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Complex Regulatory Interplay Between Multidrug Resistance and Oxidative Stress Response in Yeast: The *FLR1* Regulatory Network as a Systems Biology Case-Study

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1. Introduction

Multidrug resistance (MDR), the intrinsic or acquired ability to tolerate toxic concentrations of structurally and functionally diverse chemicals, is a widespread phenomenon that can be found in all living organisms, from bacteria to man (Hayes and Wolf 1997). Its negative consequences include the failure of many therapeutic, antimicrobial and crop protection actions. At the same time, the ability to tolerate multiple stresses is a highly desirable phenotype in organisms used as cell factories that have to cope with fermentation-related stresses (Teixeira et al. 2011b). It is, thus, crucial to understand the molecular basis underlying this phenomenon to be able to circumvent it or to explore it to design more robust industrial strains.

Oxidative stress, on the other hand, is usually considered the result from an imbalance between the generation or influx of reactive oxygen species and the cell ability to readily neutralize these molecules (Ikner and Shiozaki 2005; Lushchak 2011). Increased ROS concentration may lead, in term, to the modification of susceptible biomolecules, such as [Fe-S]-clusters-containing enzymes, proteins exhibiting reactive thiol groups, DNA and lipids, which may undergo peroxidation. These corrupted molecules may, in a small number of cases, be regenerated, but in most cases are degraded or accumulated in cells. The steady-state accumulation of ROS and associated ROS-damaged biomolecules has been linked to ageing and to the development of certain pathologies, such as diabetes mellitus, atherosclerosis and cardiovascular and neurodegenerative diseases (Lushchak 2011). Oxidative stress is usually linked to cell exposure to reactive oxygen species, including hydrogen peroxide, or to redox-cycling agents such as menadione, which leads to superoxide radical generation. However, mounting evidence appears to suggest that many

chemical compounds, including widely used pesticides and pharmaceuticals, can induce oxidative stress indirectly, acting as pro-oxidant agents, at the same time that drug resistance mechanisms are activated. The comprehension of the underlying molecular mechanisms is, thus crucial to evaluate the toxicity of these xenobiotics and to design strategies to deal with the arising of multidrug resistance.

Being now clear that these cellular protection programs, crucial to prevent or delay disease progression and ageing, are highly interconnected, it is pivotal to fully understand the underlying cross-mechanisms. Thus, this chapter integrates current knowledge of the link between oxidative stress and multidrug resistance transcriptional control in *S. cerevisiae*, extending it to pathogenic yeasts. The particular case of the regulation of the multidrug resistance transporter Flr1 is further explored as an example of the use of systems biology approaches, including the combination of experimental and computational techniques, to increase our understanding of complex regulatory networks, shedding light into the cross-talk between the MDR phenomenon and oxidative stress response.

2. The multidrug resistance network in yeast

Multidrug resistance is often acquired through the activation of multidrug efflux pumps, belonging to the ATP-Binding Cassette (ABC) or Major Facilitator Superfamilies (MFS), this activation occurring, many times, at the transcriptional level. This fact has led to years of research aiming the definition of the transcription regulatory networks that control the expression of multidrug transporters under stress. The first finding in this field was the discovery that the *PDR1* gene (Saunders and Rank 1982), latter characterized as a transcription factor (Balzi et al. 1987), confers multidrug resistance in the model eukaryote *S. cerevisiae*. Soon after, the so-called PDR (Pleiotropic Drug Resistance) network was first described (Balzi and Goffeau 1995) as a very simple network in which Pdr1, and its homologous transcription factor Pdr3, were found to control the transcription of the *PDR5* gene (Balzi et al. 1994), encoding an ABC drug efflux pump. This network was rapidly extended to include other ABC multidrug transporters, such as Snq2 (Decottignies et al. 1995), but also members of a new family of multidrug transporters of the MFS (Sá-Correia et al. 2009), predicted to function as Drug:H^+ Antiporters (DHA) and uncovered mostly upon the release of the *S. cerevisiae* genome sequence (Goffeau et al. 1996), including Flr1 (Brôco et al. 1999; Tenreiro et al. 2001) and Tpo1 (do Valle Matta et al. 2001; Teixeira and Sá-Correia 2002). The use of genome-wide expression analysis tools helped to enlarge this network, while the genome-wide targets of Pdr1 and Pdr3 were uncovered (DeRisi et al. 2000). Apparently, several unrelated drugs and xenobiotics are able to bind to the so-called xenobiotic-binding domain of Pdr1p family members in budding yeast and in the human pathogen *Candida glabrata*, resulting in the over-expression of drug efflux pumps, this finding, providing new clues for the development of novel targets for antifungal drugs (Thakur et al. 2008). Additionally, new transcription factors were also found to belong to the PDR network, based on their homology to Pdr1 and Pdr3. These include Yrr1 (Le Crom et al. 2002), Pdr8 (Hikkel et al. 2003) and Yrm1 (Lucau-Danila et al. 2003), their target-genes also being identified through microarray analysis and, more directly, through ChIP(Chromatine ImmunoPrecipitation)-on-chip analysis. Considering only the canonical PDR transcription factors and the genes encoding predicted multidrug transporters of the ABC and MFS superfamilies, we get a relatively small, but intricate network controlling

multidrug resistance in *S. cerevisiae*, as depicted in Fig. 1. However, if we consider all the targets of the same five transcription factors, the PDR network is found to include nearly 500 target genes with a broad scope of biological functions.

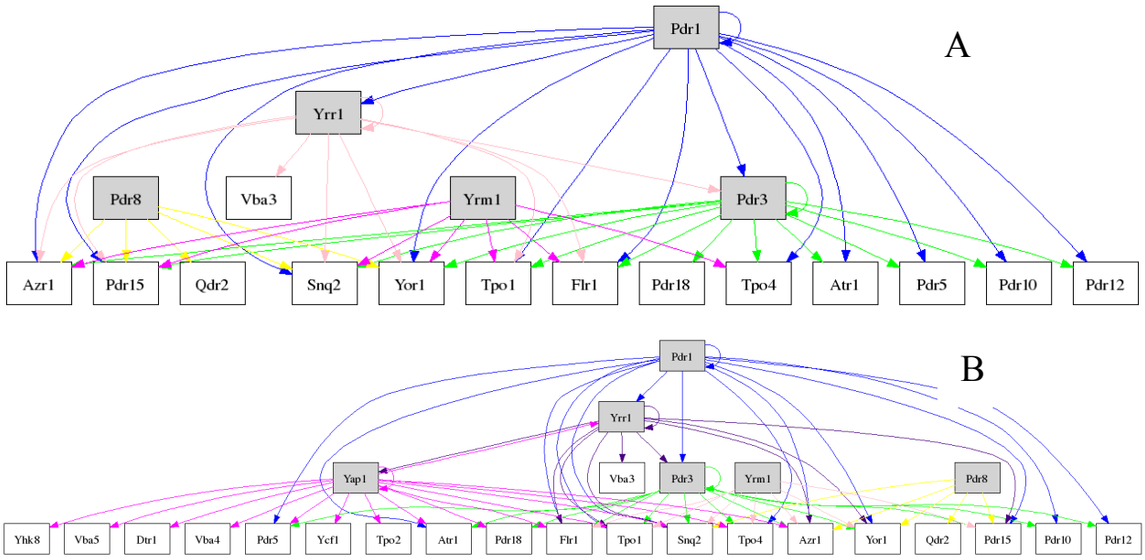

Fig. 1. A: The PDR network, considering only the canonical PDR transcription factors and the genes encoding predicted multidrug transporters of the ABC and MFS superfamilies. B: The PDR network, considering the canonical PDR transcription factors, the oxidative stress response regulator Yap1, and the genes encoding predicted multidrug transporters of the ABC and MFS superfamilies. Both networks were built based on the information gathered in the YEASTRACT database (www.yeastract.com), considering as evidence for transcriptional association between transcription factor and target genes either expression and/or DNA-binding evidence.

Further extending this network, it became clear that other transcription factors, whose function is not primarily linked to multidrug resistance, are also involved in the transcriptional control of drug efflux pumps. The first non-PDR transcription factor to join this network was Yap1, the major regulator of oxidative stress response in yeast (Rodrigues-Pousada et al. 2010), found to confer resistance to the drug diazaborine via the Pdr3 and, less significantly, Pdr1 transcription factors (Jungwirth et al. 2000; Wendler et al. 1997). Two other transcriptions factors found to relate to the PDR network are Rpn4 (Owsianik et al. 2002; Teixeira et al. 2008) and Hsf1 (Hahn et al. 2006), regulators of proteasomal genes and of the heat shock response, respectively.

3. The role of yap1 in multidrug resistance in yeast

Recent studies in this field focusing the model eukaryote *Saccharomyces cerevisiae* have shown that there seems to be a close cross-talk between the multidrug resistance regulatory network and the oxidative stress response transcription factor Yap1. Indeed, Yap1 was demonstrated to confer resistance against a wide variety of drugs, including quinine, rapamycin, trenimon and diazaborine, but also to antifungal agents, such as cerulenin,

benomyl, cycloheximide, fenpropimorph, mancozeb, to herbicides, including sulfometuron methyl, 2,4-dichlorophenoxyacetic acid (2,4-D) and paraquat and to the food preservative acetic acid. Although some of these compounds have been described as pro-oxidants molecules (Dias et al. 2010; Semchyshyn et al.; Teixeira et al. 2004), the role of Yap1 in drug resistance seems to rely not only in the control of antioxidant defenses, but also on the control of multidrug resistance transporters. In fact, Yap1 has been shown to underlie the stress-induced up-regulation of the multidrug ABC transporters Pdr5, Pdr18, Snq2, and Ycf1 (Cabrito et al. 2011; Jungwirth and Kuchler 2006; Teixeira et al. 2006) and of the drug:H^+ antiporters Atr1, Azr1, Dtr1, Flr1, Qdr3, Tpo1, Tpo2, Tpo4, and Yhk8 (Sá-Correia et al. 2009; Teixeira et al. 2011a) (Table 1). If we take a global look, it becomes clear that the role of Yap1 in the regulation of the PDR network is even broader. Indeed, using the YEASTRACT database, a repository of all demonstrated regulatory associations in *S. cerevisiae* (Abdulrehman et al. 2011; Monteiro et al. 2008; Teixeira et al. 2006), it is possible to see that Yap1 co-regulates around 18% of the Pdr1-target genes (Fig. 2A).

Interestingly, Yap1 displays two different activation mechanisms depending on the nature of the imposed stress. In both cases, the molecular events triggering Yap1 activation are apparently responsible for releasing this transcription factor from the interaction with the exportin Crm1, thus leading to its nuclear accumulation (Yan et al. 1998). One of the activation mechanisms occurs due to the increase in intracellular ROS concentration, due, for example, to cellular exposure to H_2O_2. Hydrogen peroxide appears to, indirectly, lead to the formation of an intramolecular disulfide bond between Cys303 and Cys598 of Yap1 (Delaunay et al. 2000). On the other hand, a second redox centre was later found in this transcription factor, and suggested to involve the direct binding of electrophiles such as N-ethylmaleimide (Azevedo et al. 2003) to Cys598, Cys620 and Cys629, thus inducing a conformational change that also prevents Yap1-Crm1 binding and, thus, leads to Yap1 accumulation in the nucleus. Given this differential activation mechanism, the question of whether Yap1 could regulate distinct target-gene sets under different stress conditions arose.

Microarray analysis was recently used to compare the Yap1-dependent transcriptional response to hydrogen peroxide and to the thiol-reactive compounds N-ethylmaleimide (NEM) and acrolein (Ouyang et al. 2011). The obtained results showed that 56 genes are exclusive of the response to H_2O_2, while 327 are exclusive of the response to NEM or acrolein. Although both responses were primarily under the control of the same transcription factor, in each case the elicited response resulted in the expression of protective genes specific for each of the imposed stresses (Ouyang et al. 2011). This specificity appears to result from the differential mechanisms of Yap1 activation imposed by the analyzed stress agents. The global analysis of the role of Yap1 in yeast response to benomyl induced stress had also highlighted the differences between the gene-sets up-regulated by Yap1 in response to ROS or to thiol-reactive compounds (Lucau-Danila et al. 2005). Genes required for the maintenance of redox balance were shown to be up-regulated in both cases, while specific genes such as *SOD1* and *CTT1*, encoding the cytosolic superoxide dismutase and catalase, respectively, are only responsive to ROS. An interesting discovery from this study was that the promoter occupancy by Yap1, when activated by benomyl, increases in all the promoters of Yap1 targets genes, including highly up-regulated genes such as *FLR1*, but also non-responsive genes such as *CTT1* and *SOD1* (Lucau-Danila et al. 2005). This finding

Gene	Binding evidence	Expression evidence (under stress)	References
Drug:H+ Antiporters			
ATR1	+	Arsenic, Arsenite, Hydrogen peroxide, Nitric oxide, Selenite	(Coleman et al. 1997; Harbison et al. 2004; Haugen et al. 2004; Horan et al. 2006; Kelley and Ideker 2009; Lucau-Danila et al. 2005; Salin et al. 2008; Thorsen et al. 2007; Workman et al. 2006)
AZR1	+	Arsenic	(Haugen et al. 2004; Salin et al. 2008)
DTR1	+	-	(Salin et al. 2008)
FLR1	+	Arsenic, Arsenite, Benomyl, Diamide, Diazaborine, Diethylmaleate, Hydrogen peroxide, Hydroxyurea, Methylmethane sulfonate, Tert-butyl hydroperoxide, Selenite	(Alarco et al. 1997; Brôco et al. 1999; Dubacq et al. 2006; Haugen et al. 2004; Jungwirth et al. 2000; Kelley and Ideker 2009; Lucau-Danila et al. 2005; Nguyen et al. 2001; Salin et al. 2008; Teixeira et al. 2010; Teixeira et al. 2008; Tenreiro et al. 2001; Thorsen et al. 2007; Workman et al. 2006)
QDR3	-	Spermine, Spermidine	(Teixeira et al. 2011a)
TPO1	-	Benomyl	(Lucau-Danila et al. 2005)
TPO2	-	Arsenite	(Thorsen et al. 2007)
TPO4	-	Arsenic	(Haugen et al. 2004)
YHK8	+	Nitric oxide	(Harbison et al. 2004; Horan et al. 2006; Lee et al. 2002)
Pleiotropic Drug Resistance ABC transporters			
PDR5	+	Arsenite, Benomyl, Hydrogen peroxide	(Kelley and Ideker 2009; Lucau-Danila et al. 2005; Salin et al. 2008; Thorsen et al. 2007)
PDR18	+	2,4-D	(Cabrito et al. 2011; Salin et al. 2008)
SNQ2	+	Arsenite, Benomyl	(Harbison et al. 2004; Lee et al. 2002; Lucau-Danila et al. 2005; Salin et al. 2008; Thorsen et al. 2007; Workman et al. 2006)
YCF1	+	Arsenic, Cadmium, Diazaborine	(Haugen et al. 2004; Jungwirth et al. 2000; Lucau-Danila et al. 2005; Salin et al. 2008; Wemmie et al. 1994)

Table 1. *S. cerevisiae* multidrug resistance transporter encoding genes under the control of Yap1, according to the YEASTRACT database (www.yeastract.com). Whether there is evidence (+) or not (-) for Yap1 binding to the promoter regions of the selected genes is indicated. The stress conditions leading to target gene up-regulation under Yap1 control are also highlighted. Supporting references are provided.

reinforces the possibility that the different mechanisms of Yap1 activation lead to diverse conformational changes which do not deeply affect Yap1 binding ability, but rather its action as a transcriptional activator, allowing this transcription factor to discriminate, among its target genes, those that should be up-regulated in each condition.

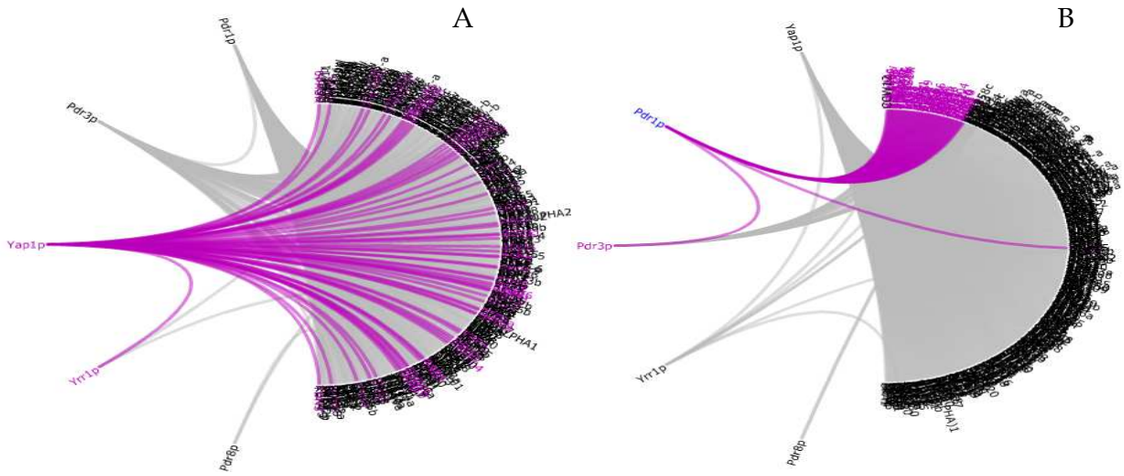

Fig. 2. Global overlapping between the Yap1 and the PDR regulatory networks. On the left, the participation of the Yap1 transcription factor in the regulation of around 18% of the Pdr1 targets is highlighted, while the right panel emphasizes the role of Pdr1 in the regulation of about 15% of the Yap1 regulon. Both networks were built based on the information gathered in the YEASTRACT database (www.yeastract.com), considering only DNA-binding evidence for the establishment of transcriptional association between transcription factors and target genes.

4. The role of the pdr network in oxidative stress response

Since there is a clear role of Yap1 in MDR, the hypothesis that the PDR network may also play a role in oxidative stress seems logical. Although, to date there is no evidence supporting that the PDR network plays a clear role in the response to oxidative stress induced by ROS, several studies have highlighted the role of this multidrug resistance network in the response to pro-oxidant drugs and xenobiotics (Lelandais and Devaux). These include the agricultural fungicides mancozeb (Teixeira et al. 2010; Teixeira et al. 2008) and benomyl (Lucau-Danila et al. 2005), the herbicide 2,4-dichlorophenoxyacetic acid (Teixeira et al. 2007), the redox-cycling agent menadione and selenite (Salin et al. 2008). Indeed, in yeast cells exposed to pro-oxidants and metalloids, a cooperation between the transcription factors Pdr1/Pdr3 and Yap1, Rpn4 and Hsf1 in the modulation of oxidative stress response appears to exist.

In the particular case of the seletine stress response, microarray analysis was used to check the transcriptome-wide effect of the deletion of the transcription factor encoding genes *YAP1*, *RPN4*, *PDR1* and *PDR3*. It was found that the absence of Pdr1 or Pdr3 affected the expression of around 20% of the Yap1 targets genes induced under selenite stress. These shared genes were found to include chemical stress response genes such as *FLR1*, as expected, but also a sub-group of oxidative stress responsive genes. When taking a global

look at the information gathered in the YEASTRACT database (Abdulrehman et al. 2011; Monteiro et al. 2008; Teixeira et al. 2006), it becomes clear that around 15% of the Yap1 target genes are also Pdr1 targets (Fig. 2B). Among these shared targets a small, but significant set of genes encoding direct antioxidant enzymes have been shown to be direct targets of the PDR transcription factors, including the cytosolic catalase Ctt1 (Devaux et al. 2002; Hikkel et al. 2003), and the alkyl hydroperoxide reductase Ahp1 (Larochelle et al. 2006) (Fig. 3).

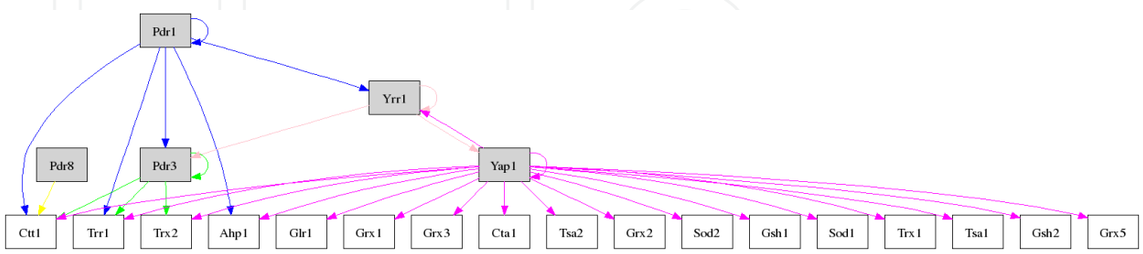

Fig. 3. The core oxidative stress response regulatory control, focused on the transcription factor Yap1 and on the yeast genes encoding antioxidant enzymes or proteins required for the maintenance of cellular redox balance. The role of the PDR network transcription factors in the regulation of these genes is displayed, based on the information gathered in the YEASTRACT database (www.yeastract.com).

In this context, it is also important to point out the specific role of Pdr3, but not of Pdr1, in the response to mitochondrial dysfunction (Devaux et al. 2002; Hallstrom and Moye-Rowley 2000), one of the main natural sources of oxidative imbalance. Indeed, upon the deletion of the mitochondrial genome, Pdr3 was seen to play a role in the activation of 14, out of 54, genes whose expression changes in these circumstances, placing Pdr3 as one of the transcription factors responsible for the retrograde response pathway (Devaux et al. 2002). Differently from what happens under chemical stress, upon mitochondrial dysfunction Pdr3 was seen to be post-translationally modified, but the exact nature of this modification was not clarified to date. The proposed role of this transcription factor in the response to mitochondrial dysfunction, as a controller of plasma membrane properties, still remains to be elucidated.

5. Cross-talk between multidrug resistance control and oxidative stress response in pathogenic yeasts

The study of multidrug resistance in pathogenic yeast species has been guided, to some extent, by the knowledge gathered for *S. cerevisiae*, as a model organism. A particularly close degree of similarity can be found when comparing this model organism with *Candida glabrata*, while the observations made for *C. albicans* and other *Candida* species reveal a lower conservation in terms of MDR regulation.

Clinical multiple antifungal drug resistance in *C. albicans* is mostly found to be based on the over-expression of the ABC multidrug efflux pumps encoded by *CDR1* (Prasad et al. 1995) and *CDR2* (Sanglard et al. 1997) genes, which share a high degree of homology with *S. cerevisiae PDR5*, and of the MFS drug:H^+ antiporter *MDR1* (Goldway et al. 1995), a close homologue to *S. cerevisiae FLR1* gene. *FLU1*, another *C. albicans* drug:H^+ antiporter encoding gene, was also found to confer fluconazole resistance, but to a lesser extent (Calabrese et al.

2000). Interestingly, the regulation of these multidrug transporters shares some similarity to that of their homologues in budding yeast. The *C. albicans* transcription factor Tac1, belonging to the *S. cerevisiae* Pdr1/Pdr3 protein family, was found to be required for *CDR1* and *CDR2* up-regulation induced by the drug fluphenazine (Coste et al. 2004). Another Pdr1/Pdr3 homologous transcription factor, Mrr1, was found to control the expression of *MDR1*, in both fluconazole-resistant clinical isolates and in benomyl-or hydrogen peroxide-challenged cells (Morschhauser et al. 2007). Of particular interest, in the context of this review, is the fact that the Yap1 homologue from *C. albicans*, named Cap1, is also involved in multidrug resistance (Alarco et al. 1997; Alarco and Raymond 1999), controlling *MDR1* expression, directly binding to its promoter region (Znaidi et al. 2009).

In *C. glabrata*, multidrug resistance relies mostly on the ABC drug efflux pumps *CgCDR1* (a *ScPDR5* homologue), *CgPDH1/CgCDR2* (a *ScPDR15* homologue) and *CgSNQ2* (a *ScSNQ2* homologue), but also on the drug-H+ antiporter *CgFLR1* (Chen et al. 2007). In this pathogenic yeast a single homologue of the budding yeast transcription factors Pdr1/Pdr3, CgPdr1, appears to control antifungal drug resistance through its action as an activator of all of the above mentioned ABC transporter encoding genes. This role is not only seen in the response of laboratory strains to suddenly imposed stress, but also in azole-resistant clinical isolates (Torelli et al. 2008; Tsai et al. 2006). The role of other *C. glabrata* CgPdr1 homologues, such as CgYrm1, has not been inspected so far. The *C. glabrata* Yap1 homologue, Cgap1, has also been related to the control of multidrug resistance transporters. Specifically, it was found to be required for *CgFLR1* up-regulation in response to benomyl-induced stress (Chen et al. 2007). Although this transcription factor was not seen to confer antifungal drug resistance its expression does increase *C. glabrata* tolerance to toxic concentrations of various oxidants and other xenobiotics (Chen et al. 2007). It is expectable that the understanding of the complex transcriptional regulation of MDR in this less well-studied organism will increase in the near future, guided by the huge amount of information that is being provided through genome-wide approaches. For example, microarray analysis revealed that ORF *CAGL0G08624g*, encoding a close homolog to the *S. cerevisiae* MFS-MDR transporter Qdr2 [T4, (Vargas et al. 2004)], is transcriptionally activated in response to fluconazole induced stress, in the dependency of the CgPdr1 transcription factor (Vermitsky and Edlind 2004). A more recent transcriptomics study showed that the expression of *CgFLR1* and ORF *CAGLOG03927g* (a *ScTPO1* homologue) genes is up-regulated in cells challenged with benomyl, under the control of the Cgap1 transcription factor (Lelandais et al. 2008). Although this subject has only now began to be unraveled in *C. glabrata*, current results already allow us to build a relatively small PDR network (Caudle et al.; Ferrari et al.; Lelandais et al. 2008; Tsai et al. 2006), including Cgap1, as depicted in Fig. 4.

Altogether, these results reinforce the notion that the transcriptional control of multidrug resistance and oxidative stress response are highly interconnected processes in yeasts and suggest that this crosstalk may be extended to other more complex eurakyotes.

6. The combinatorial regulation of the multidrug transporter Flr1: A systems biology case-study

The *FLR1* gene, encoding a plasma membrane drug:H+ antiporter, was one of the first of its family to be characterized. Although it derives its name from FLuconazol Resistance (Alarco et al. 1997), Flr1 has been shown to confer resistance to a large number of chemically and

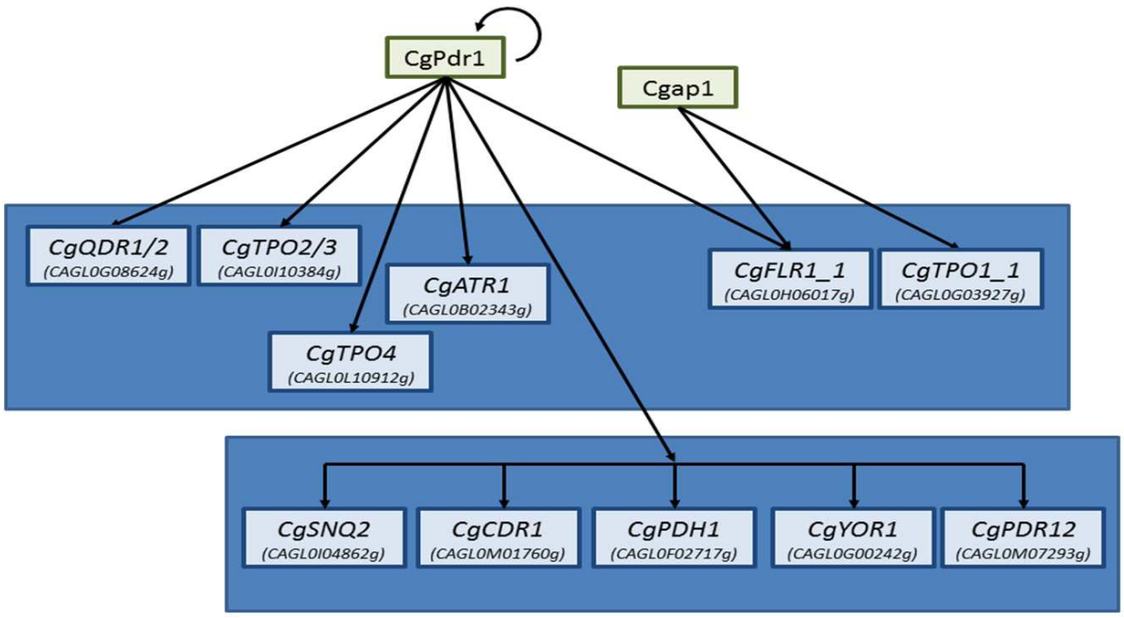

Fig. 4. The PDR network in the human pathogen *Candida glabrata*, based on the results of the few global studies carried so far on the subject (Caudle et al.; Ferrari et al.; Lelandais et al. 2008; Tsai et al. 2006). The role of the transcription factors CgPdr1 and Cgap1 in the regulation of the genes encoding predicted drug:H+ antiporters of the MFS (upper box) or multidrug resistance ABC efflux pumps (lower box) is highlighted.

structurally unrelated xenobiotics and drugs, including cycloheximide, 4-nitroquinoline-1-oxide (4-NQO), benomyl, methotrexate, diazaborine, cerulenin, diamide, diethylmaleate, menadione, paracetamol and mancozeb (Alarco et al. 1997; Brôco et al. 1999; Jungwirth et al. 2000; Srikanth et al. 2005; Teixeira et al. 2008). Unlike many of the DHA encoding genes (Sá-Correia et al. 2009), *FLR1* is highly induced at the transcriptional level when yeast cells are exposed to the stresses this gene confers resistance to. This high responsiveness to stress, made *FLR1* transcriptional control an attractive working model to study complex transcriptional regulation mechanisms. The first indication on *FLR1* transcriptional control, came very early on, when *FLR1* was identified as a Yap1 target in *S. cerevisiae* (Alarco et al. 1997). Upon Yap1 deletion, the up-regulation of *FLR1*, found to occur in yeast cells exposed to the fungicide benomyl, was seen to be completely abrogated (Brôco et al. 1999; Tenreiro et al. 2001). At the same time, maximal activation of *FLR1* under benomyl stress was found to be dependent on the presence of an additional transcription factor, Pdr3 (Brôco et al. 1999; Tenreiro et al. 2001). Interestingly, *FLR1* promoter region was found to include three putative Yap1-Responsive Elements (YRE1-3) (Fig. 6). In a detailed study of the role of each of these predicted binding sites, Nguyen and co-workers found that the three binding sites were functional, but that their relative importance depends on the imposed stress (Nguyen et al. 2001). Indeed, using site-directed mutagenesis to remove each of the Yap1-binding sites, YRE3 (-364) was found to be the major player in *FLR1* activation under stress imposed by benomyl and diethylmaleate. However, YRE2 (-167) becomes the most significant YRE in *FLR1* up-regulation induced by hydrogen peroxide, diamide and *tert*-butyl hydroperoxide. Finally, all three YREs are equally important to assure full activation of *FLR1* in response to

methylmethane sulfonate (Nguyen et al. 2001). This finding may relate to the fact that at least some of these stresses lead to different Yap1 conformations, which in term may change the transcription factor's affinity towards the possible variations of the Yap1-Responsive Element. Furthermore, the fact that YRE3 is responsible for 90% of the *FLR1* up-regulation induced by benomyl, may relate to the fact this Yap1-binding site is in the proximity of the predicted Pdr1/Pdr3-Responsive Element. The possibility that the binding of Pdr3 to the *FLR1* promoter facilitates Yap1 activity in the nearby YRE3 was then proposed by Tenreiro et al (Tenreiro et al. 2001).

Additional clues to unveil the complete *FLR1* regulatory network came from functional genomics approaches. Using all available information gathered in the YEASTRACT database, based on either microarray or ChIP-on-chip analysis, it is now possible to predict that a very complex regulatory network including 15 transcription factors is responsible for *FLR1* regulation (Fig. 5). In an attempt to understand whether this network could be working together to control *FLR1* expression in a single stress condition, an *FLR1* promoter-lacZ fusion was used to study *FLR1* expression under stress induced by the fungicide mancozeb, in the presence or absence of each of the transcription factors found to occur in the predictive network. *FLR1* activation in yeast cells exposed to mancozeb was found to depend upon Yap1 and Pdr3, as previously registered under benomyl stress. However, two additional transcription factors were found to be required for mancozeb-induced *FLR1* maximal activation: Yrr1, one of the transcription factors controlling the PDR network, and Rpn4, a proteasomal gene regulator (Teixeira et al. 2008).

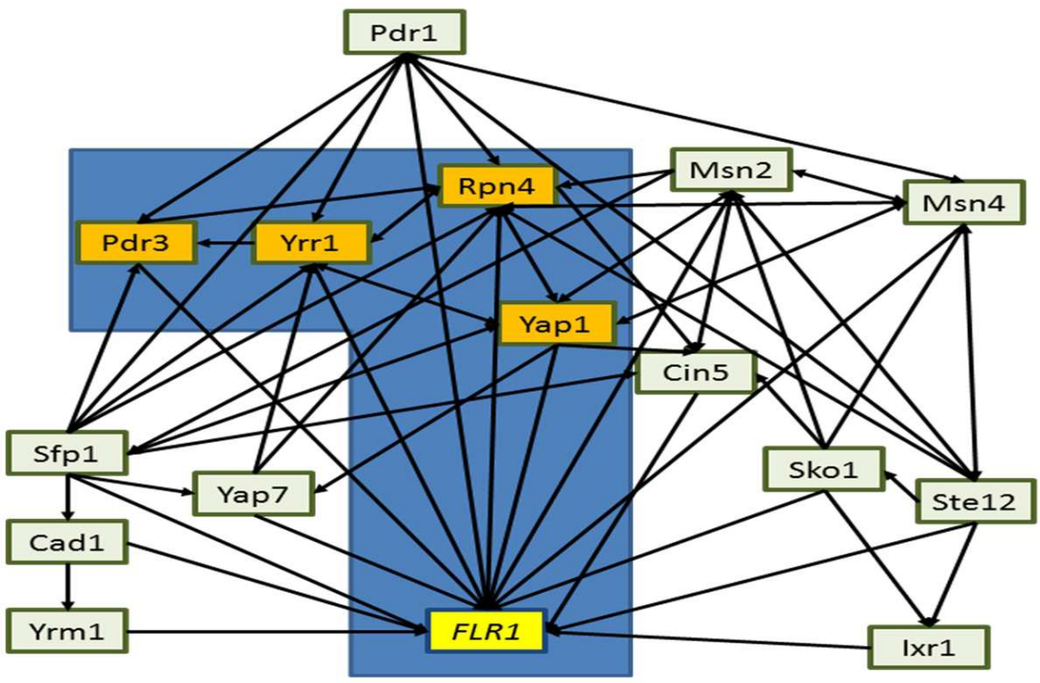

Fig. 5. Network of transcription factors documented as affecting *FLR1* expression, as retrieved from the YEASTRACT database (www.yeastract.com). The L-shaped box indicates the sub-network found to affect *FLR1* up-regulation occurring in yeast cells challenged with the fungicide mancozeb (Teixeira et al. 2008).

Given the fact that it is becoming increasingly clear that transcriptional regulation is far more complex that initially foreseen, the use of systems biology approaches seems to be the only way to pursue the goal of understanding such biological processes in all their depth. Indeed, Systems Biology aims at understanding living processes as systems of multiple interacting components, preferably at a genome-wide scale, and through the combination of experimental and computational approaches. Such an approach was undertaken to further analyze the *FLR1* regulatory network. The profiles of *FLR1*, *YAP1*, *PDR3*, *YRR1* and *RPN4* transcript levels were registered during the period of adaptation of a yeast cell population to stress imposed by the fungicide mancozeb, in wild-type cells and in mutants devoid of each of the four transcription factors (Teixeira et al. 2008). This information was used to build a mathematical description of the *FLR1* network (Teixeira et al. 2010), taking advantage of the freely available GNA software (de Jong et al. 2003). This modeling approach allowed the testing of new hypothesis in silico (Monteiro et al. 2011), providing guidance for the design of further experimental work (Teixeira et al. 2010). The comparison between simulated and experimentally obtained results led to a refined understanding of the network, including the realization that a fifth still unidentified transcription factor, denominated FactorX, has to be included in the network to fully explain the observed transcriptional profiles (Fig. 6). Furthermore, combined results suggested that Yap1 and Yrr1 may function together, eventually working as a heterodimer, in the co-regulation of their shared target genes, which include the multidrug transporter encoding genes *FLR1*, *AZR1* and *SNQ2* and the transcription factor encoding genes *PDR3*, *YRR1* and *RPN4* (Teixeira et al. 2010).

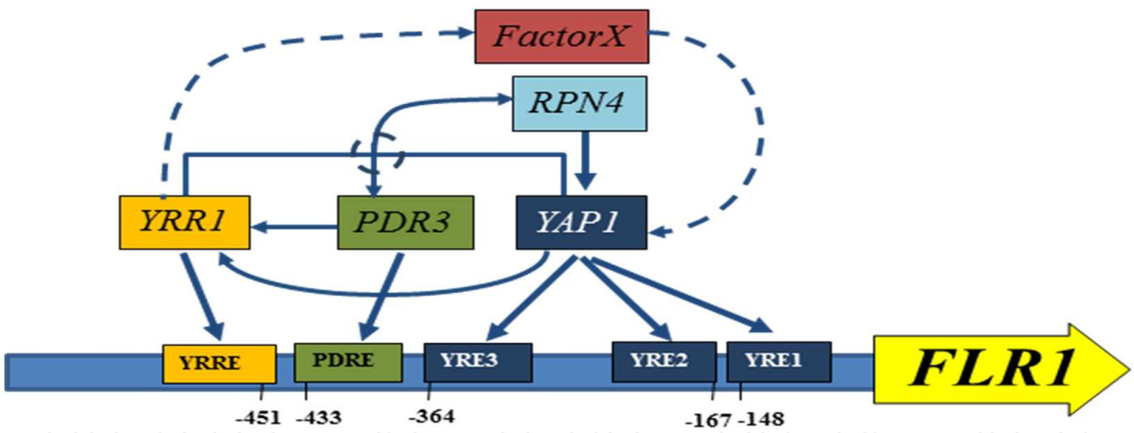

Fig. 6. *FLR1* regulatory network structure, found to be functional in yeast cells exposed to mancozeb stress, as obtained from the combination of computational and experimental approaches described by Teixeira et al (Monteiro et al. 2011; Teixeira et al. 2010). Dashed arrows indicate the new aspects of the network suggested by the used systems biology approach that are still to be validated.

Studies in *C. glabrata* and *C. albicans* showed that the *ScFLR1* homologues *CgFLR1* and *CaMDR1* are regulated by Yap1p homologs in either species (Chen et al. 2007; Znaidi et al. 2009). Whether the *C. glabrata* homologs of the *S. cerevisiae* Yrr1 and Rpn4 transcription factors also play a role in *CgFLR1* regulation is still an open question. An interesting clue comes from the fact that the *C. albicans* homologue of ScPdr3, CaMrr1, is also required for full *CaMDR1* activation (Morschhauser et al. 2007). These results strongly suggest that there

may be a significant degree of conservation between multidrug resistance control in these related yeast species.

The current version of the *FLR1* regulatory network is small but intricate. We believe that it is a good example of how complex transcription control in eukaryotes may be. Furthermore, it provides a good platform for further studies on the connection between oxidative stress response and two additional biological processes: multidrug resistance and protein degradation through the proteasome.

7. Conclusion and perspectives

Multidrug resistance control and stress response, in particular oxidative stress response, are now recognized as two complex cellular processes that coexist and interplay to allow cells to thrive in harsh environments. This chapter highlights current knowledge on the cross-talk between oxidative stress and MDR regulation in the model eukaryote *S. cerevisiae*. It emphasizes that, although being more focused on their canonical roles, both Yap1 and the PDR regulators play important functions in the regulation of multidrug efflux pumps and antioxidant enzymes, respectively.

At the moment, the logical explanation for this cross-talk appears to lie on the fact that many drugs and other xenobiotics may exert pro-oxidant effects, thus activating both multidrug resistance and oxidative stress response pathways. A question that still remains to be answered relates to why multidrug transporters should be controlled by Yap1 and more specifically what might be their role, if any, in oxidative stress response. For the single case of the vacuolar membrane ABC transporter Ycf1, there seems to be a possible connection. Ycf1, a close homologue to the human MRP1 multidrug transporter, confers resistance to chemical stress, including metal ions, antimonite, arsenite, 1-chloro-2,4-dinitrobenzene and diazaborine. The role of Ycf1 in metal ion resistance was further explored and this transporter was found to mediate the vacuolar compartmentalization of S-glutathione conjugates (Li et al. 1996). Glutathione, whose concentration is controlled by Yap1, through the regulation of the glutathione synthetase encoding gene *GSH1*, plays a crucial role in the maintenance of the intracellular redox potential. It is thus reasonable to think that the oxidative stress response regulator Yap1 may coordinately control the expression of *GSH1* and *YCF1*, to assure the maintenance of the physiological concentration of free cytosolic reduced glutathione. As for the remaining multidrug transporters controlled by Yap1, such a close link between drug detoxification and oxidative stress response remains to be established. Nonetheless, it is reasonable to think that, since many of the natural chemical stress inducers are also capable of unbalancing the cellular redox state, the oxidative stress signaling would also control the expression of membrane transporters capable of relieving the cell from the exogenous source of oxidative stress. Interestingly, in bacterial systems a rather similar coordination of the response to xenobiotics and oxidants can also be found, under the control of the SoxRS regulon. Indeed, the *E. coli* SoxRS transcription factor controls the expression of both antioxidant enzymes and also of, at least, the outer membrane protein (porin) F, OmpF, suggested to play a role in reducing cell membrane permeability towards ROS or ROS-generating compounds (reviewed in (Lushchak 2011)).

Altogether, the results reviewed herein also highlight the fact that transcriptional control is much more complex than initially foreseen. Indeed, we come to realize that it is not possible

to study individual phenomena, as if there was no influence from the surrounding, and even interconnected, cellular processes. The compilation of all the regulatory associations identified, so far, in *S. cerevisiae*, deposited in the YEASTRACT database (Abdulrehman et al. 2011; Monteiro et al. 2008; Teixeira et al. 2006), reveals that there are more than 48,000 regulatory associations between transcription factors and target genes. This number, which indicates that, on average, each yeast gene is controlled by at least 8 different transcription factors, rises up to nearly 375,000, when making in silico predictions based on the occurrence of transcription factor recognition sequences in the yeast promoter regions. Given this high degree of complexity, the study of biological networks using the new interdisciplinary approaches of Systems Biology seems to be the most suitable way to tackle this issue. The small, but intricate case-study explored herein, focused on the *S. cerevisiae FLR1* regulatory network, suggests that the use of computer modeling, as a systems biology tool, will be crucial to increase our understanding of the cross-talk between regulatory networks.

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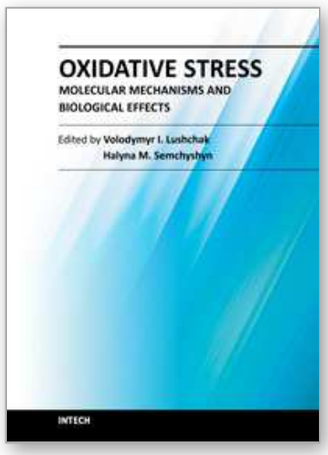

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Since the discovery of free radicals in biological systems researchers have been highly interested in their interaction with biological molecules. Denoted in 1980, and due to fruitful results and ideas, oxidative stress is now appreciated by both basic 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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