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## The Yeast Genes *ROX1*, *IXR1*, *SKY1* and Their Effect upon Enzymatic Activities Related to Oxidative Stress

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### 1. Introduction

Aerobic organisms are characterized by the use of molecular oxygen as the final electron acceptor in the process known as respiration. In the inner membrane of mitochondria the four respiratory complexes transport the electrons and protons from FADH and NAD(P)H to oxygen and produce H<sub>2</sub>O. This mechanism is coupled to energy generation, but incomplete reduction of O<sub>2</sub> causes the appearance of reactive oxygen species (ROS) such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), the hydroxyl radical (OH<sup>•</sup>) and the superoxide anion (O<sub>2</sub><sup>•-</sup>). ROS are highly reactive in the cell and interact with nucleic acids, proteins and lipids, thus causing a wide spectrum of damages. Increase in steady state ROS level leads to oxidative stress (Lushchak, 2011) and stimulates defence systems. Along evolution of aerobic organisms diverse antioxidant strategies were developed. Many proteins have the function of removing ROS or are able to correct the damage caused by them. Glutathione (GSH), a tripeptide formed by cysteine, glutamic acid and glycine, is the major non-protein thiol-based redox buffer present in the cell (Penninckx, 2002; Perrone et al., 2005). Glutathione is synthesized in its reduced form and transformed to the oxidized form (GSSG) by the formation of one inter-molecular disulfide bond. The principal function of glutathione is to maintain the intracellular redox balance, reducing oxidized molecules and detoxifying ROS, xenobiotics and heavy metals (Grant et al., 1996b; Yu & Zhou, 2007). In fact, for long time the GSH/GSSG ratio was used to describe the redox state of the cell. GSH binds to and directly reduces oxidized molecules, but more often GSH is used as a donor of reducing equivalents to other antioxidant enzymes, like glutaredoxins, glutathione peroxidases and glutathione transferases (Avery & Avery, 2001; Garcera et al., 2006; Lillig et al., 2008).

*Saccharomyces cerevisiae*, with a predominant fermentative metabolism under aerobic conditions, is considered an eukaryote model for exploring the complex response induced by oxidative stress (Li et al., 2009; Lushchak, 2010, 2011). Besides the use of oxidants, like hydrogen peroxide or menadione, other compounds containing metals also induce the oxidative stress response in yeasts cells (Martins et al., 2008; Thorsen et al., 2009). Sugar

oxidation re-routing by different metabolic pathways may also influence the oxidative stress response and, on the contrary, the onset of an oxidative stress response may open previously-blocked metabolic pathways in yeasts (González-Siso et al., 2009).

In *S. cerevisiae*, the Yap family of b-ZIP proteins is involved in a variety of stress-related programs, including the response to DNA damage and oxidative, osmotic and toxic metal stresses. To sum up, functionally, Yap1 is the major regulator of oxidative stress response, Yap2 of cadmium stress, Yap4 and Yap6 of osmotic stress and Yap8 of arsenic stress (Thorsen et al., 2009). We have recently found that in *S. cerevisiae* the transcriptional factor Ixr1 is also related to the oxidative stress response. Moreover, a cross-regulation, affecting transcription, exists between Ixr1 and Rox1, which is the aerobic transcriptional repressor of hypoxic genes (Castro-Prego et al., 2010a). Ixr1 and Sky1 are both related in mediating the cyto-toxicity of the anticancer drug cisplatin (cis-Diaminodichloroplatinum) in yeasts. Cisplatin-induced cell toxicity is also associated with oxidative stress, redox state unbalance, impairment of energetic metabolism and apoptosis (Martins et al., 2008).

As previously described, in aerobic organisms there are multiple connections between the oxidative stress-response, changes in metabolic pathways related to energy production and oxygen utilization and the onset of a cellular response elicited by metals, metalloids or therapeutic compounds containing metals such as cisplatin. In this work we have overtaken a study trying to obtain integrative information about the role of the yeast genes *IXR1*, *ROX1* and *SKY1* in the oxidative stress response induced by As (V), Cd (II) and cisplatin in terms of modulation of four enzymatic activities. The four enzymatic activities tested are glucose-6-phosphate dehydrogenase (G6PDH) that is related to the pentose phosphate pathway (PPP), catalase (CAT) that breaks down  $H_2O_2$  into  $O_2$  and  $H_2O$ , glutathione reductase (GLR) and thioredoxin reductase (TRR). The Figure 1 summarizes the metabolic role of the enzymatic activities analyzed and their relationships.

### 1.1 The relationships between Ixr1, Rox1 and the oxidative stress response

In *S. cerevisiae*, adaptation to environmental signals requires the transcriptional regulation of multiple genes organized in regulons controlled by specific transcriptional regulators. Rox1 and Ixr1 are two yeast transcriptional regulators, which share several structural and functional characteristics in common. Structurally, both contain HMG (high-mobility group) domains, which bind to and bend DNA (Deckert et al., 1995; Deckert et al., 1999; McA'Nulty et al., 1996). Functionally, both control genes that are expressed at higher levels when oxygen is low (hypoxia) or absent (anoxia) than during normoxia (Bourdineaud et al., 2000; Castro-Prego et al., 2010a, 2010b; Kastaniotis & Zitomer, 2000; Klinkenberg et al., 2005; Lambert et al., 1994; Zitomer & Lowry, 1992; Zitomer et al., 1997;). Moreover, a transcriptional cross-regulation between the genes *ROX1* and *IXR1* has been reported (Castro-Prego et al., 2010b). During aerobic growth, low levels of *IXR1* expression are maintained by Rox1 repression through the general co-repressor complex Tup1-Ssn6. Ixr1 is also required for hypoxic repression of *ROX1* and binds to its promoter (Castro-Prego et al., 2010b).

Interestingly, it has been previously reported that low-oxygen levels induce an oxidative stress response accompanied by a rise in ROS levels in *S. cerevisiae* (Dirmeier et al., 2002). Among the evidences of oxidative stress during this transient state are DNA oxidation and

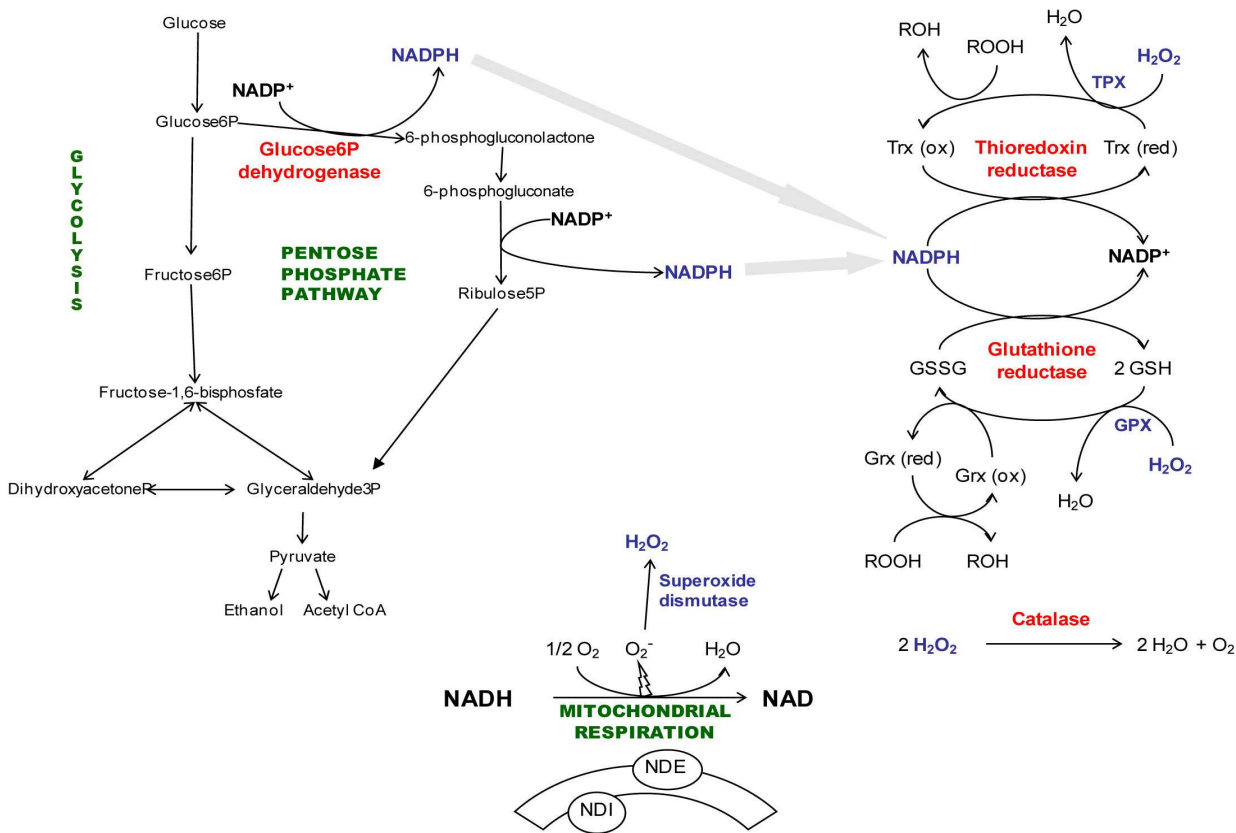


Fig. 1. Metabolic pathways producing and consuming NAD(P)H and connections to the stress response.

selective protein carbonylation. Only certain proteins, such as glyceraldehyde-3-phosphate dehydrogenase, pyruvate decarboxylase, enolase and aconitase, are the targets of oxidants generated during the shift from normoxia to anoxia. The same proteins are also modified during direct exposure of yeast cells to hydrogen peroxide. Besides, *SOD1* (encoding Cu/Zn superoxide dismutase) expression initially declines and then increases during the shift to anoxia, indicating an oxidative stress response (Dirmeier et al., 2002).

Several connections between the transcriptional regulators Rox1, Ixr1 and the yeast response to oxidative stress have been shown. Peroxiredoxins, a family of antioxidant enzymes, play an important role in the cellular defence against oxidative and nitrosative stresses. They have peroxidase and peroxynitrite reductase activities supported by thioredoxin, cyclophilin and glutaredoxin, as well as other electron donors. In *S. cerevisiae*, the transcription of *TSA2*, encoding for peroxiredoxin, is regulated by transcriptional activators, like Yap1 or Skn7, which respond to oxidative signals, but also by Rox1 and the Rox1 transcriptional activator Hap1 (Wong et al., 2003).

In a transtriptome approach comparing wild type and  $\Delta rox1$  null strains, several genes involved in mitigating oxidative stress, including *CTT1* (catalase T), *SOD1* and *TSA1* (thioredoxin peroxidase), are up-regulated in absence of Rox1. It is believed that they are not directly repressed by Rox1 because these genes are down-regulated under anoxia, when Rox1 levels diminish; but probably, they change their expression by complex interactions of regulatory networks affected by Rox1 (Lai et al., 2006). Rox1 also appears to play a role in

the control of redox balance through the genes *GPM2*, *GMP3* and *CDC19* of the late steps of glycolysis and *ADH1* or *ADH5* of ethanol biosynthesis (Lai et al., 2006).

It has been proposed that caloric restriction extends life span by a process that initially raises ROS levels. But, in turn, it produces protection from acute doses of oxidant, providing adaptation, and Rox1 is active during this adaptative response (Kelley & Ideker, 2009). The mechanisms by which Rox1 is activated after mild pre-treatment with oxidants are unknown, but it has been proposed that a fall in heme levels via degradation induced by hydrogen peroxide may be the signal (Kelley & Ideker, 2009).

During anaerobic growth, *S. cerevisiae* requires both a sterol (at or beyond zymosterol) and unsaturated fatty acids, which must be exogenously supplied. During anaerobiosis the genes required for sterol import and nearly all of the genes involved in the latter portion of sterol biosynthesis (beyond farnesylpyrophosphate) are induced. Many of them are regulated by Rox1. It has been recently shown that oxidative stress triggers repression of *ERG2* and *ERG11* transcription, two genes that are necessary for sterol biosynthesis and this response is partially dependent on Rox1 (Montañés et al., 2011).

About the regulator Ixr1, there are also some reports directly or indirectly related to oxidative stress in yeasts. Hypoxic expression of *SRP1* (*TIR1*) is dependent on Ixr1 and Yap1, the main regulator of the oxidative stress response. Besides, the effect of  $\Delta$ *ixr1* is epistatic to  $\Delta$ *yap1* (Bourdineaud et al., 2000). *IXR1* expression is moderately activated by H<sub>2</sub>O<sub>2</sub> and this induction is Yap1-dependent (Castro-Prego et al., 2010a). In multi-cellular eukaryotes connexions between the oxidative stress response and *IXR1* homologues also exist. Thus, in surgically resected hepatocellular carcinomas, TRX, a disulfide-reducing intracellular tioredoxin that functions as a cellular defence mechanism against oxidative stress, and HMG proteins type 1, with significant homology to the yeast protein Ixr1, are co-overexpressed when compared to normal tissue (Kawahara et al., 1996). Besides, active transcription of peroxiredoxins is dependent on Ets transcription factors and HMGB1 was shown to function as a coactivator through direct interactions with these Ets transcription factors (Shiota et al., 2008). By other hand, the protein HMGB1 was identified as a substrate of glutaredoxin that reduces the disulfide bond between Cys23 and Cys45. The conformational changes following this event may serve as a basis for redox-dependent control of gene expression, DNA replication, protection and repair (Hoppe et al., 2006).

## 1.2 The role of Ixr1 and Sky1 in the sensitivity to cisplatin

The yeast *S. cerevisiae* has been used as a simple eukaryotic model to identify genes related to cisplatin-sensitivity or cisplatin-resistance (Fox et al., 1994; Huang et al., 2005; Schenk et al., 2001, 2003). Among the genes that confer cisplatin-resistance are *IXR1* and *SKY1*.

Ixr1 is a yeast HMG-domain protein which binds the major DNA adducts formed with cisplatin (Brown et al., 1993). It has been demonstrated than in the excision repair mutants  $\Delta$ *rad2*,  $\Delta$ *rad4* and  $\Delta$ *radl4*, deletion of *IXR1* does not increase the resistance of *S. cerevisiae* cells to cisplatin (McA Nulty et al., 1996). This result gives support to the hypothesis that Ixr1 and other HMG-domain proteins can block repair of the major cisplatin-DNA adducts *in vivo* (McA Nulty & Lippard, 1996). Therefore, the cisplatin sensitivity in cells expressing Ixr1 might be caused by an architectural role of this HMG-protein in the chromatin assembles



that protects the area from the machinery of DNA repair, thus inducing cell death. The non-histone chromosomal protein high mobility group 1 (HMG1), which is ubiquitously expressed in higher eukaryotic cells, preferentially binds to cisplatin-modified DNA. HMG1 is overexpressed in cisplatin-resistant cell lines from human epidermoid cancer and the specific factor CTF/NF-1 regulates HMG1 gene expression (Nagatani et al., 2001).

Sky1 is a yeast rich serine-arginine (SR) protein-specific kinase and experimental data suggest that its kinase function is essential in the cytotoxicity of cisplatin (Schenk et al., 2001). SR protein-specific kinases and the SR proteins that they phosphorylate are thought to be key regulators of RNA processing and, in mammalian cells, alternative splicing through multiple mechanisms (Siebel et al., 1999). *SKY1* mRNA levels do not change after treatment with cisplatin, which suggests that its expression could be regulated by autophosphorylation or posttranslational modification by upstream components (Schenk et al., 2001). In  $\Delta sky1$  cells, lower cisplatin accumulation or DNA platination were not observed, which indicates that the resistance to cisplatin is not related to decreased drug import or increased drug export (Schenk et al., 2002). Besides,  $\Delta sky1$  cells display a mutator phenotype, which suggests that Sky1 might play a significant role in specific DNA repair pathways (Schenk et al., 2002). SRPK1, the human homologue of Sky1, is predominantly found in the testis, where it phosphorylates protamine 1 as well as a cytoplasmic pool of other SR proteins (Papoutsopoulou et al., 1999). Protamines are small highly basic proteins that replace histones during spermatogenesis, resulting in extreme chromatin condensation (Oliva & Dixon, 1991). In *S. cerevisiae* Sky1 is a key regulator of inward transport of polyamines such as putrescine, spermine and spermidine (Erez & Kahana, 2001) and it has been suggested that SRPK1 might have a role in spermatogenesis by direct or indirect regulation of intracellular concentrations of polyamines (Schenk et al., 2004). Inactivation of *SRPK1* using antisense oligo-deoxynucleotides directed against the translation initiation site of its mRNA induces cisplatin resistance in a human ovarian carcinoma cell line and *SRPK1* heterologous expression is able to complement the cisplatin-resistant phenotype of a  $\Delta sky1$  yeast strain (Schenk et al., 2001).

### 1.3 Cellular response to cisplatin and oxidative stress

Several connections exist between cellular responses to cisplatin and oxidative stress. Deletion of the yeast *QDR3* gene, encoding for a drug/H<sup>+</sup> antiporter, confers sensitivity to cisplatin while its over-expression confers resistance to this drug in yeast (Tenreiro et al., 2005). It has been shown that *QDR3* transcription is up-regulated in response to polyamines by a mechanism dependent on the oxidative stress transcriptional regulator Yap1 (Teixeira et al., 2010). *NPR2* (nitrogen permease regulator 2) is a gene whose disruption confers resistance to cisplatin and hypersensitivity to cadmium chloride (Schenk et al., 2003). In turn, Cd (II) is related to the onset of oxidative stress in yeast cells as summarized in section 1.4.

The clinical use of cisplatin is highly limited by its nephrotoxicity and this effect is caused by cisplatin-induced mitochondrial damage in kidney. It has been proposed that oxidative stress exists in the early stage of cisplatin-induced nephrotoxicity and also in hepatotoxicity (Iraz et al., 2006; Mansour et al., 2006; Pratibha et al., 2006; Satoh et al., 2000). In rats, mitochondrial dysfunction in kidney and liver was evidenced after cisplatin treatment. Impairment of mitochondrial function and structure, depletion of the antioxidant defence

system and cellular death by apoptosis were observed (Santos et al., 2008; Martins et al., 2008). In rats, cisplatin increased lactate dehydrogenase and acid phosphatase activities whereas, the activities of malate dehydrogenase, glucose-6-phosphatase, superoxide dismutase and CAT, as well as phosphate transport significantly decreased (Khan et al., 2009).

Consequently, there are reports of different antioxidants, which protect cells from the oxidative damage caused by cisplatin and whose use represents a possible strategy to minimize the nephrotoxicity induced by this antitumor agent. The hydroxyl radical scavenger dimethylthiourea (DMTU) shows a protective effect against cisplatin-induced alterations of renal mitochondrial bioenergetics, redox state and oxidative stress defence (Santos et al., 2008). Green tea consumption increases the activities of the enzymes of carbohydrate metabolism, brush-border membrane, oxidative stress and phosphate transport (Khan et al., 2009). Carvedilol, a beta-blocker with strong antioxidant properties, prevents lipid peroxidation, oxidation of cardiolipin, oxidation of protein sulfhydryls, depletion of the non-enzymatic antioxidant defence and increased activity of caspase-3 (Rodrigues et al., 2011).

ROS production in eukaryotic cells is also characterized by their ability to cause damage to DNA. The cytosolic serine peptidase tripeptidyl-peptidase II (TPPII) translocates into the nucleus of most tumor cell lines in response to gamma-irradiation and ROS production and also after treatment with several types of DNA-damaging drugs including the DNA cross-linker cisplatin (Preta et al., 2010). This demonstrates its participation in mechanisms elicited by both treatments and suggests common connections between ROS production and DNA damage. Antioxidants are also able to prevent DNA damage. Thus, lutein, the second most prevalent carotenoid in human serum and also abundant in green vegetables, reduces the formation of crosslinks and chromosome instability induced by cisplatin (Serpeloni et al., 2010). Lutein also increases GSH levels without affecting CAT activity (Serpeloni et al., 2010).

#### 1.4 Cadmium and arsenate toxicity, the role of oxidative stress

Several metals are toxic for the cell and it has been suggested that one of the mechanisms of metal toxicity might be the induction of oxidative stress (Stohs & Bagchi, 1995). In yeast, Cd (II) has been shown to induce lipid peroxidation and oxidative stress (Brennan & Schiestl, 1996; Howlett & Avery, 1997). As (III) does not produce these effects in a wild type strain, but oxidative stress and lipid peroxidation were detected in *Δyap8* or *Δyap1* mutants (Menezes et al., 2008), suggesting that As (III) also enhances ROS levels in yeast. Since As (V) is reduced to As (III) inside the cell by the action of the arsenate reductase Acr2, using GSH and glutaredoxin as electron donors (Mukhopadhyay & Rosen, 1998; Mukhopadhyay et al., 2000) similar alterations to those produced by As (III) are expected. Different metals have diverse mechanisms to induce oxidative stress (Wysocki & Tamás, 2010) and therefore we have focused on reviewing published data and hypotheses about the mechanisms that induce the oxidative stress response after treatment with Cd (II) or As (V), both with a high capacity to bind thiols. We might consider three ways by which the metals could generate the oxidative stress. First, the metal may stimulate directly or indirectly the generation of ROS; second, it may cause depletion of antioxidant pools; third, it may inhibit specific enzymes necessary to maintain the redox balance in the cell (Beyersmann & Hartwig, 2008; Ercal et al., 2001; Stohs & Bagchi, 1995).

Contrary to As (III or V), Cd (II) is redox-inactive and therefore only indirect mechanisms are possible for the generation of ROS. It has been proposed that redox-inactive metals may alter the Fe metabolism (Kitchin & Wallace, 2008a), increasing the levels of free Fe in the cell, which could be involved in Fenton-type reactions and increase ROS levels. Regarding the question whether As (III), Cd (II) and various oxidants might have similar toxicity profiles in *S. cerevisiae*, a set of genes, which products are responsible for metal tolerance (Thorsen et al., 2009), was compared to other genes previously reported to mediate tolerance to a number of ROS-generating agents including hydrogen peroxide, menadione, cumene hydroperoxide, diamide and linoleic acid 13-hydroperoxide (Thorpe et al., 2004). Some of the genes required for metal tolerance are also necessary for oxidative stress tolerance. However, from this comparison it was not possible to conclude the source and type of ROS that As (III) and Cd (II) generate in the cell and that cause their toxicity (Thorsen et al., 2009).

In relation to metal toxicity and the transcriptional regulators of metal-compounds transport, it has been reported that Rox1 represses *FET4* expression in aerobic conditions causing up-regulation in the *S. cerevisiae*  $\Delta$ *rox1* mutant and increased Cd (II) toxicity (Jensen & Culotta, 2002). Fet4 is the major importer of Cd (II) into the cell during hypoxic growth (Jensen & Culotta, 2002). GSH is the main antioxidant molecule in yeast cells but it is also used for chelating metals (Thorsen et al., 2009; Wysocki & Tamás, 2010). Cellular mechanisms for Cd (II) or As (III) detoxification depend on their chelation with glutathione, which facilitates their export outside the cell or their sequestration into vacuole. As (III) is exported by the Acr3 transporter (Ghosh et al., 1999; Wysocki et al., 1997). It has been proposed that Yor1 mediates Cd (II) efflux in the form of Cd (GS)<sub>2</sub> (Cui et al., 1996; Nagy et al., 2006). Cd (II) and As (III) GSH-conjugates are imported into vacuole by Ycf1. This ABC transporter represents the major pathway for vacuolar sequestration of metals in *S. cerevisiae* (Paumi et al., 2009) although their homologs Bpt1 and Vmr1 might also play a minor role in Cd (II) detoxification (Wysocki & Tamás, 2010). In spite of the fact that metal detoxification requires GSH consumption, it is not probable that always metal treatment causes GSH depletion. The intracellular GSH concentration is in the millimolar range in yeast, whereas Cd (II) is toxic in the micromolar range (Lafaye et al., 2005). However, As (V) is toxic in the millimolar range and therefore it cannot be excluded that some metals could decrease the GSH pool to an extent where GSH-dependent enzyme activities, such as glutathione peroxidases, glutathione S-transferases and glutaredoxins, might be affected. Other argument against this mechanism of Cd (II) or As (V) induction of the oxidative stress response by depletion of the GSH antioxidant pool is the observation that GSH levels strongly increase in response to Cd (II) (Lafaye et al., 2005) and As (III) (Thorsen et al., 2007) exposure.

Regarding Cd (II) or As (IV) inhibition of enzymes, which are necessary for redox balance and protection against oxidative stress, several data have been published. The metals can inhibit these enzymes by different mechanism. They can bind specific thiols that take part of the active site, change the redox state of the protein or diminish enzymatic activity by other complex interactions. Cd (II) inhibits human thiol transferases (GLR, TRR, and thioredoxin) *in vitro*, possibly by binding to vicinal cysteines in their active sites (Chrestensen et al., 2000). Cd (II) may also displace Zn and Ca ions from metalloproteins (Faller et al., 2005; Schutzendubel & Polle, 2002; Stohs & Bagchi, 1995) and zinc-finger proteins (Hartwig, 2001). As (III) has been shown to interact with TRR, pyruvate dehydrogenase and many other



proteins (Kitchin & Wallace, 2008b; Menzel et al., 1999; Samikkannu et al., 2003; Wang et al., 2007). Besides, certain proteins may be more susceptible to As (III)-induced protein oxidation than to direct binding of As (III) to critical thiols (Samikkannu et al., 2003).

In this study we have tested the role of the genes *ROX1*, *IXR1* and *SKY1*, as well as their interconnections, in the yeast response to oxidative stress elicited by As (V), Cd (II) and cisplatin and in terms of modulation of glucose-6-phosphate dehydrogenase, catalase, glutathione reductase and thioredoxin reductase enzymatic activities.

## 2. Materials and methods

### 2.1 Yeast strains and construction of double knock-outs

Yeast cells from the strain BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and its derivatives  $\Delta ixr1$  (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YKL032c::kanMX4*),  $\Delta rox1$  (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YPR065W::kanMX4*) and  $\Delta sky1$  (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YMR216C::kanMX4*) were obtained from EUROSCARF (<http://web.uni-frankfurt.de/fb15/mikro/euroscarf/>). The  $\Delta ixr1\Delta rox1$  (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YKL032c::kanMX4 YPR065W::URA3*) and  $\Delta ixr1\Delta sky1$  (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YKL032c::kanMX4 YMR216C::URA3*) double knock-out strains were obtained by the one-step replacement method and verified by PCR as follows. The plasmid YEplac 195 (Gietz & Sugino, 1998) digested with *EcoRI* was used as template to amplify by PCR a linear fragment containing the *URA3* gene and two flanking regions of homology to the 5' and 3' ends of the selected open reading frame (ORF) as explained in Figure 2A

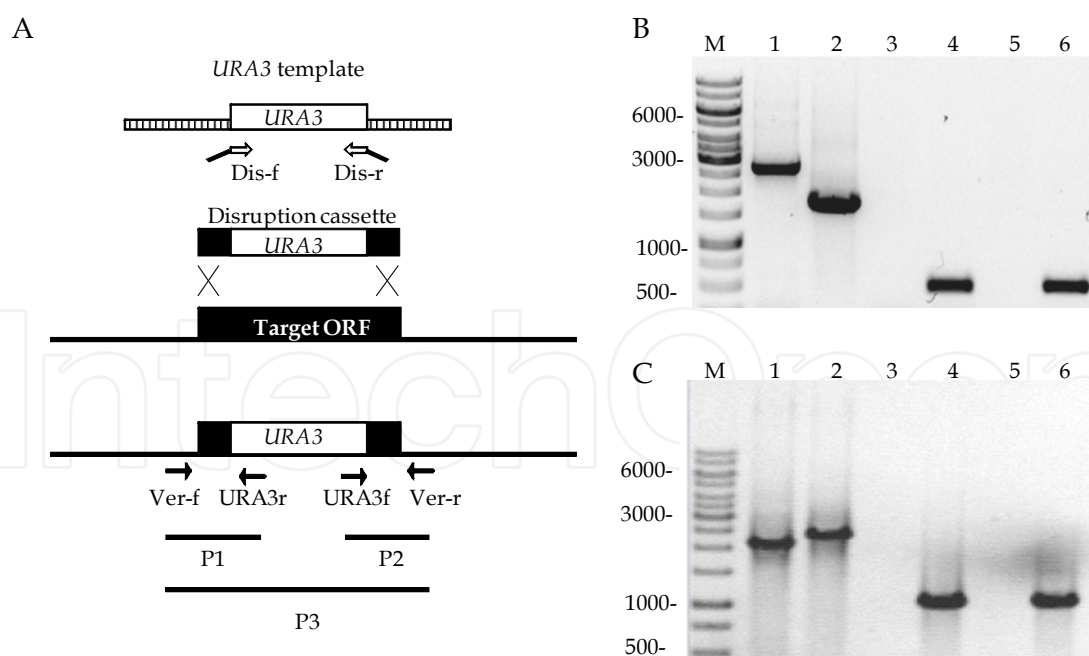


Fig. 2. (A) Strategy for construction of knock-out strains. B) Verification of the BY4741- $\Delta ixr1\Delta sky1$  strain C) verification of the BY4741- $\Delta ixr1\Delta rox1$  strain. In B and C the positions are indicated as follows, M, size marker (bp ladder); 1 and 2, P3; 3 and 4, P1; 5 and 6, P2. The reactions with the starting strain, BY4741- $\Delta ixr1$ , are shown in 1, 3, 5 and with the double nulls in 2, 4, 6.

Target ORF: SKY1						
Primer name	Code	Sequence	HP SKY1	HP URA3	PCR products	PCR size bp
Dis-f	ECV741	atgggttcatcaattaactatcctgggttgC CACCTGACGTCTAAGAAACC	+1	-312	P1 SKY1 P1 <i>Δsky1::URA3</i>	- 481
Dis-r	ECV742	tcaatgtcttttatgatcgcggaacttctcCCT TTAGCTGTTCTATATGCTGC	+2229	+996	P2 SKY1 P2 <i>Δsky1::URA3</i>	- 483
Ver-f	ECV717SR	GATCACCTGGCGCTGAGAA	-97	-	P3 SKY1 P3 <i>Δsky1::URA3</i>	2562 1701
Ver-r	ECV718SR	CGAGTATGGATTCAAAAACC GC	+2465	-		
URA3-f	ECV716SR	GAGAAGATGCGGCCAGCA	-	+780		
URA3-r	ECV715SR	GGATGAGTAGCAGCACGTTC C	-	+41		
Target ORF: ROX1						
Primer name	Code	Sequence	HP ROX1	HP URA3	PCR products	PCR size bp
Dis-f	ECV688AV	atgaatcctaaatcctctacacctaagattCC TTTAGCTGTTCTATATGCTGC	+1	-312	P1 ROX1 P1 <i>Δrox1::URA3</i>	- 1014
Dis-r	ECV689AV	tcatttcggagaaactaggctagtttagcCC ACCTGACGTCTAAGAAACC	+1107	+996	P2 ROX1 P2 <i>Δrox1::URA3</i>	- 1023
Ver-f	ECV698AV	GTGATCTTCGGCTCGGC	-557	-	P3 ROX1 P3 <i>Δrox1::URA3</i>	2091 2352
Ver-r	ECV700AV	TTGTACTTGGCGGATAATGC	+1534	-		
URA3-f	ECV699AV	AAGAGATGAAGGTTACGATT GGT	-	+570		
URA3-r	ECV701AV	ATATCTTGCAGTCCATCCTCG	-	+254		

Table 1. Oligonucleotides used in the construction and verification of knock-out strains. HP, Hybridization position. Oligo position and PCR product designations are as defined in Figure 2A.

After transformation of the *S. cerevisiae* strain BY4741-*Δixr1* with these fragments, cells were selected in complete media without uracyl (CM-Ura) and supplemented with 40 mg/mL geneticin. The correct replacement in the *S. cerevisiae* genome was verified by PCR as previously described (Tizón et al., 1999). Genomic DNAs isolated from the BY4741-*Δixr1* and the null candidates were amplified with two pairs of primers. Internal primers, URA3f and URA3r, were designed for annealing divergently inside *URA3* and external primers, were designed for convergent annealing in the sequences of the *S. cerevisiae* genome, flanking to the

knock-out ORFs, but external to the regions of homology used for the recombination event. The strategy and results obtained in the verification of the replacement of the ORFs with the *URA3* marker is summarized in Figure 2 and the primers used are shown in Table 1.

## 2.2 Yeast treatments with arsenate, cadmium and cisplatin

Stress treatments with arsenate, cadmium and cisplatin were performed as follows. Cd (II) was added to the media in the form of cadmium sulphate 8/3-hydrate and As (V) in the form of sodium arsenate dibasic hepta-hydrate. Arsenate and cadmium were added in concentrations of 500  $\mu$ M and 10  $\mu$ M respectively to the culture media, and cells were collected when OD<sub>600nm</sub> reached 1. Cisplatin was added in concentration 150  $\mu$ M in DMSO when cultures reached an OD<sub>600nm</sub> of 1. In this case, after addition, cells were incubated during 4 h before protein extraction.

## 2.3 Determination of enzymatic activities

For the determination of enzymatic activities, protein extracts were prepared as follows. Cultures were grown in Erlenmeyer flask (with a ratio flask-capacity/volume of medium of 5) at 30 °C in YPD medium. The cells from 20 mL of culture were collected by centrifugation at 3000 x g and resuspended in 1 mL of buffer A (0.2 M Tris-HCl (pH=7.0), 0.3 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 10% glycerol) per gram of wet weight. Cells were broken by vortexing with glass beads (45  $\mu$ m) in 10 seconds pulses. After centrifugation at 8000 x g during 15 minutes, the supernatant was used for enzymatic determinations. For quantification of G6PDH, GLR and TRR activities, proteins were frozen at -80°C until assays were performed. Protein extracts for measurement of CAT activity were immediately used. Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as a standard.

Enzymatic activities were determined following the methods established by Smith et al., (1988) for GLR; Holmgren & Björnstedt (1995) for TRR; Kuby & Noltmann (1966) for G6PDH and Aebi (1984) for CAT. All protocols, with the exception of CAT, were scaled down to reduce the final volume in order to measure absorbance in a 96-well microplate, using a GENios spectrophotometer (TECAN). CAT activity was assayed using a UV-1700 PharmaSpec spectrophotometer (Shimadzu).

Both methods for measuring GLR and TRR enzymatic activities were based in the reduction of 5-5'-ditio-bis (2-nitrobenzoic acid) or DTNB to 2-nitro-5-tiobenzoic acid or TNB. For measurement of GLR activity, the two coupled reactions were the following:  $\text{NADPH} + \text{H}^+ + \text{GSSG} \rightarrow \text{NADP}^+ + 2\text{GSH}$ ;  $\text{GSH} + \text{DTNB} \rightarrow \text{GSTNB} + \text{TNB}$ . The reaction mix contained (final volume = 100  $\mu$ L) 0.1 M phosphate buffer pH 7.5, 0.5 mM EDTA, 0.75 mM DTNB, 0.1 mM NADPH, 1 mM oxidized glutathione (GSSG) and the protein extracts added in aliquots of 5 and 10  $\mu$ L in the two respective replicates. The reaction was started by the addition of GSSG (5  $\mu$ L, 20 mM). The increase of absorbance was recorded at  $\lambda = 412$  nm, and at 24°C, during 2 minutes. Specific activity, enzymatic units (EU)/mg, was defined as  $\mu$ mol of TNB formed per minute and per mg of protein and it was calculated using the Lambert-Beer law, taking into account that the extinction coefficient of TNB is  $13.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ .

Determination of TRR activity was based in the comparison of the reduction of DTNB to TNB in the samples to a standard curve made, by triplicate, with different quantities (0, 10,

20, 30, 40, 50 and 70  $\mu\text{L}$ ) of 20 nM TRR from mammals, diluted in the moment of use from a stock of 88.1 UE/mg protein in TE buffer with 100  $\mu\text{g/mL}$  of bovine serum albumin. The reaction mix contained (final volume = 120  $\mu\text{L}$ ): 0.3 M HEPES pH 7.6, 0.07 M EDTA, 13.3 mg/mL NADPH, 3.3 mg/mL insulin, 5  $\mu\text{M}$  thioredoxin from *E. coli* and the protein samples added in quantities varying between 10 and 70  $\mu\text{L}$ . Reaction was started by adding thioredoxin (10  $\mu\text{L}$ , 60  $\mu\text{M}$ ) and continued for 20 minutes at 37 °C. The reaction was stopped by adding 50  $\mu\text{L}$  of 0.4 mg/mL DTNB in 6 M guanidine hydrochloride 0.2 M Tris-HCl pH 8. Absorbance was read at  $\lambda = 412 \text{ nm}$ . The specific activity (EU/mg) corresponded to  $\mu\text{mol}$  of TNB formed per minute and per mg of protein.

Determination of G6PDH activity was based in the following reaction: D-glucose-6-phosphate +  $\text{NADP}^+ \rightarrow$  6-phosphoglucolactone +  $\text{NADPH} + \text{H}^+$ . The reaction mix contained 100  $\mu\text{L}$  of 0.1 M glycylglycine buffer (pH 8.0) 0.03 M glucose-6-phosphate; 0.01 M  $\text{NADP}^+$  and 0.15 M magnesium sulphate. Aliquots of 5 or 10  $\mu\text{L}$  of the protein sample were added. Increase of absorbance was quantified in the spectrophotometer at  $\lambda = 340 \text{ nm}$  and at 30°C during 4 minutes. The specific activity (EU/mg) corresponded to  $\mu\text{mol}$  of generated product (NADPH) per minute and per mg of protein, and it was calculated using the Lambert-Beer law, taking into account that the extinction coefficient of NADPH ( $E_{\text{NADPH}}$ ) is  $6.22 \cdot 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ .

Quantification of CAT activity was based in the following reaction:  $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ . Two reaction mixes were made. The first one (A) contained 30 mM of  $\text{H}_2\text{O}_2$  in 50 mM phosphate buffer pH 7. The second one (B) contained a dilution of the protein sample in 50 mM phosphate buffer pH 7. Mix B was prepared in several dilutions, and these should be used in a time not longer than 5-10 minutes after their preparation. Reaction started when 0.33 mL of the A mix were added to 0.67 mL of the B mix. Decrease of absorbance was recorded in the spectrophotometer at  $\lambda = 240 \text{ nm}$  during 30 seconds and at room temperature. The specific activity (EU/mg) corresponded to 1  $\mu\text{mol}$  of consumed substrate ( $\text{H}_2\text{O}_2$ ) per minute and per mg of protein. Concentration of the substrate was determined by applying the Lambert-Beer law, taking into account that  $E_{\text{H}_2\text{O}_2}$  is  $3.94 \cdot 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ .

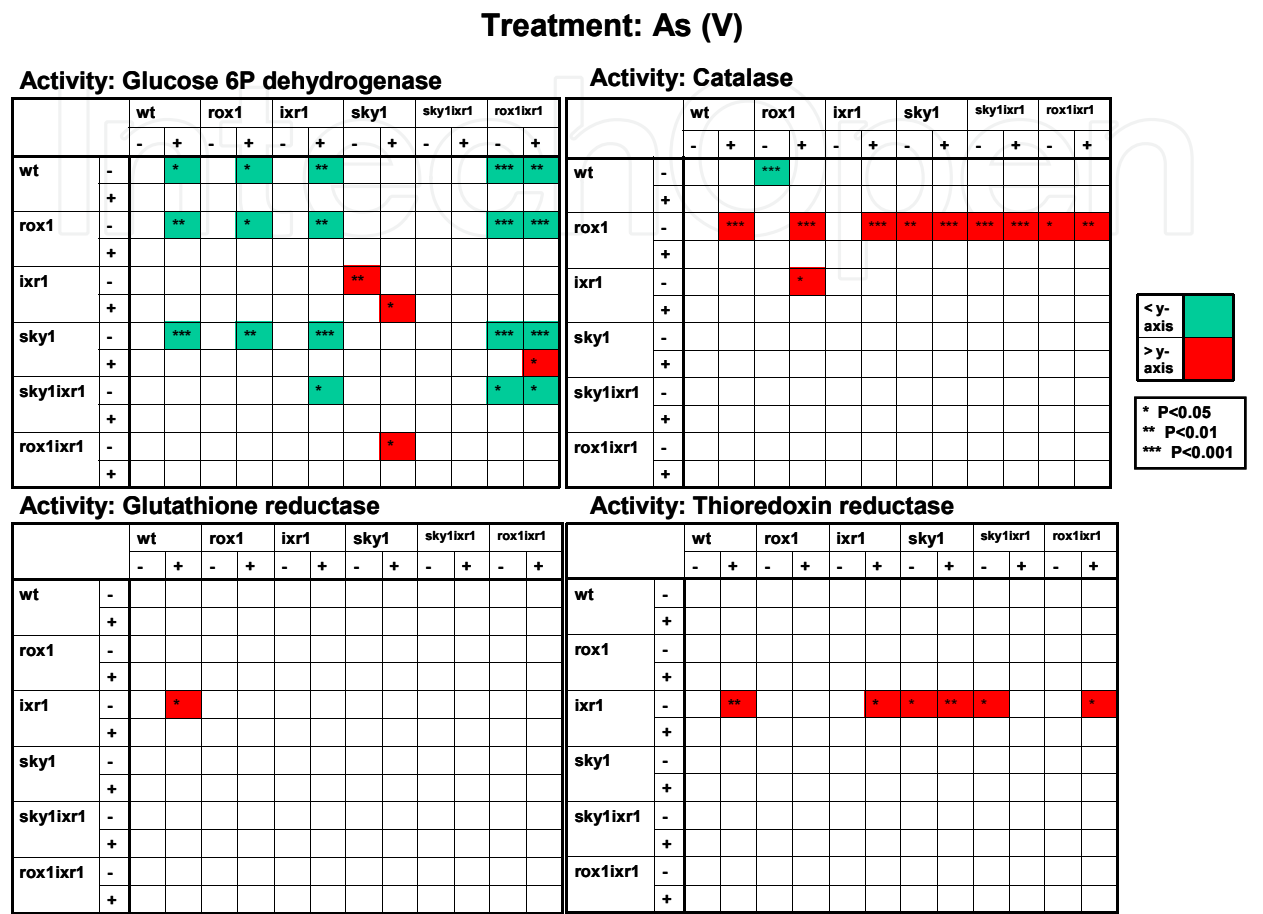
## 2.4 Statistical analyses

Data were expressed as mean  $\pm$  standard deviation (SD). The statistical significance of differences between means was evaluated by one-way ANOVA with Tukey post-test or by Kruskal-Wallis test with Dunn post-test, both at the 95% confidence level. The program GraphPad Instat was used.

## 3. Results and discussion

The *S. cerevisiae* response to As (V), Cd (II) and cisplatin was evaluated in terms of modulation of enzymatic activities related to the PPP (G6PDH); break down of  $\text{H}_2\text{O}_2$  into  $\text{O}_2$  and  $\text{H}_2\text{O}$  (CAT); or glutathione and thioredoxin reduction (GLR and TRR). The BY4741 wild type strain and its derivatives  $\Delta ixr$ ,  $\Delta rox1$ ,  $\Delta sky1$ ,  $\Delta ixr1\Delta rox1$  and  $\Delta ixr1\Delta sky1$  strains, which are described in the Materials and Methods section, were used. At least two independent cultures of each case were performed and enzymatic activities were measured in duplicates

from each. Multiple statistical comparisons of means were performed classifying the data of the six strains by enzyme activity and treatment. Significant differences found in each case are outlined in Figures 3 to 5 and Tables 2A and 2B.



< y-axis

> y-axis

\* P<0.05

\*\* P<0.01

\*\*\* P<0.001

Fig. 3. Statistical comparison of the effects of As (V) treatment on the four enzymatic activities and six strains studied in this work. N=4. Only significant differences are marked.

Figure 3 and Table 2B shows the effect of the treatment with As (V) on the four enzymatic activities in the six strains assayed. Treatment with As (V) caused, as most outstanding results, the following activity-dependent and strain-dependent responses: increase of G6PDH activity in wild type and  $\Delta$ rox1 backgrounds; decrease of CAT activity in the  $\Delta$ rox1 background; decrease of TRR activity in the  $\Delta$ ixr1 background.

The treatment with Cd (II) only affected significantly the activity of GLR among the four studied enzymes, which increased in the wild type and  $\Delta$ ixr1 backgrounds. However this increase was not observed in other single or double mutants (Figure 4 and Table 2B). The effect of Cd (II) on TRR and G6PDH activities was also statistically analyzed using a non-parametric test without finding significant differences (data not shown).

The effect of the treatment with cisplatin is represented in Figure 5 and Table 2B. The most outstanding results were the decrease of GLR activity in the double mutant  $\Delta$ ixr1 $\Delta$ sky1 and the decrease of TRR activity in the double mutant  $\Delta$ ixr1 $\Delta$ rox1.



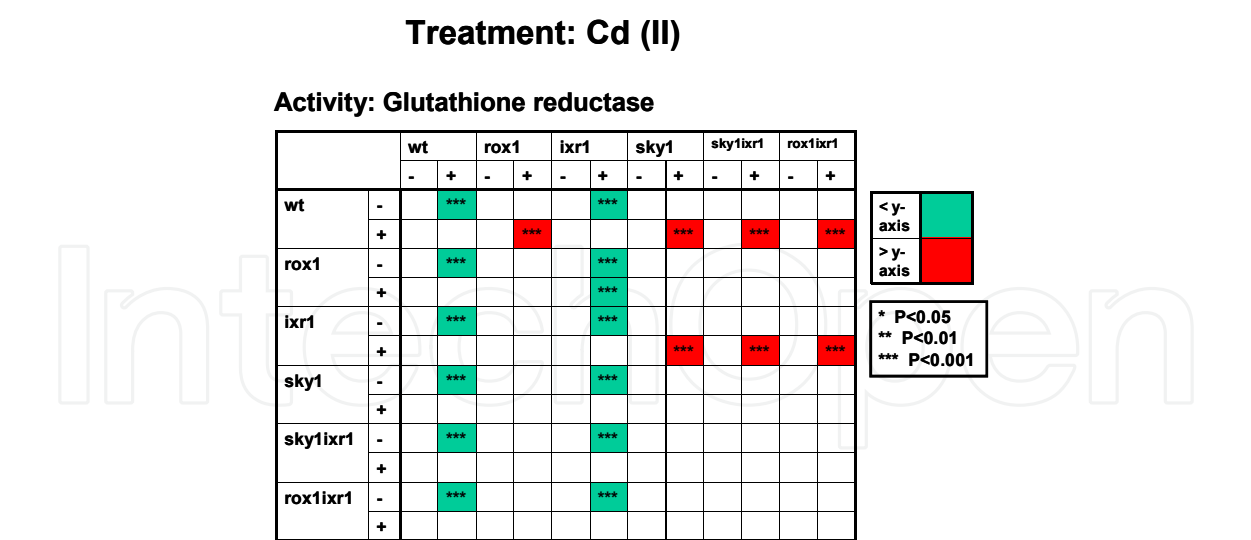


Fig. 4. Statistical comparison of the effects of Cd (II) treatment on GLR activity in the six strains studied in this work. N=4. Only significant differences are marked.

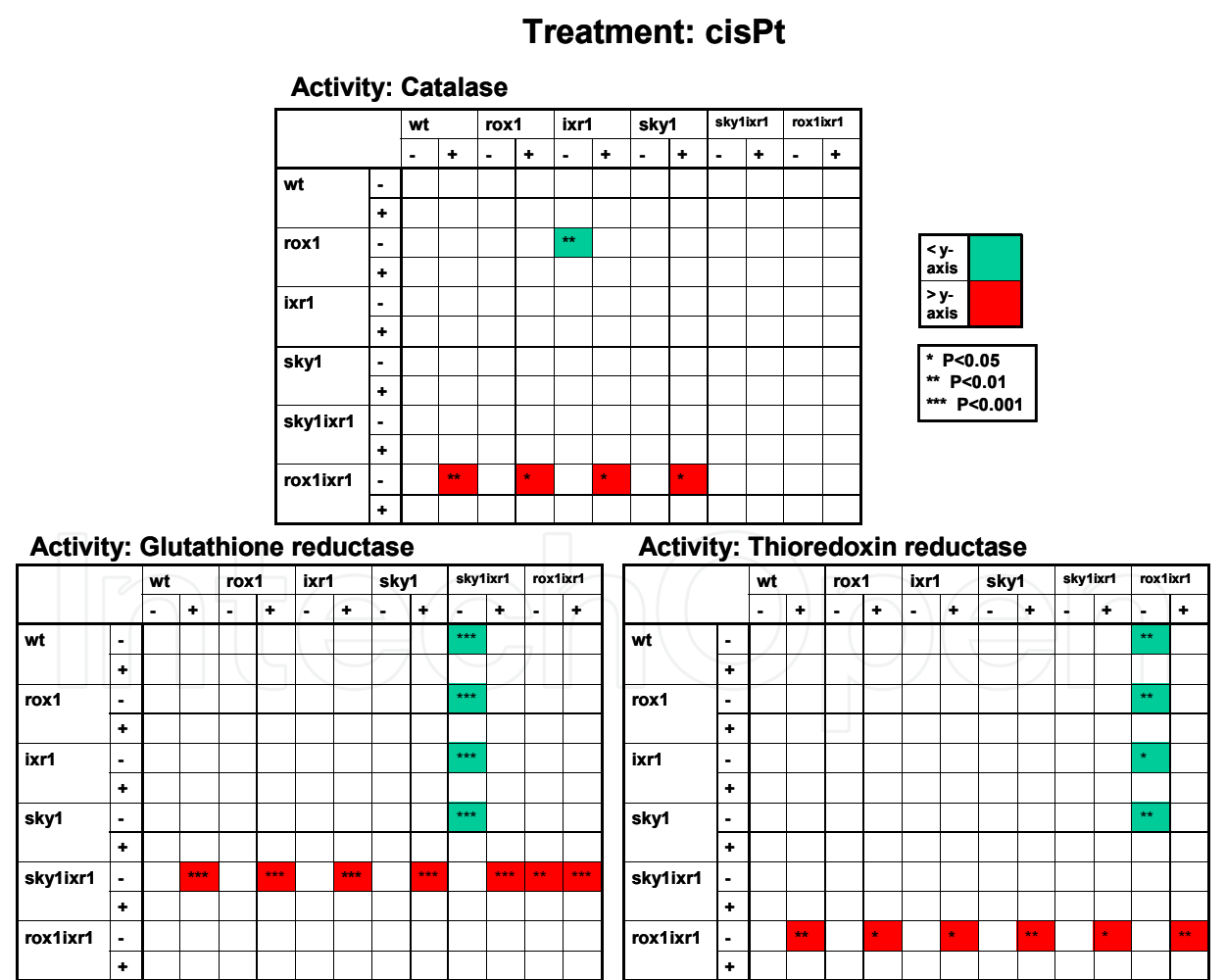


Fig. 5. Statistical comparison of the effects of cisplatin treatment on the enzymatic activities in the six strains studied in this work. N=4. Only significant differences are marked.

### 3.1 Changes in glucose-6-phosphate dehydrogenase activity

Glucose-6-phosphate dehydrogenase is the protein that catalyzes the first step in the oxidative branch of the pentose phosphate pathway (PPP), the conversion of glucose-6-phosphate into ribulose-5-phosphate. Nevertheless, the protein does not seem to be essential, since mutants in its coding gene, *ZWF1*, can still grow in both respiratory and fermentative carbon sources (Nogae & Johnston 1990; Saliola et al., 2007). The enzyme uses NADP<sup>+</sup> as a coenzyme, thus converting it to the reduced form NADPH, which is used by proteins with antioxidant functions. In fact, the PPP is the major source of this coenzyme during situations of oxidative stress (Minard et al., 2005). Mutants in *ZWF1* also show methionine auxotrophy (Thomas et al., 1991) probably caused by the interconnections between methionine biosynthesis and glutathione biosynthesis.

If we analyzed the results of this study, shown in Figures 3, 4 and 5, by enzyme activity (Table 2) we observed that G6PDH was significantly affected only by As (V) treatment (increase of activity in wild type and *Δrox1* backgrounds). A previous work (Godon et al., 1998) showed that *S. cerevisiae* treated with H<sub>2</sub>O<sub>2</sub> is able to oxidize more glucose through the PPP than through glycolysis in order to obtain NADPH necessary in the oxidative defence reactions. In fact it has been proved that G6PDH mutants are more sensitive to oxidative stress caused by H<sub>2</sub>O<sub>2</sub> (Izawa et al., 1998; Junhke et al., 1996).

Why As (V) affects G6PDH activity and probably increases the glucose utilization via PPP, while treatment with Cd (II) or cisplatin does not produce a similar effect is striking since it has been reported that all these treatments stimulate intracellular ROS generation. A possible explanation could be related to the different ways that these metals and derivative compounds use to enter into the cells. Cd (II) enters yeasts cells through proteins involved in the uptake of other bivalent cations, which are essential for cell survival. Zn (II) enters through Zrt1, Mn (II) through Smf1 or Smf2, Fe (II) through Fet4 and Ca (II) through Mid1; all these proteins are also Cd (II) importers (reviewed in Wysocki & Tamas, 2010). In *S. cerevisiae* the import of cisplatin inside the cell is mediated by the copper transporter Ctr1 and the N-terminal methionine-rich motifs that are dispensable for copper transport play a critical role for cisplatin uptake (Adle et al., 2007). The arsenate As (V) oxyanion is a structural analogue of inorganic phosphate and is taken up through phosphate transporters. Phosphate import into *S. cerevisiae* is mediated by two high-affinity permeases, Pho84p and Pho89p, and two low-affinity permeases, Pho87p and Pho90p (Persson et al., 1999; Wykoff & O'Shea, 2001). Perhaps in presence of As (V) the cellular homeostasis of phosphate change, this might affect the energetic balance and indirectly cause a redistribution of sugar utilization by different metabolic pathways.

About the target regulators investigated in the five mutants analyzed in this work we may conclude that Ixr1 and Sky1 are necessary to directly or indirectly mediate the observed increase in G6PDH activity, while Rox1 is dispensable.

### 3.2 Changes in catalase activity

Catalase catalyzes the breakdown of H<sub>2</sub>O<sub>2</sub> into oxygen and water. In *S. cerevisiae*, there are two genes for CAT, *CTA1* that encodes the peroxisomal and mitochondrial isoforms, and *CTT1*, that encodes the protein in the cytosol (Jamieson, 1998). CAT is one of the principal members that conforms the H<sub>2</sub>O<sub>2</sub> stimulon, the set of proteins induced in *S. cerevisiae* in

response to H<sub>2</sub>O<sub>2</sub> (Godon et al., 1998). Also, *Schyzosaccharomyces pombe* (Vivancos et al., 2006) and *Kluyveromyces lactis* (Becerra et al., 2004; Tarrío et al., 2008), other two yeasts used frequently in this area of research, show increased levels of CAT in response of oxidative stress conditions. It has been shown that CAT function, even though it is an important antioxidant protein, can be partially substituted by other enzymatic activities. For example, in *S. cerevisiae*, when CAT activity is inhibited with 3-aminotriazol (3-AT), a compound commonly used as herbicide, simultaneous decrease in G6PDH and increase of GLR activity is observed (Bayliak et al., 2008).

Catalase activity did not increase after the treatments assayed in this work (Table 2). Among the hypothetical mechanism by which metals produce the onset of oxidative stress defence the production of ROS has been proposed (Stohs & Bagchi, 1995; Ercal et al., 2001; Beyersmann & Hartwig, 2008). However, from our data we might assume that the production of hydrogen peroxide is not significant in these conditions and perhaps other ROS are predominant after exposure to these metal compounds. Contrary to our results, previous studies (Muthukumar & Nachiappan, 2010) found that GSH levels were increased in cells exposed to Cd (II), as well as CAT and glutathione peroxidase activities. However, these changes were observed when yeast cells were exposed to 100 µM Cd (II), ten folds higher than the concentration used in our study.

	As (V)				Cd (II)				cisPt			
	G6PDH	CAT	GLR	TRR	G6PDH	CAT	GLR	TRR	G6PDH	CAT	GLR	TRR
wt	↑						↑					
Δrox1	↑	↓										
Δixr1				↓			↑					
Δsky1												
Δixr1Δsky1											↓	
Δixr1Δrox1										↓		↓

Table 2A. Most significant effects of As (V), Cd (II) and cisplatin (cisPt) treatment on the four enzymatic activities and six strains under study in this work. Green arrow: increase, red arrow: decrease.

Activity	Strain	Treatment	-	+	Ratio
G6PDH	wt	As	0.20±0.06	0.34±0.04	+1.7
	Δrox1	As	0.19±0.03	0.32±0.04	+1.7
CAT	Δrox1	As	0.82±0.02	0.02±0.02	-41.0
	Δixr1Δrox1	cisPt	0.32±0.15	0.06±0.05	-5.3
GLR	wt	Cd	0.06±0.04	0.33±0.04	+5.5
	Δixr1	Cd	0.06±0.03	0.28±0.04	+4.7
	Δixr1Δsky1	cisPt	0.07±0.04	0.01±0.01	-7
TRR	Δixr1	As	1.35±0.35	0.66±0.16	-2.0
	Δixr1Δrox1	cisPt	0.11±0.06	0.03±0.00	-3.7

Table 2B. Numerical data corresponding to significant effects reported in Table 2A. Media ± standard deviation (0.00 = < 0.005). Enzymatic units are defined in the text (- without and + with treatment). Ratio +, fold increase; ratio -, fold decrease.

About the control exerted by the selected regulators on CAT activity, it is interesting to say that Rox1 is necessary to maintain wild type activity levels after treatment with As (V) and cisplatin (Table 2), since the enzymatic activity diminished in the mutant background.

### 3.3 Changes in glutathione reductase activity

Once GSH is oxidized to GSSG, it becomes toxic to the cell and it cannot be accumulated for long. The principal protein in charge of catalyzing the transformation of one molecule of GSSG into two of GSH is glutathione reductase (GLR). Glutathione reductase is an enzyme belonging to the family of flavoproteins with oxidoreductase activity (Mustacich & Powis, 2000), and together with GSH and glutaredoxins, it constitutes the glutathione/glutaredoxin system. GLR has a double function, since it uses NADPH as a coenzyme and reduces GSSG. Besides it also produces NADP<sup>+</sup> that can be reduced by other enzymes, such as G6PDH. *S. cerevisiae* has only one gene to codify for GLR, and it contains two in-frame start codons (Collinson & Dawes, 1995). Translation from AUG1 or AUG2 generates the mitochondrial or cytosolic isoforms of the protein, respectively (Outten & Culotta, 2004). GLR is not essential to cell survival (Collinson & Dawes, 1995) but it is required for defence against oxidative stress (Grant et al., 1996ab).

GLR was significantly increased by Cd (II) treatment in the wild type strain (Table 2), which is in agreement with previous data that indicate that the oxidative stress caused by Cd (II) and the processes related to metal detoxification are highly dependent on the GSH/GRX system (Paumi et al., 2009; Wysocki & Tamás, 2010). Comparing the effects in the wild type and the five mutants analyzed in this work we may conclude that Rox1 and Sky1 are necessary to directly or indirectly mediate the observed increase in GLR activity, while Ixr1 is dispensable.

### 3.4 Changes in thioredoxin reductase activity

Thioredoxin reductase is an enzyme that belongs to the flavoprotein family of pyridine nucleotide-disulphide oxidoreductases. Its primary function is to reduce oxidized thioredoxins (TRXs). TRXs are small peptides between 10 and 12 kDa, which can supply reducing equivalents to enzymes such as ribonucleotide reductase (Laurent et al., 1964) and thioredoxin peroxidase (Chae et al., 1994). They also produce thiol-disulphide exchange and may reduce key Cys residues in certain transcription factors, which increases their ability to bind to DNA and regulate gene transcription. TRR and TRXs form the called “thioredoxin system” and TRRs from *S. cerevisiae* are induced by H<sub>2</sub>O<sub>2</sub> (Godon et al., 1998).

In *S. cerevisiae* there are two genes encoding TRR: *TRR1* and *TRR2*, coding *TRR1* the cytosolic and *TRR2* the mitochondrial isoform. There are also three TRXs genes. *TRX1* and *TRX2* encode cytosolic forms, whereas *TRX3* the mitochondrial form. In *S. cerevisiae*, deletion of both *TRR1* and *TRR2* genes inhibits vacuole inheritance, decreases the rate of DNA synthesis, increases the cell size and the generation time and makes the cells auxotrophic for methionine/cysteine (Pedrajas et al., 1999). In a long term, cells lacking *TRR1* are unviable (Pedrajas et al., 1999).

TRR activity was not increased after the treatments applied in this work (Table 2). This observation might indicate that oxidative stress caused by these metals is counteracted principally by the glutathione/glutaredoxin system instead of the thioredoxin system. However, the thioredoxin system is important for Cd (II) tolerance, since deletion of *TRR1* or

both *TRX1* and *TRX2* results in cadmium-hypersensitivity (Vido et al., 2001). Also, it has been proved that organic arsenicals can inhibit the thioredoxin and glutathione reductases leading to the increase of ROS steady state levels in the cell (Lin et al., 1999; Styblo et al., 1997).

### 3.5 Interactions between *Ixr1*, *Rox1* and *Sky1* in the maintenance of CAT, GLR and TRR activities during response to cisplatin

Data from Table 2 clearly show that enzymatic activities in the double mutants were affected by cisplatin treatment in reference to the wild type. Glutathione reductase activity decreased in  $\Delta ixr1\Delta sky1$  strain, while CAT and TRR activities decreased in the  $\Delta ixr1\Delta rox1$  strain. These data indicate that both *Ixr1*-*Rox1* and *Ixr1*-*Sky1* interactions are necessary in the response to cisplatin, since none of the single mutants analyzed demonstrated significant effect. However, probably the interconnections of *Ixr1* with *Rox1* and *Sky1* play specialized functions in this change, as deduced from the different enzymatic activities that change in each case. The nature of these interactions, physic or genetic, has not been yet explored and constitutes an interesting subject for further studies.

## 4. Conclusions

Summarizing the results from this work (Figures 3-5 and Tables 2A and 2B), we observed in the wild type strain an increase of G6PDH activity upon As (V) treatment and increase of GLR activity upon Cd (II) treatment. In the  $\Delta rox1$  mutant, treatment with As (V) caused increase of G6PDH activity and decreased CAT activity. The  $\Delta ixr1$  mutant showed decrease of TRR activity upon As (V) treatment and increased GLR activity upon Cd (II) treatment. The double mutants were affected by cisplatin; GLR activity decreased in the  $\Delta ixr1\Delta sky1$  strain and TRR activity decreased in  $\Delta ixr1\Delta rox1$  strain.

We may conclude that changes caused by As (V), Cd (II) and cisplatin treatments could not be considered as only general oxidative stress response. On the contrary, each treatment induced changes on specific enzymatic activities without affecting the others. G6PDH was enhanced by As (V) while GLR activity was increased by Cd (II). Besides, the increase of these activities depended on different regulatory factors; G6PDH seems to be regulated by *Ixr1* and *Sky1*, while GLR by *Rox1* and *Sky1*. After treatment with cisplatin, maintenance of enzymatic activities in the levels observed in the wild type strain was also under the control of complex interaction between *Rox1*, *Ixr1* and *Sky1*. Further studies will be necessary to understand the nature of these interactions.

## 5. Acknowledgements

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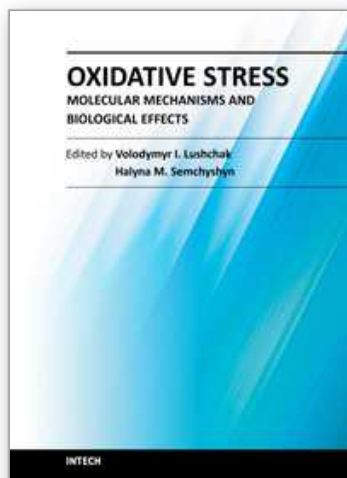
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## **Oxidative Stress - Molecular Mechanisms and Biological Effects**

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Since the discovery of free radicals in biological systems researchers have been highly interested in their interaction with biological molecules. Denoted in 1980, and due to fruitful results and ideas, oxidative stress is now appreciated by both basic and applied scientists as an enhanced steady state level of reactive oxygen species with wide range of biological effects. This book covers a wide range of aspects and issues related to the field of oxidative stress. The association between generation and elimination of reactive species and effects of oxidative stress are also addressed, as well as summaries of recent works on the signaling role of reactive species in eukaryotic organisms. The readers will gain an overview of our current understanding of homeostasis of reactive species and cellular processes they are involved in, as well as useful resources for further reading.

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