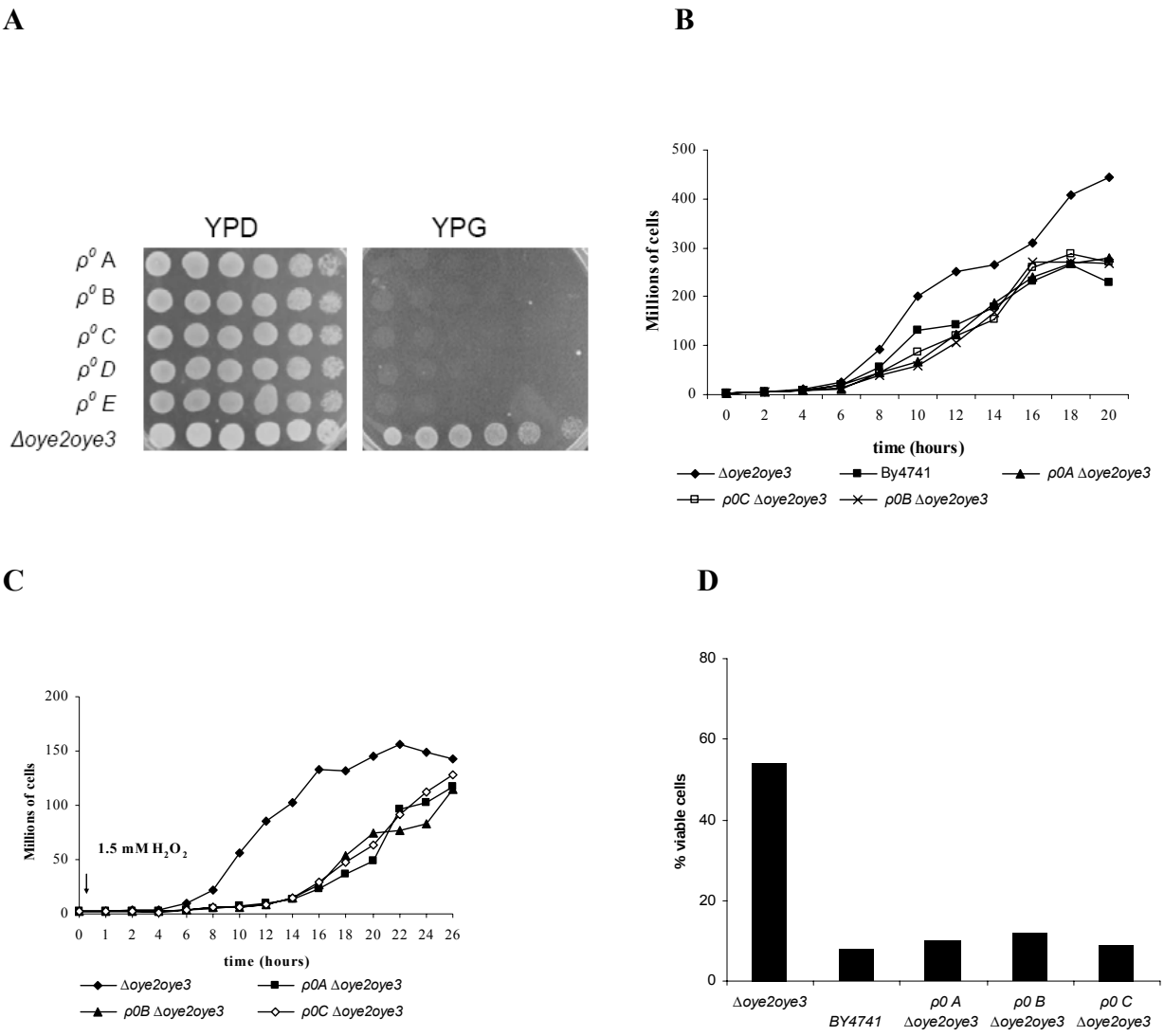


Figure 3.5. Verification of respiration incompetence in ρ^0 cells by growth assays and colony number. (A) *Δoye2 oye3* cells ρ^0 cells were generated by ethidium bromide treatment. Five strains (A-E) ,isolated from treated cells and the parental *Δoye2 oye3* strain, were plated in YPD and YPG plates. (B) BY4741 wild type cells, the parental *Δoye2 oye3* , and 3 ρ^0 *Δoye2 oye3* strains grown in YPD broth medium and the growth was monitored at regular time intervals. ρ^0 *Δoye2 oye3* behaved like wild type cells. (C) The parental *Δoye2 oye3* strain and 3 ρ0 *Δoye2 oye3* (A-C) strains were treated 1.5mM H_2O_2 pulse and the growth was monitored. (D) The same strains used in (C) were treated with 1.5mM H_2O_2 as mentioned in (materials section17) and enumerated by plating serial dilutions on YPD plates. The ρ^0 strains were sensitive to H2O2-induced PCD.

7. OYE cooperate in enhancing survival with knockouts of mitochondrial apoptotic regulators but not cytoplasmic or nuclear.

OYE2 was isolated based on restoring Bax-sensitivity to a strain with deficient localization of Bax to its site of action, the mitochondria, A full-length OYE2-GFP fusion was introduced into EGY48 cells under the control of a galactose-inducible promoter OYE2-GFP localized to distinct domains in the cytoplasm that partially overlapped with mitochondria. This indicates that OYE2 localized on the organelle, and can thus limit directly the capacity of Bax to insert and oligomerize on the mitochondrial outer membrane (OM) (Odat et *al.,* 2007). This close association is supported by the recent identification of TOM22, a component of the outer mitochondrial membrane import complex (TOM) as a mitochondrial receptor of Bax (Bellot et *al.*, 2006). In order to investigate further OYE2 association with other apoptotic regulators OYE2, OYE3, and (1-388) OYE2 were overexpressed in single knockouts of mitochondrial apoptotic gene deletions (*Δcyc1* and *Δaif1*), the cytoplasmic inducer of apoptosis (*Δyca1*) and the nuclear mediator of apoptosis (*Δnma111*), and BY4741 cells which carry empty vector as a control. Overexpression of *OYE* in strains with deletion in the mitochondrial proteins AIF1 and CYC1 enhanced cell survival but, no further enhancement of survival could be seen in the knockouts of the cytoplasmic inducer of apoptosis YCA1 and the nuclear mediator of apoptosis (Fig. 3.6).

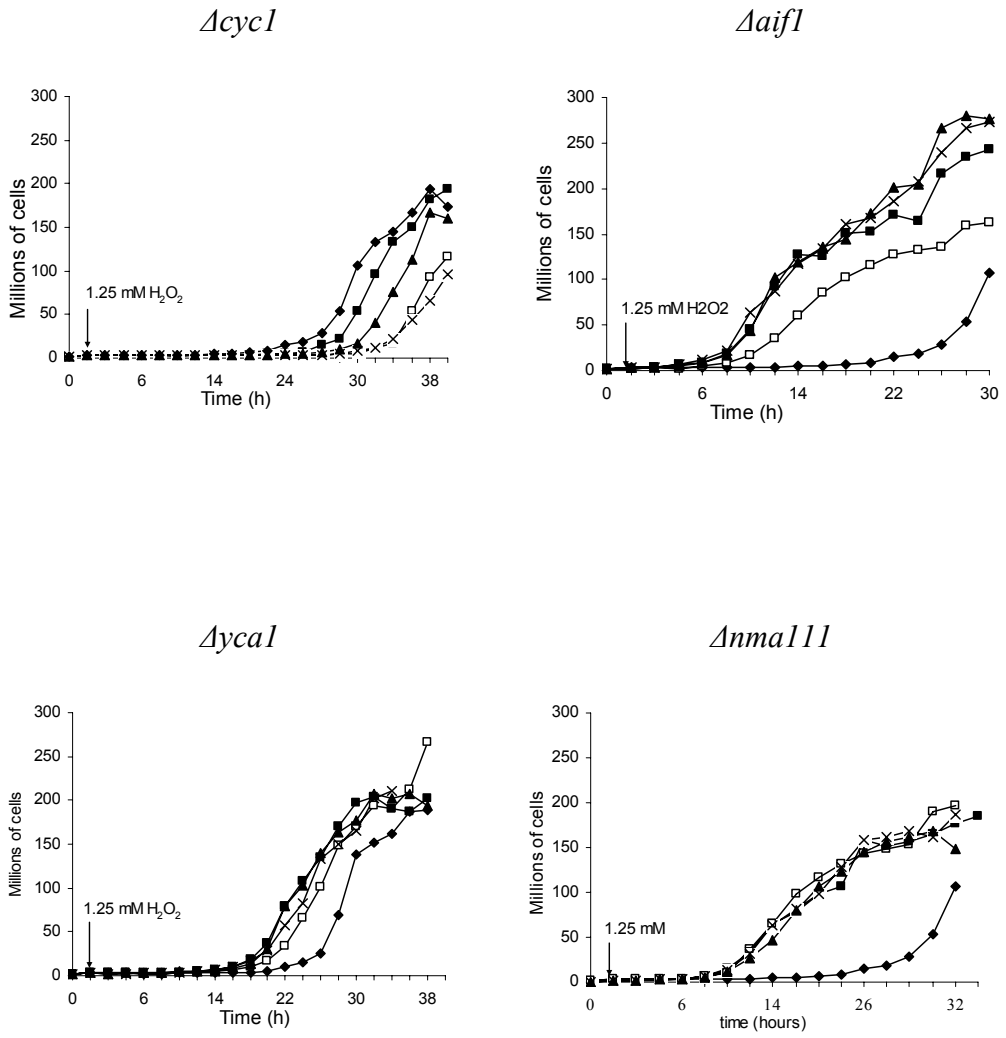


Figure 3.6. OYE system augment survival From H_2O_2–induced PCD in knockouts of mitochondrial apoptotic regulators. *Δcyc1, Δyca1, Δnma111, and Δaif1* cells carrying empty vector (-□-), overexpressing OYE2 (-■-), overexpressing OYE3 (-▲-), overexpressing (1-388)OYE2 (-x-) were treated with 1.5 mM H_2O_2 and growth recovery was monitored relative to BY4741 cells carrying empty vector (-♦-) as a control.

8. Creating a Δoye3 with a URA cassette

The mutant *Δoye3* with URA3 cassette insertion will be used to generate double knockout strains by mating with single knockout strains bearing G418 cassette, sporulating diploids and selecting them for URA+ and G418+ haploid cells. Having double knockouts, will allow us to test the cooperative effect of multiple genes involved with OYE3 in the cell-death adaptation pathway and will delineate in general the role of OYE3 in programmed cell death. The approach is based on (Materials and Methods, section 15.7) generating by PCR a

URA3 cassette of 50 bp OYE3 sequence flanking the cassette on both sides (Fig. 3.7A). After being purified by Phenol / chloroform extraction and concentrated to 10µl the product was used to transform BY4741 cells (200µl of cells). A high efficiency transformation is required, since the fragments are linear, for the successful integration by homologous recombination. Cells were selected by plating in Glu/CM-ura out of which 6 colonies were tested for the integration of the URA cassette in the proper. The integration was confirmed by extracting the genomic DNA from the transformed colonies and by PCR utilizing primers within the URA cassette. Reactions were done using the genomic DNA as template (which extracted from the positive colonies) with different combination of primers to confirm the deletion of OYE3.

a) 5' promOYE3 5'-TCC TTA GAA GGG CTC CTT AGT AAG CAG-3' and URA-D (5'AGT GGT GGA TCA GAT ATC 3')

b) 5' promOYE3, and 3' OYE3 (LOX)

c) 5'OYE3 and 3'OYE3

The product in the first two reactions was positive and negative in the third which confirm the deletion of OYE3 and the insertion of URA cassette in its locus (Fig. 3.7 B).

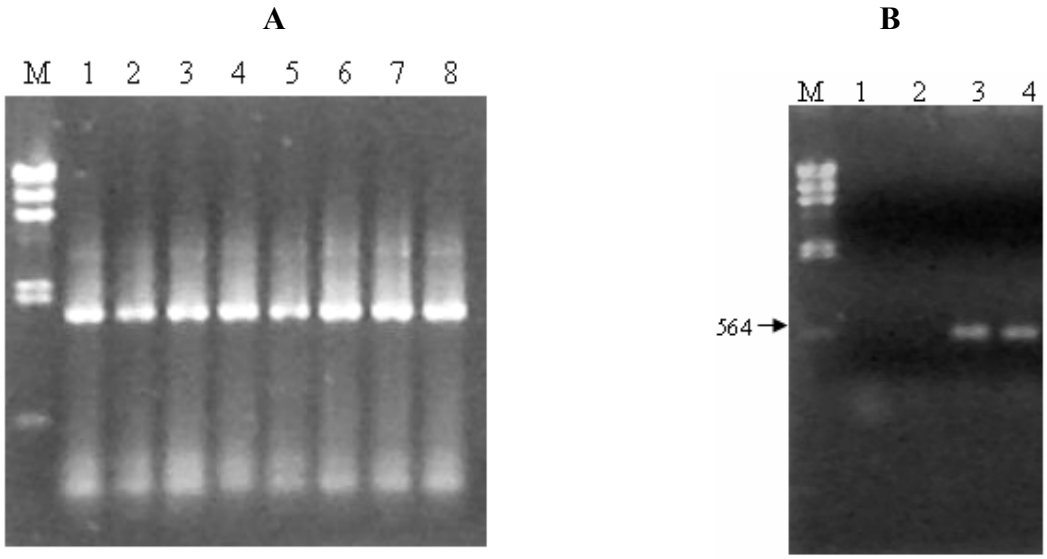


Figure 3.7. (1A) 1-8 PCR amplifications of URA3 cassette flanked by 50b oye3 on both sides were generated by preparing 8 parallel reactions. (1B) Verification of OYE3 deletion, M: Gene ruler, λ DNA/HindIII marker, 1: PCR product using the primers 5'OYE3 and 3'OYE3 the negative result is expected as OYE3 is already deleted. 2: PCR product using the primers 5' promOYE3, and URA-D using wild type BY4741 genome as a template the reaction served as a negative control. 3 and 4: PCR product using the primers 5' promOYE3, and URA-D, clear band appeared ~ 500bp which confirm the knocking out of OYE3.

8.1 Creating a Mat α, URA3, *Δoye3* strain

The cells that were transformed with the URA3 cassette were of BY4741 back ground, Mat **a** mating type, and they should be Mat **α** in order to be able to mate with the single deletion strains, which have the BY4741 background (Mat a) as well. To achieve this goal, the cells were mated with OSO13 cells which are (mat α (BY4741 *oye2*Δ::G418X EGY48) oye2::G418, ura3, his3, trp1). The diploid cells were selected on Glu/CM $G418^+$ ura^-, and then diploid cells were sporulated as before. The haploid cells (spores) which were Mat **α** URA^+, $G418^-$, trp, his, leu, were selected to be used in mating with the other single mutants in order to generate double knockouts (Fig. 3.8).

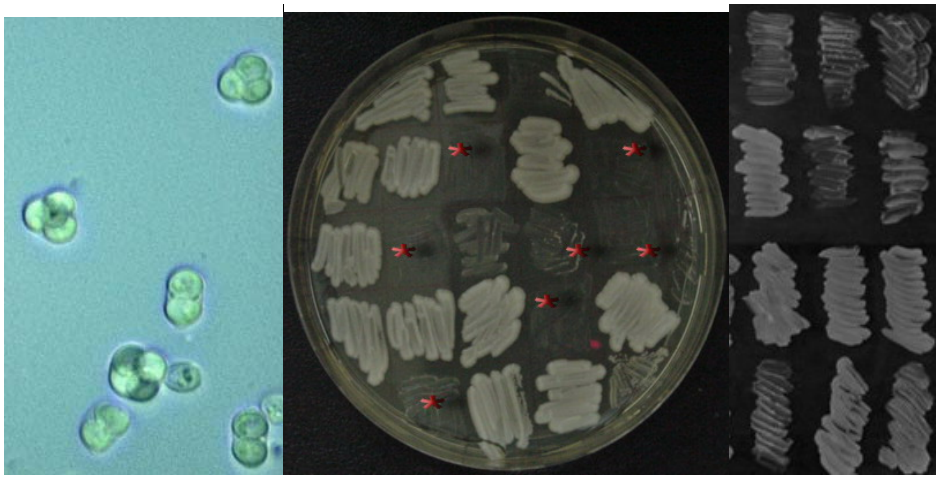


Figure 3.8. Sporulation of HKY1cells. (Left) Tetrads of HKY1 appeared on sporulation medium after 6 days incubation. (Middle) selection of HKY1 $G418^-$ colonies marked with star. (Right) checking the mating type of six selected $G418^-$ colonies mated upper row with Mat alfa and lower row with Mat a.

8.2 Generating double knockouts with *oye2*Δ::URA background

The strains (*oye2*Δ::URA), his3, trp1, leu2, and haploids with G418 cassette (*Δbud20, Δypl150w, Δapp1, Δapg1, Δcyc1, Δrad59, Δtrx3, Δhot13, Δapl1*) were mated by streaking both strains over each other on YPD plate and were incubated at 30^{o}C. Next day, the cells were subcultured in Glu/CM $G418^+$ ura^- and incubated as before. Two days later cells were transferred to new PSP media and were incubated for two days and then transferred to sporulation medium for further incubation for about 5 days. Samples of the cultures were

examined under the microscope to check for the formation of the tetrad ascospores. Once the tetrad stage had been seen, the spores were collected and treated as mentioned previously (sporulation). The double knockout spores were selected in Glu/CM G418^{+} ura^{-} plates, and the spores were mated with the mating controls to check for the mating type of each spore (Fig. 3.9).

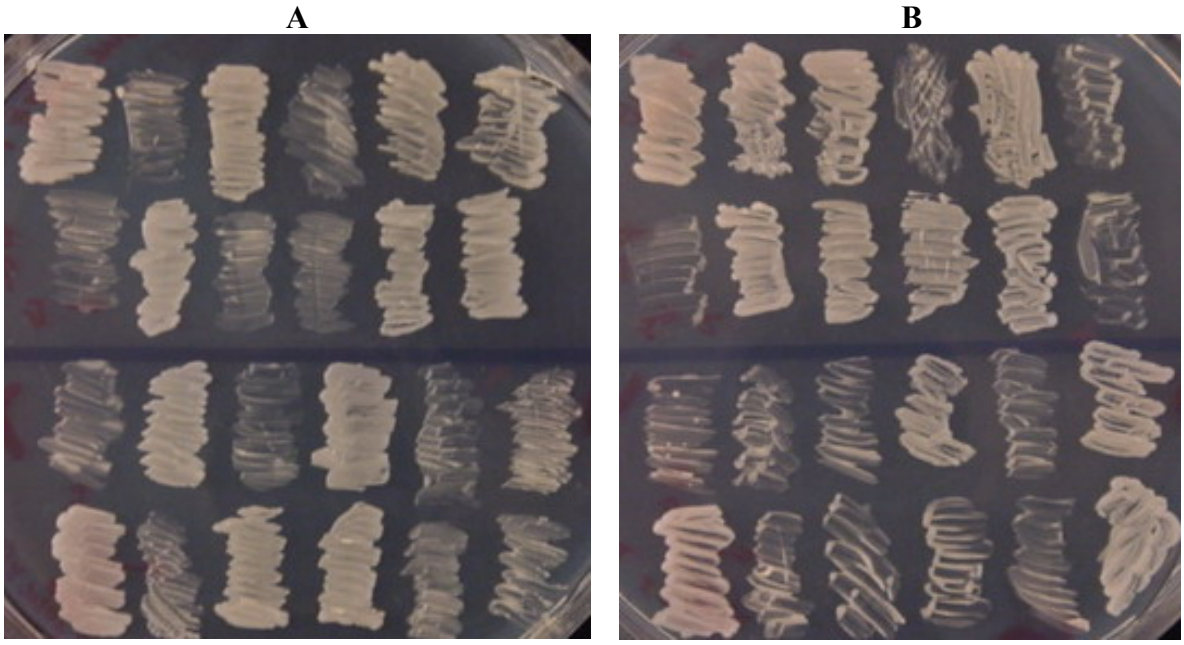


Figure 3.9. Generation of OYE2 double knock outs, (A) *Δoye2 rad59*, (B) *Δoye2arl1*. The upper 12 colonies mated with Mat a and the lower part mated with Mat α. The mirror image that appeard proves the mating type of each colony (Mat alpha mate with Mat ; haploids of the same mating type never mate together).

8.3. Generating OYE2 fusion strains

Among the most useful gene modifications is placing a gene under control of an inducible promoter and adding sequences encoding protein tags that allow facile protein detection and isolation. A PCR mediated technique that allows single step insertion of tags has been developed by (Longtine *et al.,* 1998). The primers used have 5'-ends (~40 nucleotides) that correspond to the desired gene sequence: 3'OYE2 F2, 5'-TTG ACT ACC CTA CGT ACG AAG AAG CTC TAA AAC TCG GTT GGG ACA AAA ATC GGA TCC CCG GGT TAA TTA-3, and the 3'-ends that anneal and allow amplification of the selectable marker (HA tag, GFP,or MYC tag) 3'OYE2 R1,

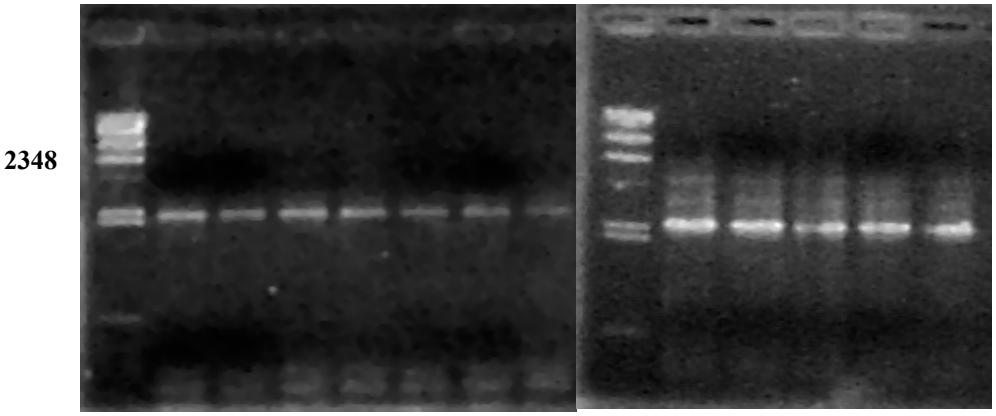
5’-AGT TCT GTT AAT ATT TCA TTA AAG GCT ATT TAT CCC AAA TAT ATA TAT TCG AAT TCG AGC TCG TTT AAA-3.

8 PCR reactions (5 µl of 10x Taq polymerase buffer, 2 µl of each primer, 1 µl of Template plasmid, 1 µl dNTPs, 1 µl Taq polymeras and 38 µl H_2O) were run together, and then the 5 µl of each reaction were run on gel to quantify the PCR procedure (Fig. 3.10).

1 hold 5 min, 94˚C	3-term 10 cycles 30sec, 94˚C 1 min, 56˚C 4 min, 72˚C	1 hold 7 min, 72 ˚C	3-term 25 cycles 30sec, 94˚C 1 min, 72˚C 4 min, 72˚C	1 hold 7 min, 72 ˚C

The products then were collected together and subjected to phenol / chloroform purification and concentration (Materials and Methods, 14.2), the concentrated linear DNA fragments were transformed directly to BY4741 wild type cells, and then the transfomants were selected on Glu/ his plates. 5 different colonies which grew on the selective medium were randomly chosen. Genomic DNA was isolated and was used a template in a PCR reaction to confirm the insertion of the cassettes.The primers used are OYE2 1110 5’- GAC AGA GAC ACT TTC TAC AAA ATG -3’ from the 3’ end of *OYE2* as a forward primer pointing downstream and pFA6a conf 5’-GCA ACC TGA CCT ACA GGA AGT AAG-3’ confirmation PCR was done using the confiramtion primer pfa6a conf. Within the cassette as a reverse primer (Fig. 3.10 C).

A- pFA6a-GFP (S65T)-HIS3MX6 **B-** pFA6a-13MYC-HIS3MX6



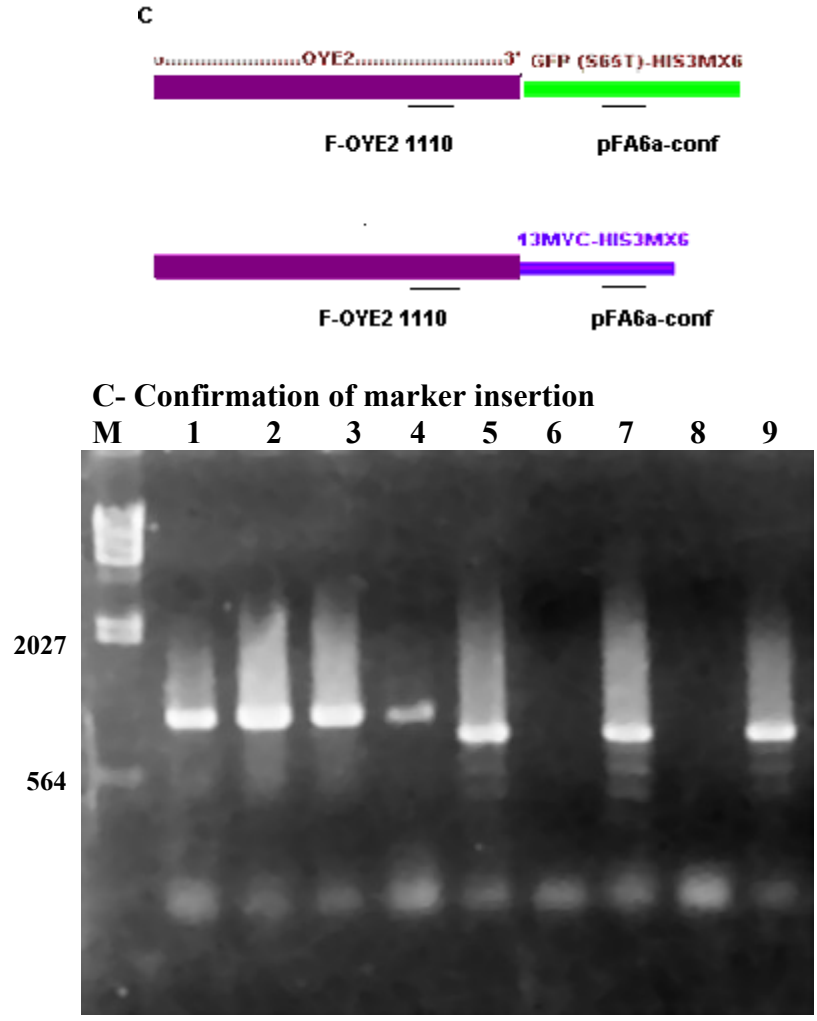


Figure 3.10. (A) PCR Amplification of GFP with HIS3. **(B)** PCR amplification of MYC tag with HIS3. **(C)** Confirmation of insertion of the amplified cassette, M: DNA Molecular weight Marker. 1, 2, 3 and 4 confirmation of insertion of GFP (S65T)-HIS3MX6. 5, 6, 7, 8, and 9 confirmation of insertion of 13MYC-HIS3MX6.

8.4. Generating double knockouts with oye3Δ::URA

The strains (*oye3*Δ::URA), G418^{-}, his3, trp1, leu2, and haploids with G418 cassette were mated by streaking both strains over each other on YPD plate and were incubated at 30°C (Appendix). Next day the cells were subcultured in Glu/CM G418^{+} ura^{-} and incubated as before. Two days later cells were transferred to new PSP media and were incubated for two days and then transferred to sporulation medium for further incubation for about 5 days.

Samples of the cultures were examined under the microscope to check for the formation of the tetrad ascospores. Once tetrad stage was seen the spores were collected and treated as mentioned previously (sporulation). The double knockout spores were selected in Glu/CM $G418^{+}$ ura^{-} plates, and the spores were mated with the mating controls to check for the mating type of each spore.

9. Δoye2oye3 double knockout are sensitive to the prooxidant Diamide

Bcl-2 prevents the induction of apoptosis by the pro-oxidant ter-butylhydroperoxide (t-BHP), but has no cytoprotective effect when apoptosis is induced by the thiol crosslinking agent diazenedicarboxylic acid his 5N,N-dimethylamide (diamide). Both t-BHP and diamide cause a disruption of the mitochondrial transmembrane potential. In contrast, Bcl-2 does not confer protection against diamide-triggered membrane potential collapse and the release of AIF and cytochrome c. This indicates that Bcl-2 is not able to suppress the permeability transition (PT) and the associated release of intermembrane proteins induced by diamide (Zamzami, et al. 1998). In order to test the effect of the OYE system in the protection against thiol crosslinking under the effect of we tested on different concentrations of diamide the mutants *Δoye2, Δoye3, Δoye2 oye3, Δglr1 and Δoye2 glr1* beside wild type cells. YPD medium was prepared, and was then autoclaved under standard conditions. After cooling down at around 55°C, the corresponding volume of diamide (1.5 Mm and 2mM and 2.5mM) was added separately. The last step involved pouring immediately the media into plates in sterile conditions, Aliquots from fresh overnight cultures diluted 1:5 in ddH_2O (1 volume of culture with 5 volumes of H_2O) were plated on the diamide prepared plates beside a control YPD plate, and incubated for three days at 30°C till growth reached saturation (Fig. 3.11). The single OYE knockouts *Δoye2* and *Δoye3* did not show any sensitivity and behaved almost like wild type cells, while the double knokout *Δoye2 oye3* was sensitive at the 1.5 mM and extremely sensitve at the 2.5 mM relative to the control. Sensitivity in the total absence of the OYE proteins indicates a role for the OYE system in cytoprotection against the induction of apoptosis by the thiol crosslinking agent diamide. The same observation was for the *Δglr1 and Δoye2 glr1,* which was expected since (GLR1 catalyses the reduction of oxidized glutathione biosynthesis GSSG to GSH) GSH is one of the important antioxidants

in the cell and its depletion or irreversible oxidation will favor inter and intra molecular thiol crosslinking in proteins.

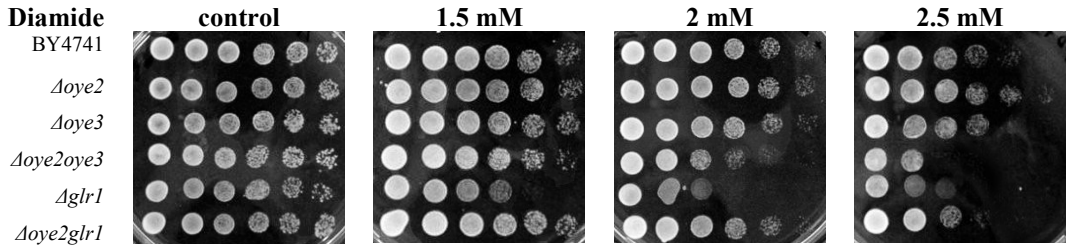


Figure 3.11. OYE system protects against the thiol crosslinking induced PCD. *Δoye2oye3* is sensitive compared to the single knock outs and the wild type cells.

10. OYE2 *mediates resistance to small α/β unsaturated compounds, Methyl vinyl ketone and Acrolein*

All aerobic organisms are exposed to reactive oxygen species (ROS), during the course of normal aerobic metabolism or following exposure to radical generating compounds in the environment. Unsaturated lipids are major targets of ROS attack, resulting in lipid peroxidation. During lipid peroxidation, reactive lipid and fatty acid radicals which can attack other fatty acids are formed, leading to oxidative chain reactions. These chain reactions proceed until they come into contact with a chain-breaking antioxidant, such as vitamin E. As a result of the oxidative damage to lipids, various lipid hydroperoxides are formed. These are relatively unstable and are converted into breakdown products which can be just as toxic as the ROS themselves. Major breakdown products include reactive aldehydes, such as malondialdehyde (MDA) and acrolein. These reactive aldehydes have a number of cytotoxic effects and inhibit a variety of enzymes which lead to membrane damage by increasing fluidity and DNA damage. In addition, they can react with nucleophiles, such as the sulfhydryl group of cysteine, therefore, reactive aldehydes are highly toxic.

10.1. OYE2 *mediates resistance to Methyl vinyl ketone*

In vitro enzyme analysis has shown that old yellow enzymes can reduce the α,β-unsaturated ketone methyl vinyl ketone (MVK). The sensitivities of strains lacking old yellow enzymes to MVK were tested and a pattern similar to that of acrolein has been seen.

Deletion of *OYE2* caused sensitivity to MVK. YPD medium was prepared, and was then autoclaved under standard conditions. After cooling down to around 55°C, the corresponding volume of MVK (0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM and 1 mM) was added separately. Aliquots from fresh overnight cultures diluted 1:5 in ddH_2O (1 volume of culture with 5 volumes of H_2O) were plated on the MVK prepared plates beside a control YPD plate, and incubated for three days at 30°C till growth reached saturation. Within the OYE system, the presence of or absence of *OYE2* appeared is the most crucial since the *Δoye2* and *Δoye2 oye3* were sensitive, while *Δoye3* was not, suggesting that the protective role is dependent on OYE2 but not OYE3 (Fig. 3.12).

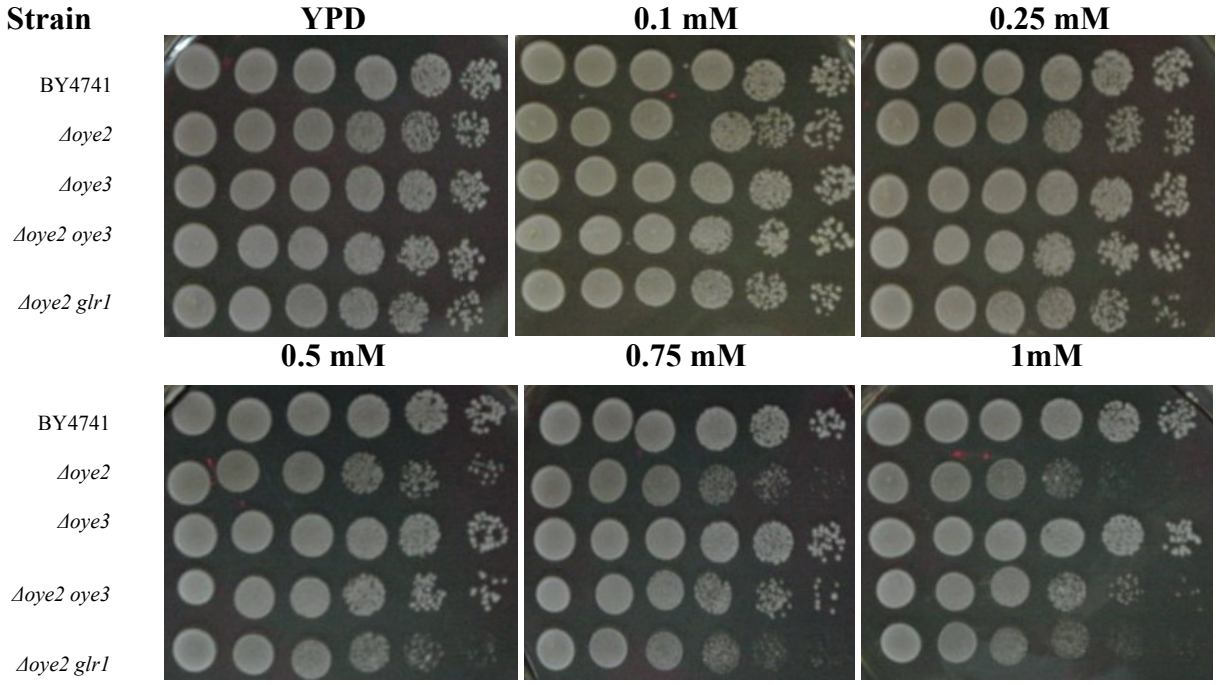


Figure 3.12. Methyl vinyl ketone sensitivity of OYE system mutants. Wild-type BY4741, *Δoye2, Δoye3, Δoye2 oye3 and Δoye2glr1* cells were grown and tested at different concentrations of Methyl vinyl ketone. At 1mM *Δoye2 oye3* cells were the most sensitive, similarly *Δoye2 showed almost the same degree of sensitivity, on the contrary Δoye3* were resistant and grew almost like wild type cells.

11. OYE2 *mediate resistance to (Acrolein)*

Acrolein has been identified as a product of lipid peroxidation in biological systems α/β-unsaturated aldehydes, acrolein is by far the strongest electrophile, reacting with nucleophiles, such as the sulfhydryl group of cysteine, the imidazole group of histidine, and the amino group of lysine. Therefore, acrolein is highly toxic towards mammalian cells and is a potent mutagen. Despite our understanding of the molecular effects underlying the toxicity

of acrolein, relatively little is known regarding the cellular systems which protect against acrolein and other reactive aldehydes. The gene products, according to their cellular components, revealed that acrolein sensitive mutants are not associated with any particular organelle and are localized throughout the cell (Trotter *et al*. 2006). Gene products are localized mainly to the cytoplasm, mitochondria, and nucleus. In addition, gene products are localized throughout the secretory pathway, including in the endoplasmic reticulum, Golgi, vacuole, and plasma membranes. Acrolein-sensitive mutants are distributed among most major biological processes, but particularly those of gene expression (transcription), metabolism, and cellular signaling. Surprisingly, the screen did not identify any antioxidants or similar stress-protective molecules. Similarly, it did not identify any components of the redox regulatory systems, including the glutathione system. A mutant lacking an old yellow enzyme (*OYE2*) was identified as being acrolein sensitive. Overexpression of either *OYE2* or *OYE3* was found to markedly increase resistance to acrolein. These previous data indicate that *OYE2* and *OYE3* mediate acrolein tolerance (Trotter, Collinson *et al.* 2006); in order to test the effect of *OYE2* in combination with other apoptotic genes, wild-type cell BY4741, *Δoye2, Δoye3, Δoye2 oye3 and Δoye2 glr1* were grown and tested at different concentrations of acrolein (Fig. 3.13), At 3mM Acrolein the OYE mutants were sensitive and the wild type grew normally. Single knock outs (apoptotic mediators) and their OYE2 double knock outs counter parts were tested (following the method, Materials section 19) at 3mM acrolein YPD plates. Most of the *oye2* double knock outs tested had showed increased sensitivity towards acrolein as compared to the single knock outs, and the control YPD plates. Out of the 36 strains tested (Fig. 3.14) the strains (*Δoye2 cta1, Δoye2 grx2, Δoye2 grx3, Δoye2 gsh1, Δoye2 tsa2, Δoye2 gcn1)* were relatively resistant. Surprisingly the single knock out strains *Δsod1 and Δglr1* were very sensitive compared to their OYE2 double knockout counterparts, suggesting a protective role favored by the double deletion, the mechanism of such protection is to be revealed.

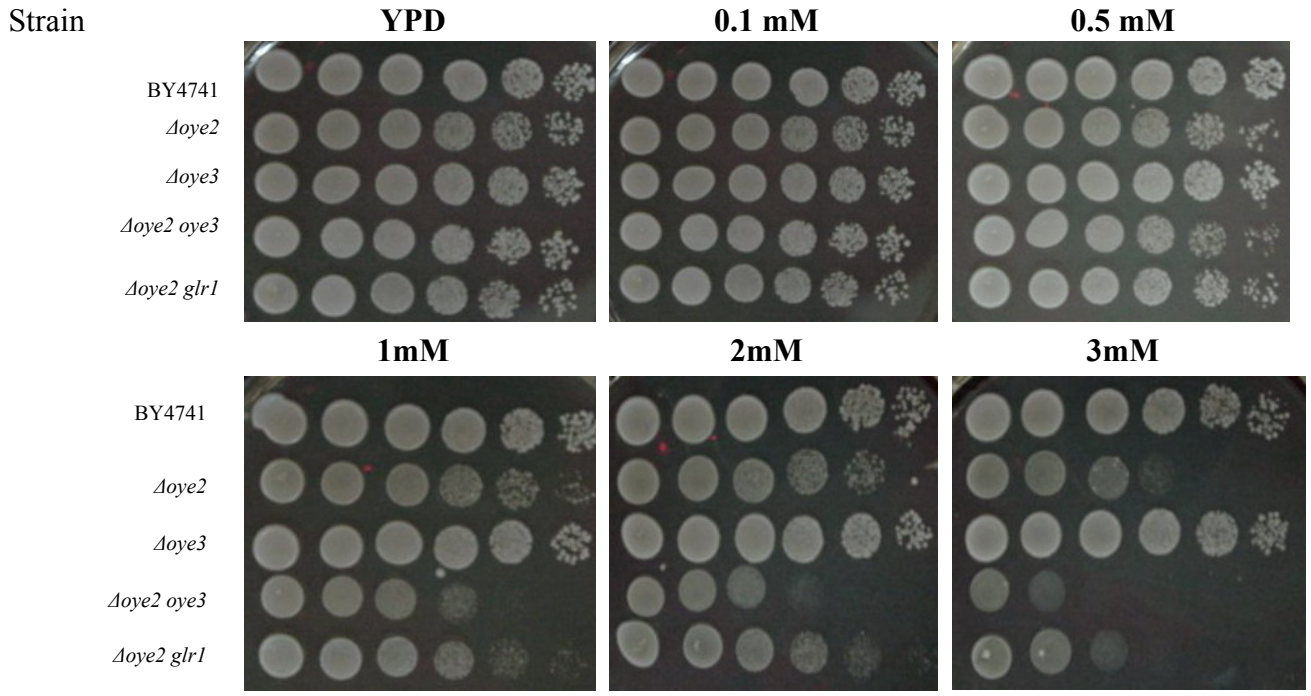
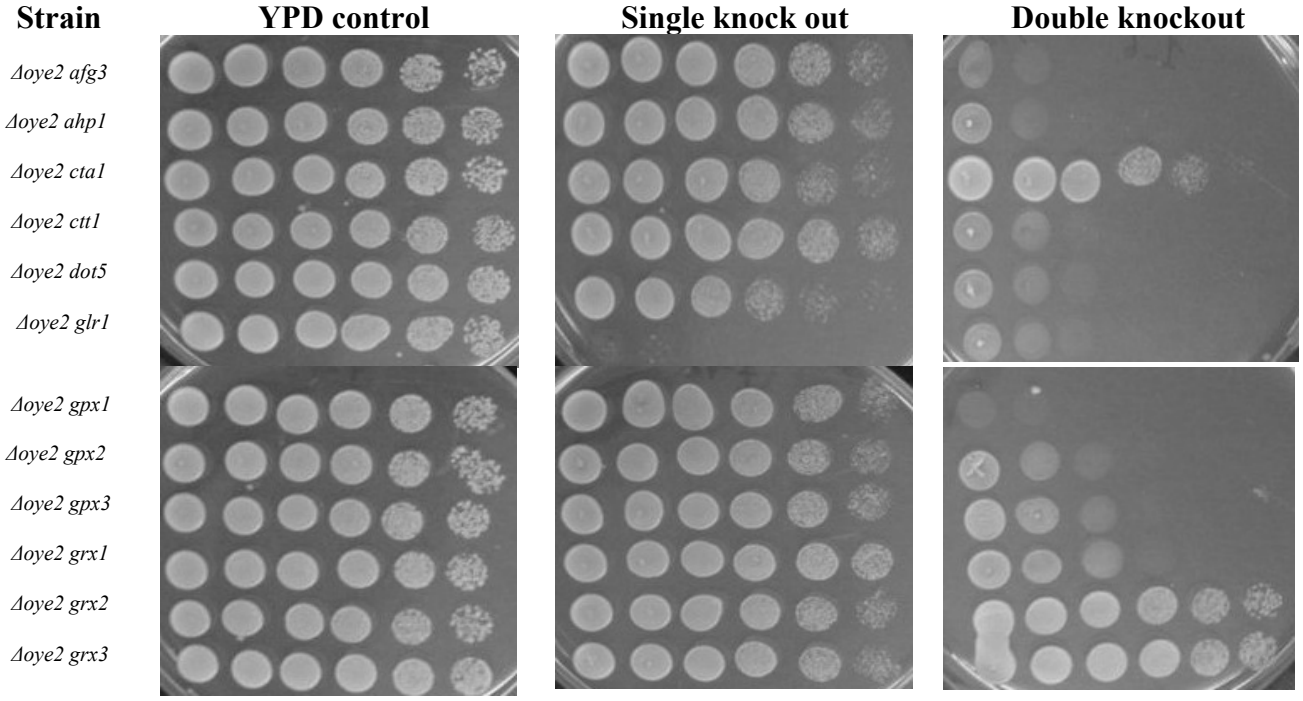


Figure 3.13. Acrolein sensitivity of OYE system mutants. Wild-type BY4741, *Δoye2, Δoye3, Δoye2 oye3 and Δoye2glr1* cells were grown and tested at different concentrations of acrolein. Starting at 1 mM up to 3mM Acrolein, *Δoye2 oye3* cells were the most sensitive. *Δoye2 showed almost the same degree of sensitivity, on the contrary Δoye3* were resistant and grew almost like wild type cells, suggesting that the resistance capacity is revealed by OYE2 but not by OYE3.



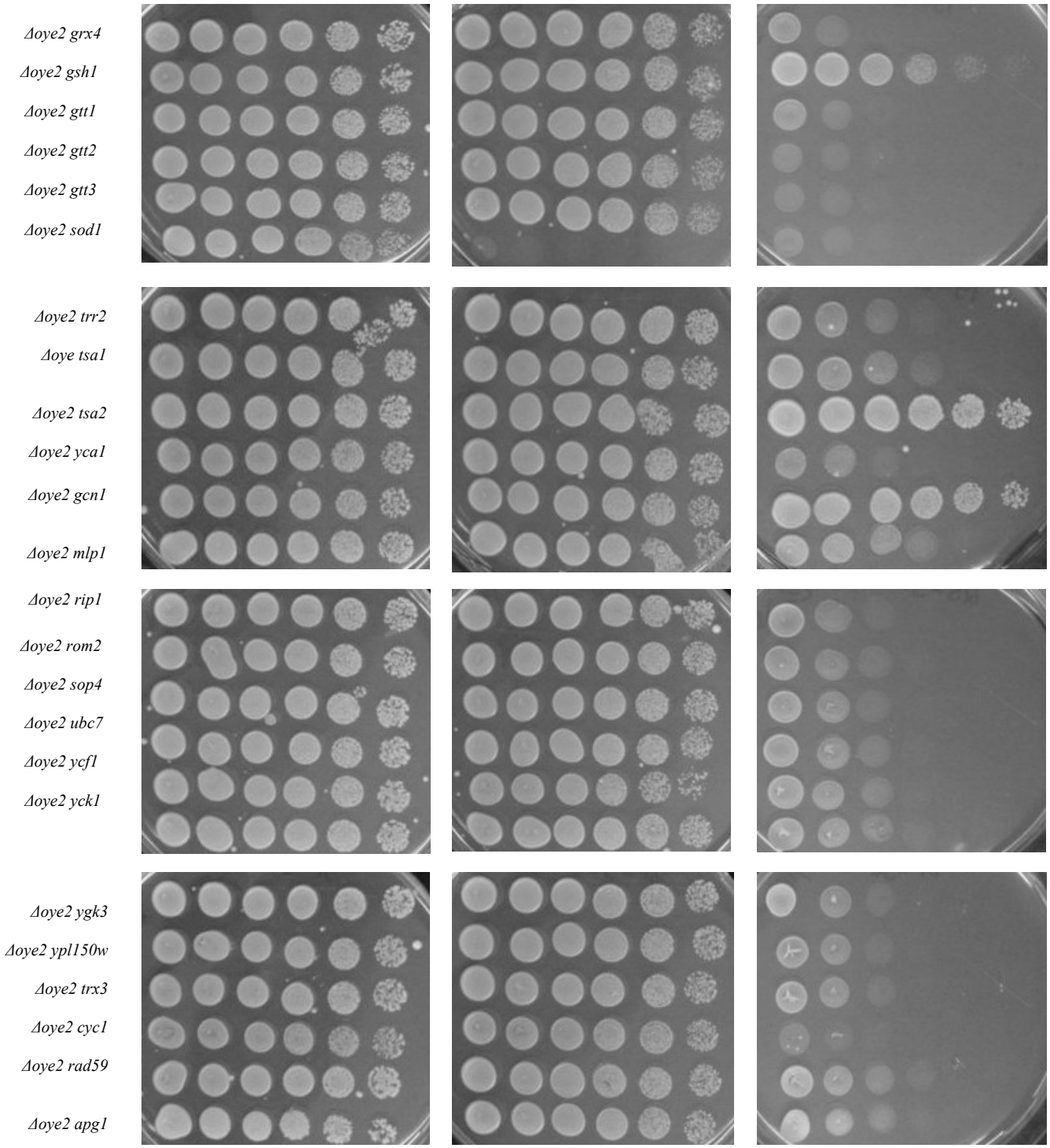


Figure 3.14. Deletion of *OYE2* causes sensitivity to acrolein. Sensitivity was determined by spotting strains on YPD plates containing 3 mM of acrolein. Cultures were grown to stationary phase and were spotted onto plates after being diluted 5 folds. Growth was monitored after 3 days of incubation at 30°C. Most of the oye2 double knockouts tested showed higher sensitivity as compared to the single knockouts.

12. The double mutant Δoye2 glr1 has morphological deformations

The double knock out *Δoye2 gsh1* contained high levels of endogenous ROS and showed sensitivity to oxidative stress (GSH1 catalyses the first step in glutathione biosynthesis). Reduced glutathione (g-glutamylcysteinylglycine, GSH) depletion has been shown to affect mitochondrial function probably via selective inhibition of mitochondrial complex I activity (Bharath et *al.* 2002). No cytoskeletal and morphological aberrations were visualized in *Δgsh1*, meanwhile in specific, the mutant strain Δ*oye2 glr1* has showed morphological aberration which did not appear in the single knockout Δ*glr1*.This observation was likely due to the increased level of GSSG which suggests that oye2p in combination with GSSG have a role in regulation of actin polymerization and cell polarity. The phenotype in Δ*oye2 glr1* was reversed completely into the normal phenotype after exogenous supplementation with 5mM of GSH. The double knockout Δ*oye2glr1* cells were grown under different conditions (at 30˚C and 37˚C, shaking or stand still) and different nutrient supplementation (in media with half or complete amino acids, in media with half of the quantity of YNB nitrogen source) and different pH (acidic 3.5 or basic 8.5) in order to enhance the appearance of the phenotype. Cells were subsequently washed twice and resuspended in 375µl 35% glycerol and 125 µl 4% Evan's blue stain. None of the above conditions enhanced the aberration of morphology except when incubated for 24 hours at 37˚C without shaking and then further incubated at 30˚C shaking for 24 hours more. The double knock outs *Δoye2 bud20, Δoye2 ypl150w, Δoye2 app1, Δoye2 apg1, Δoye2 cyc1, Δoye2 rad59, Δoye2 trx3, Δoye2 hot13, Δoye2 apl1*, *Δoye2 afg3 and Δoye2 yca1* beside the single knock out counterparts and *Δoye2, Δoye3* and the wild type cells were subjected to the same conditions mentioned above, although the double knockouts didn't show exactly the same a massive aberration in their phenotype yet they didn't have exactly a wild type appearance (Fig. 3.15).

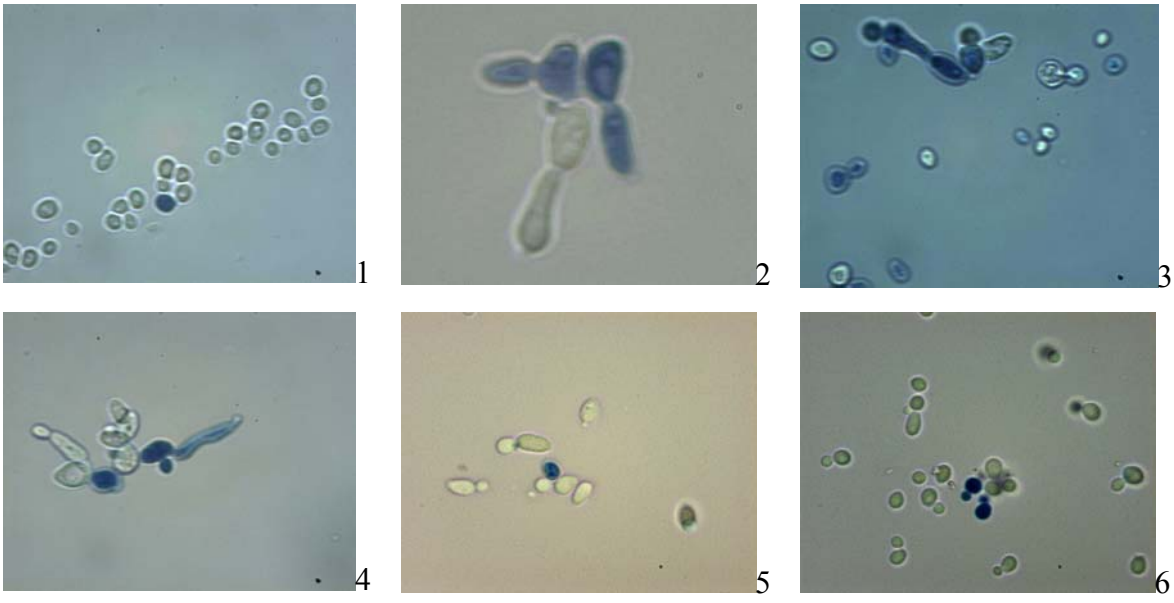


Figure 3. 15. Aberration in the *Δoye2 glr1*. (1) Wild type cells BY4741. (2, 3, 4) *Δoye2 glr1*. (5) *Δoye2 bud20*. *(6) Δoye2 ypl150w.*

12.1. *Δoye2 glr1* has 6-fold increase in oxidized glutathione levels.

Glutathione is conveniently assayed by an enzymatic recycling procedure in which it is sequentially oxidized by 5,5'- dithiobis (2-nitrobenzoic acid) (DNTB) and reduced by NADPH in the presence of gluthatione reductase. The extent of 2-nitro-5-thiobenzoic acid formation is monitored at 412 nm, the glutathione present is evaluated by comparison with a that result with a standard curve **(**Griffith 1980) BY4741, *Δoye2*, *Δoye3*, *Δoye2 oye3* and *Δoye2 glr1* cells were grown till late log phase in Glu/CM. Total glutathione and glutathione disulfide were measured according to a method described before (Material and Methods, section 12). Cells were grown to $A_{600} > 0.5$ using glucose as the carbon source, and then cells were subsequently washed and resuspended in galactose-containing medium in which they were cultured with shaking for an additional 8-h period. Total glutathione and glutathione disulfide were measured in equal amounts of cellular material. The cells were pelleted, resuspended in 5 ml of 1% oxalic acid, and broken by sonication. After the samples were centrifuged, 200 ml of the supernatant was removed. To the samples analyzed for glutathione disulfide (GSSG), 4ml of 2-vinyl pyridine was added. 45 mM final concentration of triethanolamine was then added to all the samples. 1-ml samples containing 125 mM sodium phosphate (pH 7.5), 6.3 mM EDTA, 0.21 mM NADPH, 0.1 mM 5,5-dithiobis-2-nitrobenzoic

acid (Sigma) and 200 ml of the samples were equilibrated at 30 °C for 5 min. 0.5 units of glutathione reductase was added, and the absorbance at 412 nm was monitored. The concentration of glutathione was calculated by comparing the rate of the reaction to a glutathione standard curve (Kampranis et al. 2000). GSH levels are significantly lower only in *Δoye2* cells, whereas in GSSG there is a striking 6-fold increase in the *Δoye2 glr1* cells (Fig. 3.16). This further validate the importance of GSSG, as have been seen through attempts to reverse the GSH/GSSG ratio in the cells, by exogenous supplementation of media with 5 mM GSH had completely reversed the aberrant morphology of the *Δoye2 glr1* cells. These results confirm the participation of oxidized glutathione GSSG together with the absence of oye2, in eliciting actin cytoskeletal changes and in the regulation of actin polymerization.

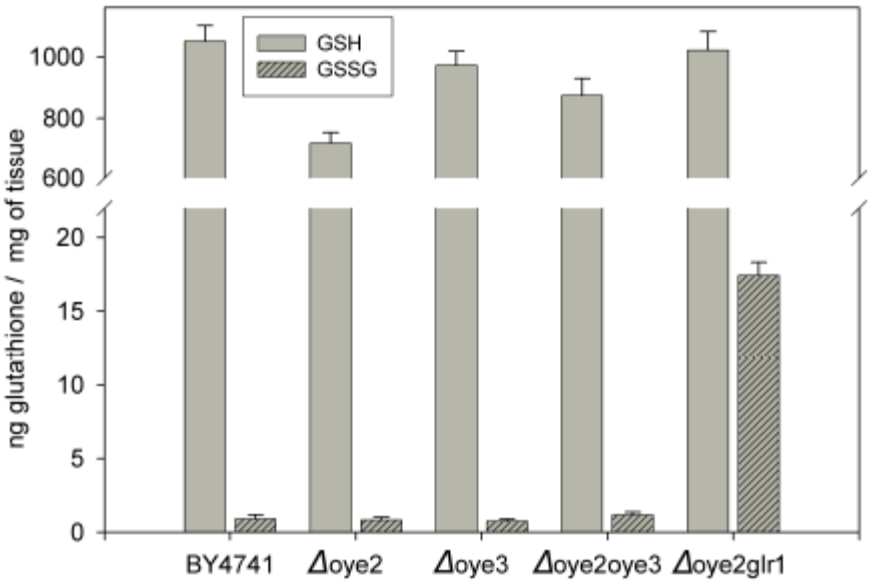


Figure 3.16. *Δoye2 glr1* exhibit a 6-fold increase in GSSG levels .wild type cells, single knockouts *Δoye2, Δoye3*, and the double knockouts *Δoye2 oye3* and *Δoye2 glr1* were measured for reduced glutathione (GSH) and oxidized glutathione (GSSG), the double mutant *Δoye2 glr1* exhibited 6-fold increase in GSSG relative to the other stains being detected.

13. Immunodetetion of OYE3 by HA tag.

In order to check the expression of *OYE3* cloned to pYX143-HA, the total protein lysates from the wild type BY4741 cells over-expressing the proteins OYE2, OYE2 Y197F and OYE3 subjected to the SDS-PAGE electrophoresis followed by western blot (Fig. 3.17) to detect and visualize the expression of these proteins (Materials and methods, section 11).

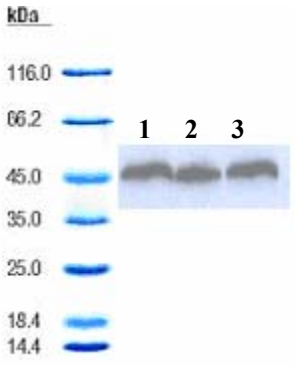


Figure 3. 17. Immunodetection of OYE proteins by HA tag. Total protein lysates from the wild type BY4741 cells over-expressing the proteins OYE2, OYE2 (Y197F) and OYE3 subjected to the SDS-PAGE electrophoresis followed by western blot (utilizing the HA tag) to detect and visualize the expression of these proteins. (1) oye2p, (2) oye2p (Y197F), (3) oye3p.

14. Δtor2 sporulation

The target of rapamycin (TOR) is a large conserved Ser/Thr protein kinase, which functions as a master controller of cell growth. TOR assembles into two distinct multiprotein complexes TORC1 and TORC2. Two genes, *TOR1* and *TOR2*, were identified in yeast. TORC1 is activated by growth factors, hormones, nutrients and energy signals to regulate cell growth (Guertin and Sabatini, 2005). TORC1 is sensitive to inhibition by rapamycin; in contrast TORC2 is resistant to rapamycin and regulates actin the cytoskeleton. Parallel experimentation identified mild nutritional stress as a factor sensitizing wild type cells to apoptotic H_2O_2 –induced cell death in yeast (Ikram, M.Sc thesis 2007).

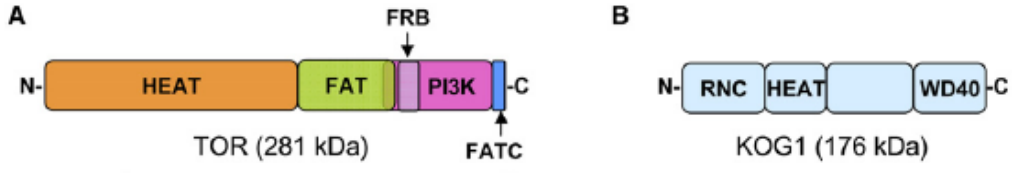


Figure 3.18. TOR is defined by several conserved domains, the amino terminus is characterized by multiple Huntingtin, elongation factor 3, a subunit of pp2A and TOR1 (HEAT) repeats. The N- terminal HEAT repeat domain is followed by a C-terminal region conserved in PIKK proteins, comprising (1) ~600 amino acid helical FRAP/TOR, ATM,TRRAP,(FAT) domain, (2)the catalytic kinase domain, and the short C-terminal FATC domain. The carboxy terminal comprises FKBP12/FPR1- rapamycin binding domain (Perry and Kleckner, 2003). TORC1 interacts with Kontroller of growth 1 (KOG1) (B) characterized by amino terminal Raptor N-terminal conserved domain RNC, four HEAT repeats, and seven C-terminal WD40 motifs (Loewith *et al.,* 2002).

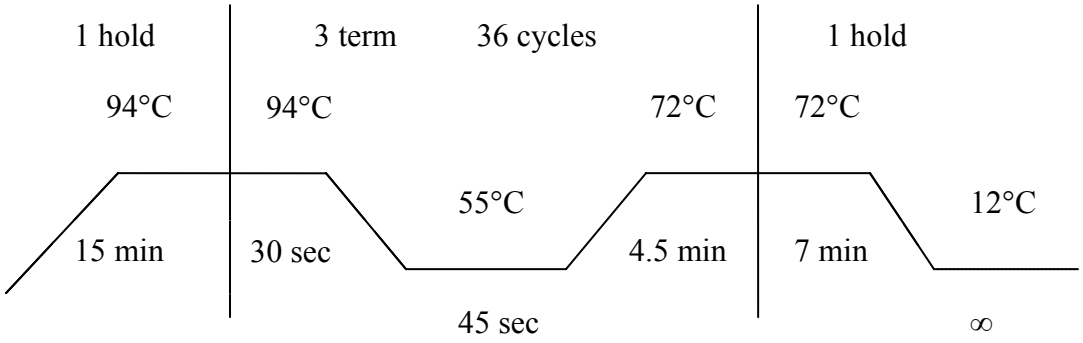
To test the involvemet of TOR complexes in apoptotic cell death, we had to develop strains harboring alteration in TOR genes in a genetic background similar to our previous work. *Δtor2* (*Δtor*:: (900)Tn HA LacZ URA) mutant was originally diploid, and contained a transposon insertion at codon 900 in the HEAT domain. The aim of this sporulation procedure is to select haploids which are close in their genetic background to the wild type BY4741 strain yet still carrying a selectable auxotrophic marker, the uracil. Fresh patches of *Δtor2* diploids were grown on YPD plates (24h) then the growing cells were transferred to presporulation rich medium (48h), and later transferred to sporulation medium to induce spore formation (after 8 days the spores were few around 5-8 % as seen under the microscope. The usual sporulation procedure (materials section 15) was carried out. Spores were selected at Glu/ CM, ura plates where 90 colonies appeared in 5 different plates. 26 colonies were tested on different media as shown below. Of these only four colonies were *Δtor2* (his, trp, leu, URA). The haploids that were mated with Mat a and Mat alpha his 5 and selected on on Glu / CM his to determine the mating type. *Δtor2* Mat alpha were mated with BY4741 (Mat a, TRP) with *Δtor2* (Mat alpha, URA) the selective medium is Glu/CM, - ura, trp. The diploids were selected and then sporulated (after one week spores appeared more that 70%). The spores were grown on Glu/ CM -ura, and colonies which are Mat alpha, URA, trp were selected, this mating and sporulation was repeated twice and *Δtor2* mutants of the third backcross were selected and characterized. *Δtor2* (Mat alpha, ura, trp, his) was selected and characterized, this strain is useful in future mating process which need a close BY4741 genetic background should be as close as possible.

15. Cloning of NMA111 to pTOPO

To further investigate yeast programmed cell death, it was necessary to obtain full length cDNAof the apoptotic mammalian orthologues. Genomic DNA from wild type BY4741 was isolated following the procedure mentioned in the materials and methods "Yeast Genomic DNA Isolation". *NMA111* was amplified using the parameters listed below, and using the primers:

5'(BamHI)5'GGA TTC ATG ACC ATA TCG TTG AGC AAT ATA AAG AAA AG-3', and 3'NMA111 (XhoI) 5'-CTC GAG CTA TTT TTC ACT TTG GCT GTT GCC GGT-3' In the reaction mixture (5 µl template genomic DNA extracted from wild type BY4741, 2 µl of each primer, 16 µl H_2O and 25 µl hot start Taq polymerase master mix (contains Taq

polymerase, Taq polymerase buffer and dNTPs). The PCR products were run on 1% gel (Fig. 3.19), clear band appear which confirm the size of NMA111, the DNA fragments were gel extracted and then cloned to pTOPO vector following the instruction manual (www.invitrogen.com), Catalog Number: K4500. pTOPO cloning products were transformed to competent E. coli cells. Growing colonies were plasmid extracted and the insert was verified by digestion and sequencing.



PCR amplification of NMA111

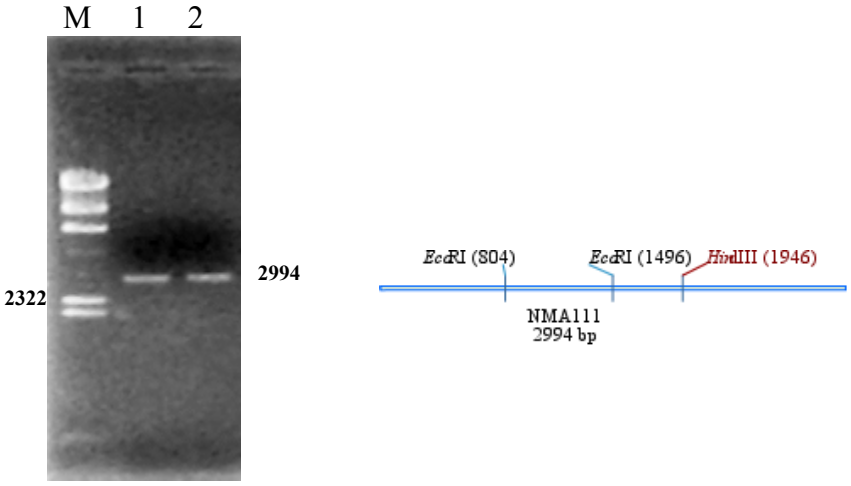


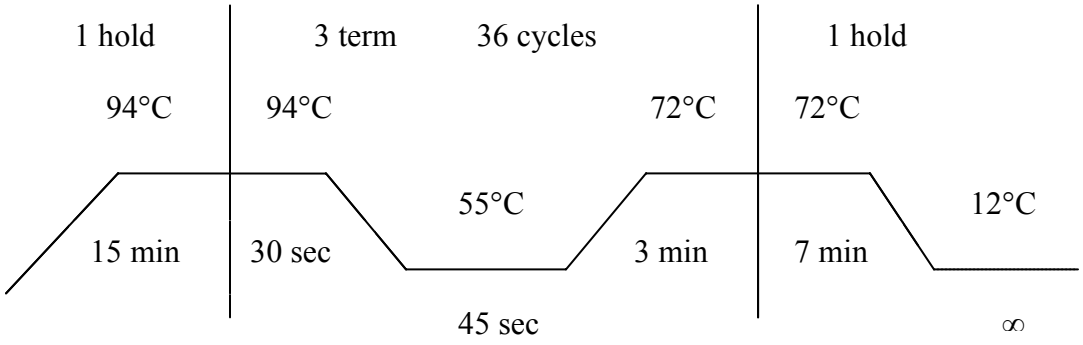
Figure 3.19. PCR amplification of NMA111. M: Gene ruler, λ DNA/HindIII marker, 1 and 2, NMA111 Amplified by PCR.

16. Cloning of AIF1 to pTOPO

Apoptosis-inducing factor (AIF 57 kDa) is a key regulator of cell death, and is also essential for normal mammalian development and participates in pathological apoptosis. AIF, as a novel mitochondrial intermembrane flavoprotein, has a significant homology to bacterial and plant oxidoreductases (Susin *et al.,* 1999**).** To clone the AIF1 into TOPO vector the genomic DNA from wild type BY4741 was isolated following the procedure mentioned in the materials and methods "Yeast Genomic DNA Isolation". *AIF1* was amplified following the PCR parameters listed below and using the primers:

5'-AIF1(EcoRI) 5'-GAA TTC ATG ACA ATT AAC AAA AGA ACA TAG TT-3' and

3'-AIF1(XhoI) 5'CTC GAG CTA TTT TTT GAA TAA AGG TTC CAT TTT GTC GGA G-3', in the reaction mixture (5 µl template genomic DNA extracted from wild type BY4741, 2 µl of each primer, 16 µl H_2O and 25 µl hot start Taq polymerase master mix (contains Taq polymerase, Taq polymerase buffer and dNTPs). The PCR products were run on 1% gel (Fig. 3.20).



Clear band appear which confirm the size of *AIF1,* the DNA fragments were gel extracted and then cloned to pTOPO vector following the instruction manual (www.invitrogen.com) Catalog Number: K4500. pTOPO cloning products were transformed to competent *Mach I* cells. Growing colonies were plasmid extracted and the insert was verified by digestion and sequencing.

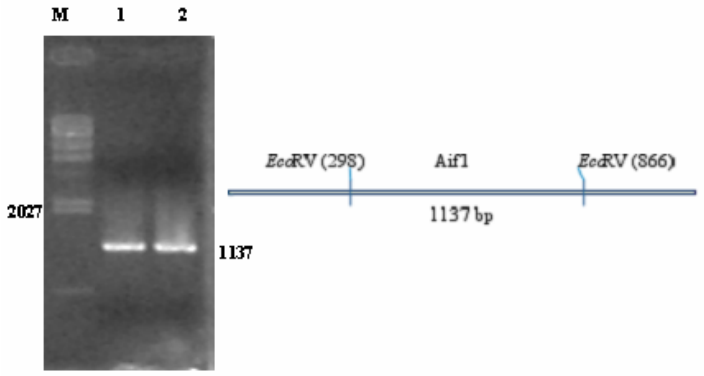


Figure 3.20. PCR amplification of AIF1. M: Gene ruler, λ DNA/HindIII marker, 1 and 2, AIF1 Amplified by PCR.

17. Discussion and future work

The past few years uncovered yeast apoptotic components that are direct counterparts of the standard mammalian apoptosis regulators, which supports the presence of a conserved suicide program in unicellular organisms. The Old Yellow Enzyme of yeast was the first flavoprotein to be discovered, in 1932. Theoye1p was initially purified from *S. carlsbergensis*, and later from *S. cerevisiae* (oye2p and oye3p). Old yellow enzymes form homodimers, but can also form heterodimers of 45kDa subunits with one monovalently bound FMN per subunit. The enzyme is rapidly reduced by NADPH and can be reoxidised by oxygen. Both OYE proteins have been shown to catalyze the NADPH-dependent reduction of quinones and of several α and β unsaturated carbonyl compounds (Karplus, Fox *et al*., 1995).

The use of heterologous proteins Bax and Bak in yeast pointed early on to the involvement of mitochondria and oxidative stress in eliciting cell death. EMS-mutant yeast strains that are resistant to Bax lethality exhibit additional aberrations in mitochondrial morphology and defects in proper targeting of proteins to the mitochondrial matrix (Belhocine *et al*., 2004). This close association is supported by the recent identification of TOM22, a component of the outer mitochondrial membrane import complex (TOM) as a mitochondrial receptor of Bax (Bellot *et al.,* 2007). In the R13 mutant strain examined, Bax failed to localize to mitochondria enabling cells to survive. The presence of the C-terminus of OYE2 restored Bax sensitivity and additionally enabled proper mit-GFP targeting. In the current study, the two OYE proteins modulate oxidative stress and the propensity of cells to

undergo H_2O_2-induced apoptosis. The oye2p and oye3p proteins exert an opposing effect in oxidative stress in wild type cells. The oye2p protein clearly maintains a protective antioxidant role, efficiently removing ROS generated from organic prooxidants, while the oye3p protein enhances ROS levels under the same conditions. The study of H_2O_2-induced PCD in overexpression and deletion strains confirmed these activities and identified the oye3-oye2 heterodimer as being responsible for the enhanced sensitivity to H_2O_2 induced PCD. The oye2p is an important participant in the antioxidant machinery, as its simultaneous deletion with other antioxidant genes exacerbates oxidative stress. OYE2 and OYE3 share extensive homology (82% identity). The catalytic activity of OYE2 is important for the function of the complex, as seen by the inactivating mutation at Y197F of OYE2. The C-terminus of OYE2 is also shown to participate in PCD modulation. A 12 amino acid truncation at the C-terminus of OYE2 substantially elevated the protection levels conferred in H_2O_2-induced apoptosis. Overexpression of the Y197F OYE2 mutant exhibited the same protective response as the truncated OYE2 protein in wild type cells but was lethal in H_2O_2 insulted cells lacking OYE3, therefore indicating that the protective effects of the altered proteins were due to the obstruction of the oye2p-oye3p heterodimer. Deletion of both OYE genes brought about a qualitative change in the cells, elevating respiratory activity, which led to their increased resistance to PCD as well as the reduction of cell death during senescence prior to adaptive regrowth. Incapacitation of respiration in the *Δoye2 oye3* cells diminished their resistance to cell death from oxidative damage. The importance of functional mitochondria in resistance to oxidative stress has been known for some time (Grant *et al.* 1997). The changes in resistance to H_2O_2 seen in *Δoye2 oye3* cells are not caused by elevated ROS levels upon increased respiration, since those are identical to the parental wild type cells.

Flexibility in the C-terminus of yeast actin and the red blood cell actin, allows two cysteine residues (Cys374 and Cys285) to come into proximity, and in a sufficiently oxidizing environment form a disulfide bond (Wang *et al*. 2001). The oye2 protein, but not the oye3 protein, was shown to interact with actin possibly in the proximity of the Cys285-Cys374 disulfide bond (Haarer and Amberg 2004). The *Δoye2 glr1* strain exhibited an exacerbated phenotype which was very similar to the *αct1-123* mutant, not only confirming

the participation of the oye2p protein in actin polymerization, but also underscoring the importance of additional players participating, such as oxidized glutathione. (Pruyne *et al*. 2004). S-glutathionylation offers the cell the advantage of a reversible mechanism that prevents the protein Cys thiol group from irreversible oxidation. The effects seen in the case of the *Δoye2 glr1* cells clearly suggest the presence of an enzymatic mechanism that mediates glutathionylation of actin in the presence of high GSSG levels. The specific cooperative phenotype caused by the simultaneous deletion of *oye2* and *glr1* can be explained by the absence of the protective function of the oye2p on actin which renders cells with high levels of GSSG highly susceptible to actin glutathionylation, altering the polymerization dynamics. The effect seen is dependent on the presence of high levels of GSSG, since the presence of increased ROS levels does not by itself cause the dramatic cytoskeletal rearrangements. The double deletion strain *Δoye2 gsh1,* despite having high ROS levels and extreme sensitivity to oxidative stress, does not show the characteristic morphology. These data show that increased ROS do not automatically translate into depleted GSH levels, and that the presence of high GSSG is important on its own right. Evidence from several studies suggests crosstalk between the dynamics of the actin cytoskeleton, the release of ROS by mitochondria and RAS signaling, in controlling PCD in eukaryotic cells (Gourlay *et al.* 2004). The results shown further reinforce this model and place Old Yellow FMN-oxidoreductases in a center stage modulating oxidative stress and programmed cell death in a positive or negative manner, while oye2p together with GSH regulate actin dynamics and organelle segregation. Experimental examination of catalytic stereospecificity of OYE2 and OYE3 using α,β-unsaturated carbonyl compounds identified differences between them (Muller *et al.* 2007). *In vivo* assays of yeast cells exposed to the toxic α,β-unsaturated carbonyl acrolein, a product of lipid peroxidation in biological systems, identified OYE2 but not OYE3 for its contribution to acrolein tolerance (Trotter *et al.* 2006). When the oye2 double knock outs and their single knockouts counter parts were tested, the double knockouts were shown to be sensitive. OYE3 double knock outs were generated and will be subsequently tested towards acrolein tolerance, and they should also be checked for any morphological aberrations. OYE3 prooxidant activity is estimated to be diminished in the OYE3 double knockouts; this should be assessed experimentally under stressful conditions like H_2O_2 –induced PCD. The OYE2 and OYE3 fusions with selectable markers (HIS3, G418) and fusion tag markers (HA and MYC tag) by

amplification and homologous recombination will be useful tools for tracking the expression of these genes *in vivo* and can help in assessing endogenous protein levels under varying conditions. Other fusions can be done and the expression of the fusions can be monitored in different deletion backgrounds. TOR is activated by growth factors, nutrients and energy signals, and it regulates the actin cytoskeleton (Loewith *et al.*, 2002) Further characterization for *Δtor2* mutant with other apoptotic genes will be carried out since mild nutritional stress was identified as s factor sensitizing wild type cells to apoptotic death in yeast (Ikram , M.Sc. thesis 2007).

The generated oye2 double knockouts were tested with other genes of the antioxidant machinery. In most cases, there was a cooperative effect in enhancing the sensitivity of the double knockouts. However, certain double deletions, contrary to expectations, rescued from the oye2 sensitivity by acting as compensatory mediators against the defects. The mechanism is not understood and future work should address this.

Appendix

References