Characterization of a family of yeast transcriptional regulators: The zinc cluster proteins

by

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ABSTRACT

Members of the zinc cluster (or binuclear cluster) protein family are characterized by a Zn(II)Cys6 zinc finger involved in DNA recognition and binding. These fungal proteins are transcriptional regulators of genes involved in a wide variety of cellular processes. One member, Gal4p, is involved in galactose metabolism, while others play a major role in primary and secondary metabolism, control of meiosis, and multidrug resistance. Sequencing of the *Saccharomyces cerevisiae* genome has revealed that 55 genes encoding putative zinc cluster proteins are present in budding yeast. However, the roles of many of these zinc cluster proteins are unknown. In order to better understand their functions, we have performed a phenotypic analysis of these putative zinc cluster proteins. We have implicated a number of them in a variety of processes in the cell, including multidrug resistance. Stb5p has been shown to be a major player in regulating the expression of multidrug resistance genes. Other zinc cluster activators of multidrug resistance genes include Pdr1p, Pdr3p, and Yrr1p. These regulators of multidrug resistance appear to interact with each other, forming many different sub-populations of homo- and heterodimers. Stb5p is found predominantly as a heterodimer with Pdr1p. It also appears that Pdr1p is a master regulator able to interact with many different partners, enabling it to mediate control over multidrug resistance genes.

RÉSUMÉ

Les membres de la famille des protéines Gal4p sont caractérisés par un doigt de zinc de type Zn(II)Cys6 impliqué dans la reconnaissance et la fixation à l'ADN. Ces protéines fongiques sont des facteurs de régulation de la transcription de gènes impliqués dans une grande variété de processus cellulaires. Un membre, Gal4p, a été impliqué dans le métabolisme du galactose. D'autres membres jouent une rôle majeur dans le métabolisme primaire et secondaire, le contrôle de la méiose, et dans la résistance à de multiples drogues. Le séquençage du génome de *Saccharomyces cerevisiae* a révelé que 55 gènes codant pour des protéines de type Zn(II)Cys6 potentielles sont présents dans la levure. Cependant, le rôle de plusieurs de ces protéines n'est pas élucidé. Pour une meilleure compréhension de leurs fonctions, nous avons procédé à une analyse phénotypique de ces protéines. Nous avons impliqué un nombre d'entre elles dans une variété de processus dans les cellules, incluant la résistance à de multiples drogues. Nos résultats montrent que Stb5p agirait comme un régulateur majeur de l'expression des gènes de la résistance aux multidrogues. D'autres membres de la famille des protéines Gal4p sont des activateurs des gènes de la résistance aux multidrogues et incluent Pdr1p, Pdr3p, et Yrr1p. Ces régulateurs semblent interagir entre eux, formant plusieures sous-populations d'homo- et d'hétérodimeres. Stb5p forme de façon prédominante des hétérodimeres avec Pdr1p. Il apparait aussi que Pdr1p est un régulateur clé, capable d'interagir avec differents partenaires ce qui lui permet d'exercer un contrôle sur les gènes de la résistance aux multidrogues.

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This thesis is a culmination of many years of work. However, none of this work would have been possible without the support of many people. I would like to thank my family for their constant support. I would also like to express a debt of gratitude to the various members of the lab and department who have taught me a lot, especially my supervisor, Dr. Bernard Turcotte, who gave me the opportunity and support that I needed. I would also like to thank FRSQ for their generous support.

PREFACE

This thesis is assembled according to McGill guidelines (available at http://www.mcgill.ca/gps/programs/thesis/guidelines/preparation) and consists of:

1) An abstract in both English and French
2) Acknowledgements
3) Rationale and objectives
4) Literature Review
5) The experimental research
6) Discussion and conclusion
7) References
8) Appendix containing radioactive license

The thesis includes three original manuscripts that have been either published or submitted for publication. Section 2 has been published in Nucleic Acids Research, 2001, 29 (10): 2181-2190. Section 3 has been published in Journal of Biological Chemistry, 2002, 277 (24): 21254-21260. Section 4 has been submitted for publication to Molecular and Cellular Biology. Since this thesis contains original manuscripts, there is some repetition of textual material. In addition, I am second author on another published paper: Karen Hellauer, Bassel Akache, Sarah MacPherson, Edith Sirard, and Bernard Turcotte. Zinc Cluster Protein Rdr1p is a Transcriptional Repressor of the *PDR5*

Gene Encoding a Multidrug Transporter. The Journal of Biological Chemistry. 2002, 277 (20): 17671-17676.

Dr. Bernard Turcotte has supervised the entire work presented in this thesis and assisted in the writing of the manuscripts. K. Wu constructed some of the deletion strains used for the analysis described in section 2. I. Massey constructed the GST-Stb5p expression vector used in Sections 3 and 4. K. Hellauer performed Southern blot analysis and constructed some of the lacZ reporters used in Section 3. S. MacPherson assisted me with the native co-immunoprecipitation and GST pull-down assays described in Section 4. Dr. Bernard Turcotte performed the EMSA depicted in Section 4.

This thesis contains five sections:

Section 1: consists of a comprehensive literature review relevant to the work presented in the thesis.

Sections 2-4: consists of the experimental research.

Section 5: consists of a discussion and final conclusion of the work presented in this thesis.

Sections 2, 3,and 4 each contain their own reference section, which is found at the end of each corresponding section. The references for sections 1 and 5 are found together at the end of the thesis.

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RATIONALE AND OBJECTIVES

The sequencing of the genomes of many organisms provides a wealth of information. It allows for the separation of the genome into non-coding intergenic regions, and into transcription units, that putatively encode all of the organism's genes. Upon analysis of the sequences of these transcription units, they can be classified according to their protein code into various protein families that include proteins whose roles and functions have already been identified. The identification of functional motifs in these newly discovered putative genes may indicate what type of proteins they encode, i.e. whether they are membrane transporters, DNA-binding proteins, kinases, etc. However, the precise role of these putative proteins can not be determined without further experiments. For example, the *S. cerevisiae* sequencing project has allowed for the identification of many new members of the zinc cluster protein family due to the recognition of a characteristic Zn(II)2CYS6 binuclear cluster motif within their open reading frame (ORF) sequence (148). This motif has been shown to be involved in the binding of many transcriptional regulators, such as Gal4p, to DNA (96). Therefore, based solely on the ORFs' sequence, it can be hypothesized that these newly identified putative zinc cluster proteins are DNA-binding proteins that may be involved in transcriptional regulation. However, even if the newly identified zinc cluster proteins prove to be sequence-specific transcription factors, their role in the cell can only be determined after their target genes and their mechanism of action are identified. In order to better understand the roles of some of the uncharacterized zinc cluster proteins in the cell: 1) A systematic phenotypic analysis will be used to implicate them in certain

processes within the cell, 2) Target genes of specific zinc cluster proteins implicated in multi-drug resistance will be identified, and 3) The ability of various zinc cluster proteins shown to be involved in multi-drug resistance to form homo- and heterodimers will be tested.

SECTION 1: LITERATURE REVIEW

PROMOTERS

Transcription is critical for any cell to function, but it is a process that becomes increasingly complicated when organisms become more complex, usually coinciding with a larger number of genes. Since all cells in an organism contain the same genes, it is the regulation of transcription which will allow cells to function differently, and allows the organism to adapt and respond to a dynamic environment's various stimuli, and thereby survive. The transcription of a gene is dependant on: 1) the type of promoter preceding the gene, 2) the factors which act on that promoter, and 3) the structure of chromatin surrounding the gene.

One general definition of a gene promoter is: "the region of DNA containing all the transcription factor binding sites required to support transcription of that gene at the normal efficiency and with the proper control (88)." There are three types of promoters in eukaryotic cells. These promoters are differentiated according to which RNA polymerase, I, II, or III, will transcribe the gene. Large ribosomal RNAs (rRNAs) are transcribed by RNA polymerase I. Messenger RNAs (mRNAs) and some small nuclear RNAs (snRNAs) are transcribed by RNA polymerase II, while 5S rRNA, tRNAs, and other snRNAs are transcribed by RNA polymerase III. The three RNA polymerases are localized differently within the nucleus reflecting their different roles within the cell. RNA polymerase I is found in the nucleolus, where the ribosomal DNA (rDNA) genes

are found, and RNA polymerase II and III are found in the nucleoplasm. In order for the promoter to initiate transcription by recruiting the appropriate general transcription factors and RNA polymerase, each type of promoter must contain DNA elements specific for each RNA polymerase and its associated transcription factors (88).

RNA POLYMERASE I

RNA polymerase I acts at the ribosomal DNA promoters, which are the least variable promoters in the eukaryotic nucleus. In human cells, two regions within the promoters are critical for proper transcription: 1) the core promoter (-45 to +20 nucleotides relative to the transcription start site (all numbers are relative to the transcription start site)), and 2) the upstream control element (UCE) (-180 to -107). The core promoter is sufficient for the initiation of transcription, but the UCE drastically increases its efficiency. Both regions are 85% identical and rich in Guanidine (G) and Cytosine (C). For transcription to occur, a stepwise binding of transcription factors to the rDNA promoter is needed. First, UBF1 binds to both the core promoter and UCE. This will then allow the subsequent binding of the factor, SL1. Finally, RNA polymerase I is able to bind the core promoter, and initiate transcription. SL1 is composed of four proteins, which include TATA-binding protein (TBP), a protein that is also involved in the activity of RNA polymerase II and III. In this case, TBP does not bind DNA, but it is still required for the proper functioning of RNA polymerase I at the transcription start site. TBP associates with all three RNA polymerases; however, it acts differentially by associating with different proteins specific to each type of promoter (88).

RNA POLYMERASE III

The promoters targeted by RNA polymerase III fall within two classes, each recognized by a different group of transcription factors. The first class includes the promoters for tRNAs and 5S rRNA. Their promoters lie downstream of the transcription start site. These promoters are bipartite, with two short DNA elements separated by a variable number of nucleotides. At the 5S rRNA promoter, two factors, TFIIIA and TFIIIC, bind these elements, and subsequently allow TFIIIB to bind the DNA around the start site. TFIIIA is not required for the transcription of tRNA genes, but TFIIIC does bind to tRNA promoters, allowing for the recruitment of TFIIIB. Once TFIIIB, which consists of TBP and two other proteins, binds DNA, the presence of TFIIIA and TFIIIC is no longer needed to initiate transcription. Only DNA-bound TFIIIB is needed to recruit RNA polymerase III and allow it to bind to DNA. Again, the TBP-containing factor is responsible for the interaction of the RNA polymerase complex with DNA and its proper positioning. The second class of RNA polymerase III promoters control the transcription of snRNAs, and they are found upstream of the start site. Three elements in the promoter are involved in the activation of transcription: 1) TATA element, 2) Proximal sequence element (PSE), and 3) OCT element. These elements are found in the promoters of snRNA genes transcribed by RNA polymerase II as well, and the TATA element is thought to determine which RNA polymerase will be recruited. The TATA element, a region rich in adenosine (A) and thymine (T) residues, is recognized and bound by TBP, which is associated with different factors depending on whether it is a

RNA polymerase II or III promoter. TBP and these associated factors form a pre-initiation complex and recruit the RNA polymerase to initiate transcription. In this case, TBP binds DNA directly, as opposed to the TATA-less promoters, where other factors bind the DNA first and then recruit TBP through protein-protein interactions (88).

RNA POLYMERASE II

RNA polymerase II is responsible for the transcription of most genes in the cell into heterogeneous nuclear (hnRNA). This RNA will be processed into mRNA, which will then be translated into all the proteins that make up the cell and allow it to function properly. This one RNA polymerase controls the expression of a large number of different genes with varying functions, which are therefore transcribed at significantly different levels. Hence, many different transcription factors of many different types are involved in the control of transcription by RNA polymerase II. These transcription factors are classified as either 1) general or basal transcription factors or 2) sequence-specific transcription factors. The general transcription factors act along with RNA polymerase II to form the basal transcription apparatus, which will initiate transcription at these promoters. Sequence-specific factors mostly activate transcription, but some act as repressors. Their role is to act on specific genes and regulate the activity of the basal transcription apparatus on that gene. This allows some genes to be transcribed at low levels, others at high levels, and some to be transcribed ubiquitously. Others are inducible due to factors such as ligand availability, cell cycle, tissue specificity, etc.

BASAL TRANSCRIPTIONAL APPARATUS

The basal transcription apparatus acts on two elements in the promoter found within the first thirty nucleotides upstream of the transcription start site. Firstly, the region encompassing the start site is called the initiator. The first base of mRNA is usually an A, and it is usually surrounded by pyrimidines, leading to an initiator sequence of Py_2CAPy_5, which is -3 to +5 relative to the start site. The second element, which is also found in some RNA polymerase III promoters, is the TATA box which consists of 8 basepairs (bp) of A or T residues, usually surrounded by GC rich sequences. It is usually found 25 bp upstream of the start site. TATA-less promoters constitute a very small percentage of RNA polymerase II promoters. The TATA box is recognized and bound by TBP in the initial step of the initiation of transcription, allowing the proper positioning of the RNA polymerase. TBP is one of the few DNA-binding proteins to bind DNA in the minor groove, and is the only general transcription factor to make sequence specific contact with DNA (88). TBP binds as part of the TFIID complex that includes proteins called TBP-associated factors (TAFs). The binding of TFIID is thought to be the rate-limiting step of transcriptional initiation, with the binding of TBP causing a drastic bend of DNA, allowing the other general transcription factors to be recruited to the promoter. These complexes are not homogenous, with different subpopulations of TFIID present in the cell (16, 129). After the binding of TFIID, TFIIB, whose recruitment is critical for transcriptional initiation, stabilizes the TBP-TATA complex through contacts with TBP and DNA upstream and downstream of the TATA box, which has been made available by TBP's binding of DNA (111, 152). TFIIA is also recruited to the transcription start

site, stabilizing the complex by interacting with TBP and DNA upstream of the TATA element (43, 147).

The binding of these factors allows the recruitment of TFIIE, TFIIF, TFIIH and RNA polymerase II. However, there are two models for the binding of these factors: 1) The stepwise model, where there is an ordered recruitment of these factors, or 2) The holoenyzme model, where a large complex composed of these factors and RNA polymerase II, are recruited in one step to form the pre-initiation complex. The discovery of a hypophosphorylated form of RNA polymerase II along with a variety of factors in holoenyzme complexes that usually include TFIIE, TFIIF, and TFIIH not bound to DNA, has challenged the idea of an ordered recruitment of these factors (93, 116). In either case, these factors are critical for efficient transcription to occur. TFIIE and TFIIH are required for promoter melting. TFIIH contains two DNA helicases, XPB and XPD (22, 138), that use ATP to unwind DNA at the promoter and allow RNA polymerase II to leave the promoter to begin mRNA elongation (39). TFIIH also contains a cyclin H/CDK7 kinase pair that phosphorylates the carboxy-terminal domain of the largest subunit of RNA polymerase II. This probably disrupts the interaction of the RNA polymerase II with the components of the pre-initiation complex, allowing it to begin the elongation step of mRNA transcription (59). The phosphorylated polymerase and TFIIF leave the promoter and begin elongation, TFIIA and TFIID remain bound to the promoter, while TFIIE and TFIIH dissociate from the promoter (169). However, a recent study has shown that in yeast, TFIIE, TFIIH, and other components of the pre-initiation complex remain bound to the promoter, indicating that subsequent rounds of initiation at

the promoter would only require the re-incorporation of TFIIB, TFIIF, and RNA polymerase II (168).

At TATA-less promoters, all these same factors are needed, including TFIID and TBP (88). However, mutations in the DNA binding domain of TBP, which inhibit transcription at TATA-containing promoters, do not affect basal or activated transcription at TATA-less promoters (100, 101). Therefore, contacts between TAFs and the DNA at TATA-less promoters may be sufficient to recruit TFIID to the initiator sequence. In the formation of the basal transcription apparatus, both the protein-protein interactions and the recognition of the promoter elements by the proteins are critical for RNA polymerase II to be correctly positioned and activated to initiate transcription.

Transcription is a complicated process, with a complete pre-initiation complex containing at least 43 distinct proteins having a total mass of 2.2 MegaDaltons (MDa). In addition to these proteins, RNA polymerase II has been found to associate with components of a multi-subunit cofactor complex termed mediator. This complex, which is composed of 20 different proteins, can associate with the unphosphorylated carboxy-terminal domain of RNA polymerase II and support transcription *in vitro* in the absence of TAFs (93). Tissue-specific TAFs, homologues of TBP and TFIIA, and cofactors that can modulate the activity of the complex in a core promoter-selective manner have been identified (99), further adding to the complexity of the model of transcription. Therefore, it is not surprising that this is only a very general model of the formation of the pre-

initiation complex, and that many other factors may be involved, which might allow the complex to form differently on some promoters, and in certain cell types or tissues.

CO-ACTIVATORS AND CO-REPRESSORS

The ability of the basal transcription apparatus to bind to the promoter, and initiate transcription efficiently, is regulated by sequence-specific transcription factors. The basal transcription apparatus is ubiquitously expressed and is ready to initiate transcription, but it can not activate gene expression until it is recruited to the initiator and until the structure of the chromatin is modified around the gene so as to not impede DNA-protein interactions. Sequence-specific transcription factors regulate the activity of RNA polymerase II and therefore the expression of a specific gene by binding to a target promoter that contains its recognition sequence and recruiting other factors. These factors are classified as either: 1) Co-activators and co-repressors, and 2) Chromatin-modifying enzymes.

Co-activators and co-repressors are thought to act as bridging molecules, connecting promoter-bound sequence-specific transcription factors with other factors that can affect gene expression such as components of the basal transcriptional machinery, or chromatin-modifying enzymes. The original indication that co-activators were present in the cell was the discovery of a phenomenon termed "squelching", or transcriptional interference. This phenomenon was characterized by the observation that in a transient transfection assay using different nuclear receptors, the addition of one nuclear receptor

repressed trans-activation of a given promoter regulated by another given receptor. This was later found to be due to competition between the receptors for the same co-activators. It also has been shown that in some cases, the co-activator transactivation domains retain their activity when they are fused to the DNA-binding domain of Gal4p (7). This indicates that the role of the sequence-specific transcription factor is to bind and recruit the co-activator, bringing it to the promoter where it can activate transcription.

Many families of co-activators exist, and different co-activators help different transcription factors via various mechanisms. Both sequence-specific transcription factors and co-activators contain specific domains through which they interact, and this interaction can be regulated. For instance, nuclear receptors contain a C-terminal AF-2 trans-activation domain whose activity is dependant on the presence of its ligand. This domain contains a conserved consensus motif that allows the receptor to interact with co-activators. This motif is conserved among most members of the nuclear receptor family, and mutations in this motif do not affect ligand binding, but do generate dominant-negative mutants that are transcriptionally silent (38). However, differences in this motif determine which specific co-activator will be recruited by a given nuclear receptor. Upon binding of the ligand to the receptor, the receptor undergoes a conformational change, leading to the exposure of a hydrophilic surface, thus enabling its interaction with the co-activator (7). Therefore, the ligand is able to activate transcription by binding a promoter-bound receptor, permitting a crucial step in the activation of the target genes to occur: the recruitment of the co-activator to the receptor.

There are several families of co-activators that can differ in their mechanism of activation. Some, such as the mammalian p160 family, have intrinsic histone acetyltransferase activity (HAT) domains. This activity allows the proteins to modify chromatin and activate transcription (25, 142). Other co-activators, such as PPAR-γ co-activator-1 (PGC-1), activate transcription once bound to the nuclear receptor PPAR-γ, by recruiting other co-activators or the co-integrator CBP/p300 (123). An RNA co-activator has been recently identified, and it has been suggested that it might act as a scaffold and recruit the p160 co-activator SRC1 (83). Other co-activators have been shown to activate transcription through their involvement in protein degradation pathways, RNA stability, or nuclear transport (7).

Co-integrators are co-activators that can also integrate extra-cellular and intra-cellular signaling pathways. For example, CREB-binding protein (CBP) and p300 are two related proteins that bind to transcription factors. CBP associates with CREB in response to cAMP-mediated phosphorylation (80). This phosphorylation could be mediated by an extra-cellular signal. CBP/p300 also interacts with many other transcription factors inside the cell including STATs, NF-κB, Jun, and Fos, aiding them in activating transcription (140). CBP/p300 has also been shown to interact with members of the basal transcriptional apparatus such as TBP, and TFIIB. Therefore, once it binds a sequence-specific transcription factor on the promoter, it can help recruit members of the pre-initiation complex in order to initiate transcription. PCAF, a HAT protein, has also been shown to associate with CBP/p300. PCAF also interacts with other co-activators that belong to the p160 family (63, 151), allowing CBP/p300 to either

interact either directly with sequence-specific transcription factors or indirectly, by forming a ternary complex through the p160 proteins. CBP/p300 also contains a domain with intrinsic HAT activity, which is crucial for its ability to initiate transcription (13, 115). Therefore, CBP/p300 interacts with different types of proteins, all shown to be involved in transcriptional activation, indicating that 1) it may act by bringing all these proteins together at an active promoter and 2) many different mechanisms may be responsible for its activity. However, since limited amounts of CBP/p300 are competed for by many different transcription factors, the activation of one transcription factor can have an antagonistic effect on the activation by another factor, such as with nuclear receptors and CREB (63, 133).

Only a small number of transcription factors are repressors, but they are important for a cell to function properly. They have different mechanisms of action, which include: 1) Competition with activators for DNA-binding sites, 2) Binding activators to form an inactive heterodimer, and 3) Binding to a promoter leading to the recruitment of co-repressors. As with co-activators, co-repressors bridge sequence-specific transcription factors to other proteins involved in transcription, but in the case of co-repressors these proteins will mediate gene repression. For example, some nuclear receptors repress transcription in the absence of their ligand. However, binding of the ligand to the receptor transforms it into an activator of transcription. Two co-repressors, nuclear co-repressor (NCoR) and silencing mediator for retinoic and thyroid hormone receptors (SMRT), have been shown to interact with the unliganded thyroid (TR) and retinoic acid receptors (RAR). However, upon addition of the ligand, this interaction is inhibited. The

addition of antibodies against NCoR or SMRT results in the alleviation of gene repression (134). Therefore, it appears that these co-repressors interact directly with these receptors to inhibit gene expression. NCoR, SMRT, and other co-repressors inhibit transcription by interacting with chromatin-modifying complexes, such as Sin3-HDAC (histone deacetylase), recruiting them to the sequence-specific transcription factor (55, 105).

CHROMATIN-MODIFYING ENZYMES

Core nucleosomes, which consist of two turns of DNA wrapped around two subunits each of histones H2A, H2B, H3 and H4, impede the binding of proteins to the DNA incorporated in the nucleosome. Therefore, genes found within a nucleosome would have their transcription restricted, since the basal transcriptional apparatus would not be able to bind the promoter. Chromatin-modifying enzymes are recruited to genes as either co-activators or co-repressors in order to either increase or decrease protein-DNA binding by altering the chromatin structure around the gene. These enzymes can act by modifying the histones post-translationally to either stabilize or destabilize chromatin. They can also disrupt the nucleosomes by using ATP to remodel the chromatin.

Covalent modifications of histones have an important role in gene regulation. Modifications, such as acetylation, phosphorylation, methylation, and ubiquitination, are involved in gene-specific regulation. Therefore, one histone could have multiple

covalent modifications, and these modifications seem to occur in patterns and on specific residues. This led to the model that there is a 'histone code', which integrates all the modifications into one biological outcome: either an 'off' or 'on' state for gene transcription. There are two non-exclusive models that explain how histone modifications affect gene expression. The first is that the chromatin structure is affected directly by the histone modification. The second model states that modified histones can be recognized and bound by other proteins (17).

Of all the histone modifications, the process of histone acetylation is the best understood. Hyper-acetylated histones are linked to transcriptional activity, while hypo-acetylated histones are linked to transcriptional repression. The histone NH_2 tails contain positively charged lysine residues, which have high affinity for the negatively charged DNA, creating a compact structure that is inaccessible to most transcription factors. HATs acetylate these lysine residues, rendering them neutral and decreasing their affinity for DNA. This leads to nucleosome unfolding and a less compact chromatin structure which is more accessible to proteins. Nucleosomes also contact each other through their histone tails to form higher order chromatin structures, so it is possible that the acetylation can disrupt these structures as well. It is also probable that the newly modified tails can be bound by transcription factors that specifically recognize acetylated histones (7). In fact, acetylated histones are specifically recognized by bromo-domains, which are found in many co-activators (74). HATs are usually found as large multi-subunit complexes, such as mammalian PCAF, and its yeast homologue, the SAGA complex, which contains Gcn5p (49). These complexes are recruited to promoters by

interacting with transcriptional activators, such as co-activators or sequence-specific transcription factors that are bound to a promoter. It has also been shown that many factors in these complexes act as co-activators by recruiting members of the basal transcriptional apparatus to the promoter (50). In fact, some promoters require certain components of the SAGA complex, which do not have HAT activity, to recruit TBP in order for full transcriptional activation to occur (18, 84).

HDACs counteract the effect of HATs and repress gene transcription by deacetylating the histone tails. As with HATs, HDACs do not bind DNA, but are recruited by co-repressors. They are also found in large multi-subunit complexes that appear to be conserved from yeast to mammals. For example, a well-known complex is the yeast Sin3p-Rpd3p complex, and its mammalian homologue mSin3-HDAC1/2. In both cases, Sin3 interacts with sequence-specific DNA binding repressors and acts as a scaffold upon which the rest of the complex forms (8, 72). The Sin3 complex, which contains the co-repressors NCoR and SMRT, and the histone deacetylase, HDAC1/2, can then repress transcription by deactylating the histones in the nucleosomes surrounding the gene (55, 105). Interestingly, a new class of HDACs has been identified (class II deacetlyases), and some of these class II HDACs interact with NCoR and SMRT in a complex that does not contain mSin3 (60, 65). Therefore, it appears that NCoR and SMRT can interact either directly or indirectly with two classes of HDACs, allowing them to recruit different complexes to mediate repression at specific promoters.

Phosphorylation of histones also occurs on specific residues of H3. This modification has been shown to be important for activating transcription and for chromosome condensation during mitosis (27). Since phosphorylation of H3 can cause chromatin to unfold during transcriptional activation, and to compact during mitosis, it is hypothesized that phosphorylation does not alter histones directly, but creates a binding site to recruit other factors. One identified histone kinase in yeast, Snf1p, has been shown to associate with transcription factors, indicating that they may be recruited as co-activators (90).

Histone ubiquitination has been recently implicated as an important modification involved in regulating transcription. The ubiquitination state of Lys-123 of the H2B carboxy-terminal tail, a substrate for Rad6 ubiquitin ligase, has been shown to be important in mitotic and meiotic growth (128). In addition, a subunit of the TFIID complex, TafII250, possesses H1 ubiquitination activity (120). However, it is not yet clear whether these modifications are involved in transcription.

Methylation of histones, as well as the methylation of DNA, can also have an effect on transcriptional activation. Histone methylation involves two types of residues, lysine and arginine. Arginines can be mono- or di-methylated. Methylation of arginine by histone methyl-transferases (HMTs) leads to activation of transcription (74). As with HATs, these HMTs are recruited to the promoter as co-activators. However, histone methylation is also important in heterochromatic gene silencing. In this case, the HMT Suvar3-9 methylates Lys-9 of H3, leading to the recruitment of the silencing protein HP1,

which binds the methylated histone through a chromodomain, which is also found in many HMTs and other factors (14, 81, 124). When Suvar3-9 is recruited by co-repressors such as the retinoblastoma protein, this modification is also involved in gene-specific repression (109, 154). Other HMTs act in opposition to Suvar3-9 by methylating Lys-4 of H3, leading to gene activation (89, 112). Therefore, methylation can lead to gene-specific activation and repression, as well as to heterochromatic gene silencing, through the activity of different families of HMTs. In addition, DNA methylation is another method used by cells to repress transcription (107). Specific DNA methylases associate with HP1, which is recruited by methylated histones, suggesting that histone and DNA methylation cooperate in transcriptional silencing (9).

As mentioned previously, a 'histone code' has been suggested to determine whether a promoter is activated or repressed. Basically, when multiple modifications affect one histone protein, all these modifications are integrated according to a 'code' to give one outcome: either the gene near the histone is active or inactive. So far, a number of modifications seem to work according to this model. For example, histone H3 has many residues that can be modified by phosphorylation, methylation, and acetylation. It has been shown that Lys-14 has to be deacetylated, before Lys-9 can be methylated, resulting in an inactive state. In addition, for gene activation to occur by Lys-14 acetylation, Ser-10 must be phosphorylated first. Arg-3 must be methylated, before Lys-8 and Lys-12 can be acetylated, and result in an 'active state' (17). In some cases, two modifications target the same residue. For example, Lys-9 of H3 has to be deacetylated, before it can be methylated (74). In conclusion, this shows that all these histone-

modifying enzymes do not work independently. Rather, different types of modifying enzymes must act sequentially in order to get a proper signal and proper activity.

ATP-dependant chromatin remodeling complexes are another group of enzymes that alter DNA-histone contacts to activate or repress transcription. They use the energy from ATP to twist and slide the nucleosome, changing the position of the histone relative to the DNA. This results in DNA that is now accessible to binding by proteins, such as co-activators and general transcription factors. Chromatin remodelers are also found as large complexes, such as SWI/SNF, ISW2 and RSC (6, 156). Isw2p represses transcription of early meiotic genes, once it is recruited by the sequence-specific transcription factor, Ume6p, by creating chromatin inaccessible to proteins in the Ume6p target promoters (46).

Both types of chromatin-modifying enzymes, ATP-dependant chromatin remodelers and histone-modifying enzymes, do not act separately. Instead, their activities are coordinated and can occur in a sequential manner. For example, in *S. cerevisiae*, SWI/SNF must remodel the DNA before the HAT, Gcn5p, can access and activate genes during mitosis, when the structure of chromatin is highly condensed (30, 76, 118). At the IFN-β promoter however, HATs must be recruited before chromatin remodelers can bind. The binding of activators to this promoter induces the recruitment of Gcn5p, which acetylates the histones. This allows the recruitment of the chromatin remodeler SWI/SNF, which can now move the nucleosome, allowing for gene expression

(2). Therefore, in some cases, chromatin remodelers and histone-modifying enzymes can not bind DNA and alter gene expression, until the other acts first.

A large number of factors belonging to the groups mentioned above function in regulating transcription. Some, such as factors in the basal transcriptional apparatus, are ubiquitously expressed and regulate the expression of most genes in the genome. Others, such as chromatin-modifying enzymes, work at a smaller number of promoters. Many factors are involved in the transcription of a specific gene, but each gene will have a different set of factors recruited to its promoter. Sequence-specific transcription factors will bind to promoters of genes containing the specific residues that correspond to its binding site, and recruit a certain group of factors that can include general transcription factors and co-activators. This step is crucial in initiating the formation of an assembly of factors that will act on a specific promoter. For example, an enhanceosome, an assembly of several activators and architectural proteins, will form on an enhancer region of a promoter that contains a binding-site for a sequence-specific transcription factor (Fig. 1) (40). The specific factors that make up an enhanceosome differ depending on which sequence-specific transcription factor bound the enhancer and therefore recruited the factors. But the activity of sequence-specific transcription factors, and thereby the composition of the proteins it recruits, can be affected by other factors. It has been demonstrated that HATs also acetylate non-histone proteins, such as transcriptional activators (26). However, one promoter usually contains binding sites for multiple sequence-specific transcription factors, with each binding site being able to associate with different transcription factors. The collection of binding sites a gene has in its promoter

will determine all the factors that will be recruited, and how they interact with each other will determine how that gene is transcribed.

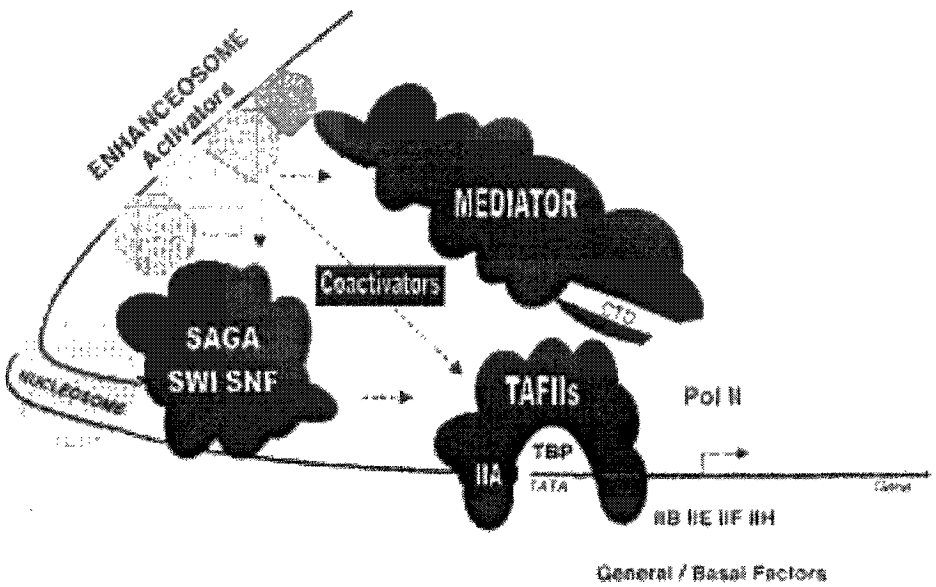

***Figure 1**: The regulation of gene expression involves various co-activators and protein-protein interactions (99).*

ZINC CLUSTER PROTEINS

Sequence-specific transcription factors belong to different families. Members of these families are characterized by specific functional domains and mechanisms of action. In the budding yeast, *Saccharomyces cerevisiae*, the largest family of sequence-specific transcriptional regulators is the C6 zinc binuclear cluster (or zinc cluster) family. Zinc cluster proteins are zinc finger proteins that are characterized by a DNA-binding domain containing the well-conserved motif, $CysX_2CysX_6CysX_{5\text{-}16}CysX_2CysX_{6\text{-}8}Cys$. The cysteine residues bind to two zinc atoms, which coordinate folding of the domain

(153). The zinc cluster motif binds DNA in the major groove, making contact with three base pairs (96). Zinc cluster proteins have been identified only in fungi, including *S. cerevisiae*, and *Schizosaccharomyces pombe*. They have also been found in the pathogenic fungal species, *Candida albicans* and *Aspergillus nidulans* (137, 148).

The first and best characterized zinc cluster protein is Gal4p. It activates genes involved in the metabolism of the carbon source, galactose (19, 141). Since the discovery of Gal4p, many other zinc cluster proteins have been identified, and they function in a wide range of processes, including primary and secondary metabolism, drug resistance and meiotic development. For example, Hap1p activates genes involved in cellular respiration, while Leu3p is involved in the regulation of leucine biosynthetic genes. Other zinc cluster proteins activate the expression of genes required for gluconeogenesis, or metabolism of lysine, arginine, pyrimidine, thymidine, etc. Ume6p regulates genes involved in meiosis (148). Pdr1p and Pdr3p are activators of multi-drug resistance genes (73). All of the characterized proteins have been shown to be transcriptional regulators, except for Cep3p, which forms part of the kinetochore and functions in chromosome segregation. *CEP3* is an essential gene as well, since deletion of the gene results in cell inviability (86). Almost all of the zinc cluster proteins are transcriptional activators. However, some, such as Ume6p, and Rgt1p, are able to both activate and repress transcription (61, 117). Homologues of some zinc cluster proteins identified in *S. cerevisiae* have also been found in other species, indicating that the study of these proteins in budding yeast will help us in determining the roles of these proteins in other fungal species, including the pathogenic *C. albicans*.

ZINC CLUSTER PROTEIN DOMAINS

Like other transcriptional activators, zinc cluster proteins have separate DNA-binding and activation domains (Fig. 2). Other domains important for protein function have also been identified. The DNA-binding domain, which contains the zinc cluster motif, is usually at the N-terminus of the protein. However, some proteins, such as Ume6p, have a C-terminal DNA-binding domain (143). Mutagenesis studies have shown that the cysteine residues are essential for DNA binding and proper protein function (34, 143, 149). Other residues in this region are conserved as well, and appear to be important for function. The zinc cluster motifs usually bind trinucleotide sequences corresponding to CGG or CGA triplets (148). If the DNA-binding domain of one zinc cluster protein is replaced by another, the binding specificity of the protein will be altered, while the exchange of the zinc cluster motif alone does not affect the binding specificity (94, 125). Therefore, it is not surprising that there is not much variability in the zinc cluster domain of different proteins, since they bind similar triplets, with other regions within the DNA-binding domain determining the specificity of binding. A linker region has been identified in zinc cluster proteins and is found at the C-terminal end of the zinc cluster domain. This region shows no conservation between members of the zinc cluster family, and it has been shown to be important in determining DNA binding specificity (148). Therefore, the variability of this region ensures that the various proteins bind to different sequences in order to fulfill their different functions.

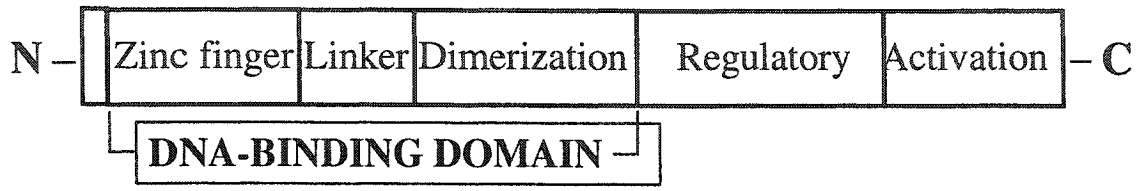

***Figure 2**: Functional domains of zinc cluster proteins.*

Immediately C-terminal to the linker region, zinc cluster proteins usually contain leucine zipper-like heptad repeat motifs. These motifs, which form coiled-coil structures shown to be important in protein-protein interactions, mediate homodimerization of Gal4p and Ppr1p (96, 97). Since different zinc cluster proteins have different dimerization partners, there is little homology between the heptad repeat motifs of different zinc cluster proteins. However, not all zinc cluster proteins bind as homodimers. In *Aspergillus*, the zinc cluster protein AlcR, an activator of ethanol oxidation genes, binds to DNA as a monomer (110). In addition, two *S. cerevisiae* proteins, Oaf1p and Pip2p, have been shown to regulate the activity of genes involved in peroxisome proliferation as heterodimers (66, 132). Zinc cluster proteins are also capable of dimerizing with transcriptional regulators from other families. The zinc cluster protein ArgRIIp forms heterodimers with ArgRIp and Mcm1p, two members of the MADS family, to activate arginine metabolism genes (4).

Zinc cluster proteins can bind to different DNA binding sites as either monomers, homodimers or heterodimers in order to perform their function. Since the zinc cluster motifs of different proteins have been shown to bind similar trinucleotides, the binding sites of various zinc cluster proteins differ in: 1) the orientation of the trinucleotides, and

2) the spacing between the triplets. The linker region of the proteins determines the exact sequence the protein will bind to by probably providing a rigid structure that prevents binding to sites with different spacing and with different orientations (94). Three types of binding sites for zinc cluster proteins have been identified: inverted, direct and everted repeats (Fig. 3) (139). These binding sites contain CGG triplets that are oriented in different directions with respect to each other. For example, Gal4p binds as a homodimer to inverted repeat sequences, causing the two zinc clusters in the homodimer to have a head to head orientation (96). In addition, the Hap1p homodimer binds to a direct repeat, with the zinc cluster motifs facing the same direction (51, 71, 170). Leu3p has been shown to bind everted repeats, which are oriented in opposite directions. This implies that the two zinc fingers of the Leu3p homodimer would be oriented in opposite directions (94). The spacing between the repeats is also critical. Gal4p binds inverted CGG triplets separated by 11 bp, CGG N_{11} CCG (91). In contrast, Put3p, a zinc cluster protein that activates genes involved in proline metabolism, binds inverted CGG triplets separated by 10 bp, CGG N_{10} CCG (145). Even though their sites are very similar and only differ by an extra base pair separating the CGG repeats in the Gal4 binding site, Put3p can not bind the Gal4p binding site, and conversely, Gal4p can not bind the Put3p binding site. Sequences in between and outside of the CGG triplets have also been shown to influence binding. However, the main factors in determining binding are the sequences of the CGG triplets, the orientation of the triplets, and the spacing between them.

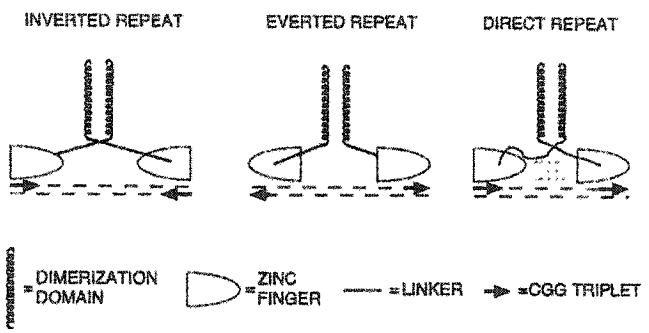

***Figure 3**: Model for binding of zinc cluster proteins to inverted, direct, or everted repeats (94).*

The activation domain of zinc cluster proteins is generally found in the C-terminus, and is usually acidic. A region of weak homology located between the DNA-binding and the activation domains has been recently identified. This region has been termed the middle homology region (MHR), and is comprised of around 80 amino acids. The MHR is thought to have a role in regulating the transcriptional activity of zinc cluster proteins (137). In many cases, deletion of the region that connects the DNA-binding domain to the activation domain results in constitutive activity. For example, deletion of this region in Hap1p renders the protein active, even in the absence of its inducer, heme (119). Leu3p also becomes constitutively active when the same region is deleted (42, 172).

Crystal and solution structures of the DBDs of some zinc cluster proteins have helped define some of the previously mentioned domains. For example, the C6 binuclear cluster motif of Gal4p is comprised of two α-helical structures, each containing three of the six cysteine residues, separated by a loop (Fig. 4) (10, 75, 96). Interestingly, the

structure of the zinc finger of Ppr1p is almost identical to that of Gal4p, reflecting the high degree of homology that exists amongst the zinc cluster motifs of the various zinc cluster proteins (96, 97). The cluster binds to the major groove of DNA by making contact with three base pairs. In addition, Gal4p homodimerizes via a coiled-coil dimerization domain composed of heptad repeats located at the C-terminus of the zinc finger (10, 75, 96). Similar coiled-coil domains have been predicted in the DNA-binding domains of a number of other zinc cluster proteins, indicating that they may mediate dimerization of these proteins (137). However, it remains to be proven whether these domains do indeed mediate dimerization, or whether other types of motifs may be responsible for dimerization in certain zinc cluster proteins.

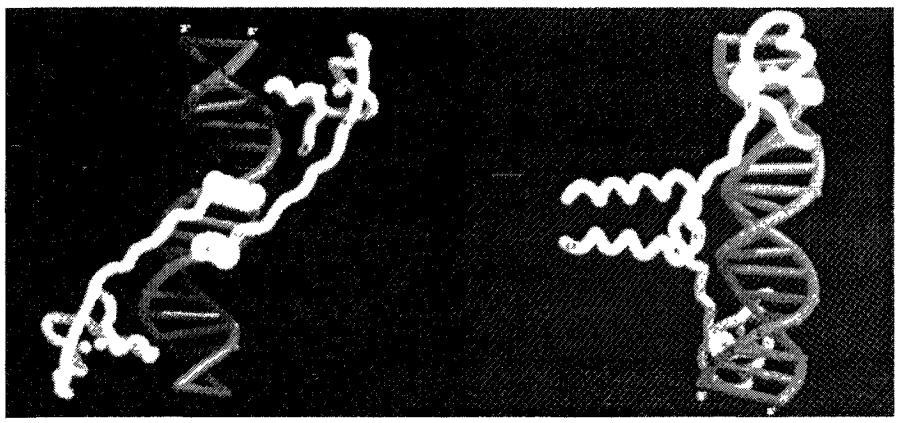

***Figure 4**: The X-ray structure of the Gal4p DNA-binding domain in complex with a palindromic 19 bp DNA containing the protein's consensus binding sequence (96).*

GENOME SEQUENCING PROJECT

The genomes of many organisms have been or are in the process of being sequenced. The *S. cerevisiae* genome was completely sequenced in 1996, providing a

huge amount of information regarding the organization of the genome of this model eukaryotic organism (44). There are 16 chromosomes in the yeast genome, which consist of 12 million bps. It is estimated that 70% of the genome consists of approximately 6000 open reading frames (ORFs), with genes having an average length of 2000 bp, and with an average of 200 bp separating the ORFs (37, 103). This indicates that the yeast genome is very compact and is in stark contrast to the human genome, whose sequence has also been completed (82, 155). Many of the identified ORFs encode putative proteins of totally unknown function. New tools are being developed in order to determine the functions of these proteins, and hopefully these tools can be applied to the study of the genomes of higher eukaryotes, such as the worm *Caenorhabditis elegans*, the fruitfly *Drosophila,* and humans (1, 29, 82, 155). In addition, many genes are conserved among eukaryotes, and the determination of their role in yeast may also indicate their role in higher eukaryotes.

The systematic deletion of all genes in yeast has been performed, leading to the determination that 17% of the genes are essential (164). Analysis of the genome has resulted in the identification of fifty-five members of the zinc cluster protein family (148). Some of these members have been previously identified and their role in the cell is well-understood. However, many of these putative proteins are either poorly characterized, or are of unknown function. Phylogenetic analysis of the zinc cluster motifs in proteins from various fungal species has revealed that the motifs from proteins with similar functions in different species are more closely related than motifs from proteins in the same species that have different functions (148). The sequencing of the

genomes of the pathogenic fungal species, *C. albicans*, and *A. nidulans*, has also been completed. Homologues of *S. cerevisiae* zinc cluster proteins can be identified in these species by analyzing the sequences of their genomes, and since zinc cluster proteins are only found in fungi, these proteins are a potential target for anti-fungal drugs (137, 148).

MULTIDRUG OR PLEIOTROPIC DRUG RESISTANCE

Toxic compounds such as drugs are used to treat many diseases by killing the harmful target cells, which can be either foreign pathogenic organisms or the patient's own tumor cells. However, both prokaryotic and eukaryotic cells can acquire the ability to become resistant to toxic compounds through the phenomenon of multidrug (MDR) or pleiotropic drug resistance (PDR). *Saccharomyces cerevisiae* also can acquire PDR, making it a valuable tool in the study of this phenomenon, allowing us to gain insight into the mechanisms behind PDR in pathogenic fungi and in higher eukaryotes.

Yeast cells have evolved different pathways and mechanisms to allow them to respond to environmental stresses and toxins. Many proteins have been identified as players in drug resistance, and they belong to either one or both of two interconnected networks: 1) The PDR network, and 2) The stress response network. These networks respond to different types of stimuli. While the PDR network is involved in resistance to drugs such as cycloheximide, the stress response network allows the cell to respond to oxidative stress such as hydrogen peroxide (166). Many proteins are involved in both networks, and are therefore critical to a cell's ability to respond under many different

circumstances. These proteins have various roles within the cell, and interestingly, some of them have homologues in mammalian cells.

Cells that have acquired PDR have consistently shown higher levels of expression of drug efflux pumps. Their increased expression allows these cells to expel the drugs from within the cell, and therefore ensure their survival in the presence of these drugs. These pumps fall within two protein families: ATP-Binding Cassette (ABC) transporters and Major Facilitator Superfamily (MFS) transporters. ABC proteins are characterized by having at least one ATP-binding cassette, or nucleotide binding domain (NBD). They also contain several predicted α-helical membrane-spanning segments, or trans-membrane segments (TMS). ABC proteins usually contain a duplicated arrangement of 6 TMS and 1 NBD, with either a forward $(TMS_6\text{-}NBD)_2$ or reverse $(NBD\text{-}TMS_6)_2$ order (166). However, there are a number of half-size transporters, which contain only one NBD and 6 TMSs (15). Yeast ABC transporters are classified into 6 distinct subfamilies. They have many roles in the cell, as demonstrated by the fact that all organelles and cellular compartments contain at least one ABC protein, except for the nuclear membrane and the endoplasmic reticulum (166). The PDR subfamily is the largest ABC subfamily in yeast, and contains many ABC proteins shown to be important in PDR, including Pdr5p and Snq2p (12, 33). Pdr5p and Snq2p are found on the plasma membrane and mediate PDR by using the energy from ATP to pump out hundreds of structurally and functionally unrelated compounds, including the drugs cycloheximide and 4-nitroquinoline N-oxide (4-NQO) (11, 166). Pdr5p is a true functional homologue of mammalian P-glycoproteins, which are also involved in PDR, since they share numerous

drug substrates, are inhibited by the same compounds, and require ATP hydrolysis for drug transport (166).

MFS transporters are also important in PDR. They are found in both prokaryotic and eukaryotic cells. However, they are not as well-characterized as ABC drug efflux pumps. They do not use ATP to transport substrates across a membrane; instead they are energized by proton-motive force to uniport, symport, or antiport substrates such as sugars, organic acids, or drugs. They also contain twelve TMS domains. However, the TMS are separated into six TMS halves by a dispensable central cytoplasmic loop, which does not contain an ABC. There are several families of MFS transporters, one which includes proteins involved in MDR and is termed the MFS-MDR family (11, 45). Members of this family include Atr1p, a plasma membrane transporter, shown to provide cells resistance to the toxic compounds 4-NQO and 3-aminotriazole (3-AT). 3-AT also induces Atr1p gene expression, while 4-NQO does not (47, 64).

In PDR, the cells can become resistant to the drugs by over-expressing the ABC or MFS transporters, allowing for more drugs to be expelled from the cell. These higher levels of expression are often due to mutations in the transcription factors that regulate the expression of these pumps. A complex network of various transcription factors has been shown to be involved in the regulation of the expression of genes encoding ABC or MFS proteins. There are two major families of transcription factors involved in PDR: 1) the bZip protein family (Yap family), and 2) the zinc cluster protein family. The Yap family of proteins is characterized by a DNA-binding domain containing a leucine zipper

that mediates dimerization, and a basic region that binds DNA directly. Eight Yap proteins have been identified in yeast (Yap1p-8p), but only the functions of Yap1p and Yap2p are well understood (41). Yap1p and Yap2p have been shown to bind and activate transcription through a YRE (yeast Ap-1 response element): TTA G/C TAA (41). Yap1p is involved in the response to oxidative stress such as hydrogen peroxide (11, 48, 104, 167). It is shuttled into the nucleus from the cytoplasm during this response in order to activate transcription of target genes which contain the YRE in their promoters (78). These target genes include the MFS, Atr1p, and the ABC transporter, Ycf1p, which is required for the resistance of the cell to the toxic heavy metal cadmium (28, 161). Yap2p is also involved in the regulation of Ycf1p (167). Two non-Yap transcription factors are also involved in the stress response and multidrug resistance; Msn2p and Msn4p. These two proteins upregulate the expression of *PDR15* (166).

Another class of transcription factors involved in PDR is composed of zinc cluster proteins. Two zinc cluster proteins, Pdr1p and Pdr3p, positively control the expression of genes involved in multidrug resistance (73). Target genes of Pdr1p and Pdr3p include the ABC transporters, *PDR5*, *SNQ2*, and *YOR1,* as well as the MFS hexose transporters, *HXT9* and *HXT11* (33, 68, 92, 114). However, it is the regulation of ABC drug efflux pumps that implicates Pdr1p and Pdr3p in PDR (Fig. 5). Pleiotropic drug response elements (PDREs) present in the promoters of these ABC transporters' genes, as well as in the Pdr3p promoter are important in the regulation of these genes. Pdr1p and Pdr3p act through this element, with Pdr1p and Pdr3p able to bind to the everted repeat CCGCGG within the PDRE (35, 57, 68). The presence of PDREs in the *PDR3* promoter

indicates that autoregulation can occur at this promoter. Many hyperactive Pdr1p and Pdr3p mutants have been identified, some of which cause various drug resistance phenotypes (21, 92, 113, 126, 162). Pdr1p and Pdr3p are constitutively phosphorylated, and their localization at the nucleus does not appear to be regulated or induced (95). Another zinc cluster protein, Yrr1p, also positively regulates the expression of *SNQ2* (31). Yrr1p also appears to act through a PDRE (85, 171). A PDRE has been identified in the *YRR1* promoter, and Yrr1p has also been shown to bind to its own promoter, indicating that autoregulation can occur at the *YRR1* locus as well (171). Even though they all act through PDREs, these three zinc cluster proteins have different roles in the cell.

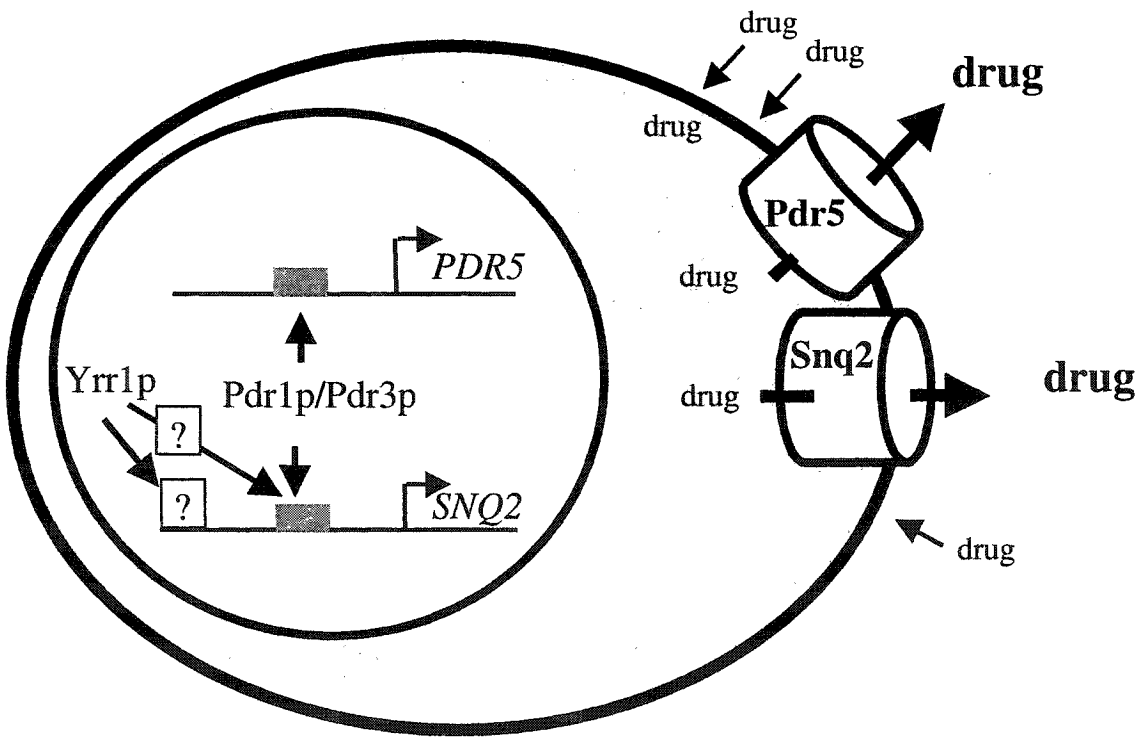

***Figure 5**: The expression of PDR5 and SNQ2 is regulated by Pdr1p, Pdr3p, and/or Yrr1p.*

It has been demonstrated that Pdr1p and Pdr3p are able to form homo- and heterodimers (95). The different combinations of homo- and heterodimers may regulate the expression of different genes, which may help explain how these two proteins act differently. In addition, Ngg1p is able to bind to the activation domain of Pdr1p, and inhibit the activity of a chimeric protein containing the Pdr1p activation domain (98). So Pdr1p, Pdr3p, and Ngg1p can interact with each other and modulate one another's activity directly. Two other zinc cluster proteins have recently been implicated in multidrug resistance. The zinc cluster protein, War1p activates the expression of Pdr12p, an ABC transporter involved in the stress response due to its ability to act as an efflux pump of the weak acids sorbate and benzoate. War1p binds and activates transcription through a weak acid response element (WARE) in the promoter of *PDR12*. A WARE is composed of everted CGG repeats separated by 23 basepairs (CCG- N_{23}-CGG) (77). Pdr8p is another zinc cluster protein that has been shown to bind to the promoters of certain genes implicated in PDR such as *YOR1* and *PDR15*. However, this binding was demonstrated using a chimeric Pdr8p; therefore, the exact role of the wild-type protein in PDR is not clear (58).

The goal of this work is to better understand the roles of the putative zinc cluster proteins identified in the *S. cerevisiae* sequencing project. With 55 putative members, the zinc cluster family appears to be the largest family of transcriptional regulators in yeast. Some of these members are involved in the important phenomenon of PDR. However, the functions of thirty-three of them remain to be determined. This work attempts to determine what role they play in the cell, with an emphasis on PDR.

SECTION2:
PHENOTYPIC ANALYSIS OF GENES ENCODING YEAST ZINC CLUSTER PROTEINS

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ABSTRACT

Zinc cluster proteins (or binuclear cluster proteins) possess zinc fingers of the Zn(II)2Cys6-type involved in DNA recognition as exemplified by the well-characterized protein Gal4p. These fungal proteins are transcriptional regulators of genes involved in a wide variety of cellular processes including metabolism of compounds such as amino acids and sugars, as well as control of meiosis, multi-drug resistance etc. The yeast (*Saccharomyces cerevisiae*) sequencing project has allowed the identification of additional zinc cluster proteins for a total of 54. However, the role of many of these putative zinc cluster proteins is unknown. We have performed phenotypic analysis of 33 genes encoding (putative) zinc cluster proteins. Only two members of the GAL4 family are essential genes. Our results show that deletion of 8 different zinc cluster genes impairs growth on non-fermentable carbon sources. The same strains are also hypersensitive to the antifungal calcofluor white suggesting a role for these genes in cell wall integrity. In addition, one of these strains (ΔYFL052W) is also heat sensitive on rich (but not minimal) plates. Thus, deletion of YFL052W results in sensitivity to a combination of low osmolarity and high temperature. In addition, 6 strains are hypersensitive to caffeine, an inhibitor of the MAP kinase pathway and phosphodiesterase of the cAMP pathway. In conclusion, our analysis assigns phenotypes to a number of genes and provides a basis to better understand the role of these transcriptional regulators.

INTRODUCTION

In yeast (*Saccharomyces cerevisiae*), a major class of transcriptional regulators is composed of a sub-family of zinc finger proteins called zinc cluster proteins or binuclear cluster proteins. These proteins contain a DNA binding domain which possesses the well-conserved motif $CysX_2CysX_6CysX_{5\text{-}16}CysX_2CysX_{6\text{-}8}Cys$ with cysteines binding to two zinc atoms which coordinate folding of the domain (1).

This type of transcriptional regulator has only been identified in fungi and these proteins have been shown to be involved in a wide variety of cellular processes (reviewed in ref. 2; also see Table 3). For example, Gal4p is involved in activation of genes that encode enzymes for galactose metabolism (3) while Hap1p activates genes involved in cellular respiration (4, 5). In addition, other zinc cluster proteins increase expression of genes required for gluconeogenesis or metabolism of leucine, lysine, arginine, pyrimidine, thiamine, etc. (2). Other roles of zinc cluster proteins include the control of expression of genes required for use of gamma-aminobutyric acid (GABA), serine, threonine, or proline as a nitrogen source (2). In addition, some members of the family, such as Pdr1p and Pdr3p, are responsible for controlling expression of multi-drug resistance genes (6).

Quite often, the DNA binding domain (comprising the cysteine-rich region) of zinc cluster proteins is located at the N-terminus while an acidic activating domain is located at the C-terminus. A region of low homology of about 80 amino acids, termed middle homology region, is found among many zinc cluster proteins and is located between the DNA binding and activation domains and may be involved in controlling the transcriptional activity of zinc cluster proteins (7). In many cases, deletion of the region that bridges the DNA binding domain to the

activation domain results in constitutive activity of the transcriptional activator. For example, deletion of the middle region of Hap1p renders the protein active even in the absence of the inducer heme (4). Similar results were obtained with Leu3p, a protein implicated in leucine biosynthesis (8, 9). Many of the characterized zinc cluster proteins are transcriptional activators. Well-known exceptions are Ume6p and Rgt1p which are both repressors and activators of early mitotic and glucose transport genes, respectively (10, 11).

Many zinc cluster proteins bind to DNA as homodimers through a coiled-coil dimerization domain located at the C-terminus of the zinc finger. Three types of DNA binding sites have been identified: inverted, direct and everted repeats (reviewed in ref. 12). For example Gal4p binds as a homodimer to inverted repeat DNA sequences (13 and refs. therein) while Hap1p binds to a direct repeat (14-16). Analysis of the binding sites of Leu3p showed that it recognizes repeats oriented in opposite directions, an everted repeat (17). These observations imply that the two zinc fingers of Leu3p must be oriented in opposite directions unlike those of Gal4p where they have been shown to face each other (13).

Alternate modes of DNA binding by zinc cluster proteins have also been described. For example, AlcR, a transcriptional activator of ethanol oxidation genes in *Aspergillus*, binds to DNA as a monomer (18). In addition, the two zinc cluster proteins Oaf1p and Oaf2p (Pip2p) bind as heterodimers to target sequences of genes for peroxisome proliferation (19, 20). Heteromeric formation is also observed between members of different families of transcription factors. For example, ArgRII, a member of the family of zinc cluster proteins, heterodimerizes with members of the MADS family, ArgRI and Mcm1p, to activate genes for arginine metabolism (21). In summary, zinc cluster proteins perform a wide variety of functions through

transcriptional activation or repression by binding to target genes as homodimers, heterodimers or monomers.

The *S. cerevisiae* sequencing project has allowed the identification of additional zinc cluster proteins for a total of 54 (2). However, the function of many of these putative zinc cluster proteins is unknown. In an effort to understand the roles of the uncharacterized zinc cluster proteins, we examined the phenotypes of 33 strains carrying deletions of genes encoding zinc cluster proteins under various growth conditions.

MATERIALS AND METHODS

Strains

Wild type *S. cerevisiae* strains used to generate the gene deletions were: FY73 (22), MATα *his3*-Δ200 *ura3*-52; YPH499 (23), MATa *ura3*-52 *lys2*-801 *ade2*-101 *trp1*-Δ63 *his3*-Δ200 *leu2*-Δ1; YPH500 (23), MATα *ura3*-52 *lys2*-801 *ade2*-101 *trp1*-Δ63 *his3*-Δ200 *leu2*-Δ1; YPH501 (23), a cross between YPH499 and YPH500; BY4742 (24), MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0. Deletions were obtained by the PCR method of Baudin et al. (25) using *HIS3* as a marker for selection (template for PCR was pMHIS3, ref. 26). Oligos had the sequence N_{43}CAGGGTTTTCCCAGTCA and N_{43}GCGGATAACAATTTCAC with N corresponding to sequences of the target genes. Some open reading frames (ORFs) were entirely deleted and, for others, the deletion spanned the zinc finger (cysteine-rich region) located at the N-terminus of the putative ORFs, as indicated in Table 1. Deletions in FY73 were obtained by direct transformation of the haploid strain. Deletions in the YPH background were obtained by transforming the diploid strain YPH501 followed by sporulation and selection of *HIS*$^+$ spores. For sporulation, diploid strains were plated on sporulation plates (1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 2% agar) for 1 week followed by random spore analysis (27). Diploid strains carrying deletions of the ORF YPR094W or YDR303C were obtained from Research Genetics. Haploid strains were obtained by sporulation.

Proper recombination events were verified by PCR using two pairs of primers. One set consisted of the primer GCCTCGTTCAGAATGACACG (located in the 3'-end of the *HIS3* marker) and a primer in the promoter region of the target gene and the second set of a primer located downstream of the ORF and the primer TTACTCTTGGCCTCCTCTAG (located in the

5'-end of the *HIS3* marker). Single integration events were verified by Southern blot analysis for all strains using the *HIS3* or the kanamycin markers as probes. In all cases, the sizes of the detected bands were in agreement with homologous recombination at the targeted gene. Genomic DNA was isolated according to ref. 28. Southern blots were done according to standard procedures (27). Hybridizations were done at 42°C in 50% formamide, 1M NaCl, 2.8X Denhardt's solution, 0.5% SDS and 10% dextran sulphate.

Media

Media were prepared according to Adams et al. (29). YPD contained 1% yeast extract, 2% peptone, 2% glucose. SD contained 2% glucose, 0.67% yeast nitrogen base without amino acids. Adenine, histidine, leucine, lysine, tryptophan and uracil were added to the media at a final concentration of 0.004%. Nitrogen source requirements were tested on minimal media as described above except that yeast nitrogen base lacked ammonium sulphate. A source for nitrogen was supplied by adding γ-aminobutyric acid (GABA) at a final concentration of 2% or 1 mg/ml of either urea, serine, threonine, proline as specified in Table 2. YEP contained 1% yeast extract, 2% peptone with either 2% glycerol or 2% lactic acid. Sensitivity to drugs was assayed on YPD plates supplemented with 0.15% caffeine (Sigma) or 70 μg/ml calcofluor white (Sigma).

Growth assays

Wild type and deletion strains were grown overnight in liquid (YPD), spun and resuspended in water. Cells were then serially diluted (approximately $5X10^4$, $5X10^3$, $5X10^2$, $5X10^1$ cells) and spotted on appropriate plates. For assay of growth under anaerobic conditions,

an anaerobic chamber was used along with a BBL GasPak (Becton Dickinson) and a palladium catalyst activated by heating at 160^0C for 2h. An oxygen indicator (BBL Becton Dickinson) showed that cells were actually grown under anaerobic conditions.

Requirement for specific compounds

Requirement for specific compounds were tested in a minimal medium (Halvorson, ref. 29) containing the following components: 2% glucose, 2% ammonium sulphate, 0.25M K_2HPO_4, 0.25 M succinic acid, 0.002% sodium carbonate, 14 mM $CaCl_2$, 21 mM $MgSO_4$, 3.8 μM $FeCl_3$, 8.3 μM $MnCl_2$, 7.7 μM $ZnSO_4$, 7.8 μM $CuSO_4$, $1X10^{-7}$ % D-biotin, $3X10^{-5}$ % calcium pantotheanate, $3X10^{-5}$ % folic acid, $3.3X10^{-4}$ % myo-inositol, $3.3X10^{-4}$ % pyridoxine-HCl, $3.3X10^{-4}$ % nicotinic acid, $3.3X10^{-4}$ % p-amino benzoic acid, $3.3X10^{-4}$ % thiamine-HCl, 0.004% adenine, 0.004% histidine, 0.004% leucine, 0.004% lysine, 0.004% methionine, 0.004% tryptophan and 0.004% uracil. Specific components were omitted as specified in Table 2.

RESULTS AND DISCUSSION

Many yeast genes encode putative zinc cluster proteins of totally unknown function while others have not been well characterized. Thus, we performed a phenotypic analysis of yeast strains carrying deletions of genes encoding members of the Gal4p family of zinc cluster proteins. The yeast genome contains 54 ORFs that potentially encode zinc cluster proteins containing the consensus sequence $CysX_2CysX_6CysX_{5\text{-}16}CysX_2CysX_{6\text{-}8}Cys$. Two "zinc cluster-like proteins" were excluded from our analysis since they do not conform to the consensus amino acid sequence given above. YPR009W and YGL162W (SUT1) have 63 and 68 amino acids between the 3rd and 4th cysteines of the zinc cluster, respectively as opposed to 5 to 16 for other zinc cluster proteins found in *Saccharomyces cerevisiae* as well as other fungi (2). YGL162W was shown to be involved in sterol uptake (30). In addition, the cysteine-rich regions of YGL162W and YPR009W share homology to the glucose transporters encoded by the SUT1, SUT2, and SUT3 genes of yeast *Pichia stipitis* (ref. 31 and unpublished results). Thus, the YPR009W and YGL162W genes may not encode DNA binding proteins. In addition, well-characterized zinc cluster proteins such as Gal4p, Hap1p, Leu3p, Uga3p etc. were also excluded from our analysis. Thus, we phenotypically analyzed 33 genes encoding (putative) zinc cluster proteins under various conditions including alternate carbon sources, growth temperature, presence of caffeine etc.

We deleted ORFs of genes encoding putative zinc cluster proteins by the PCR method of Baudin et al. (25) using *HIS3* as selectable marker and haploid strains (see materials and methods). Deletions were verified by using two pairs of primers for PCR analysis (data not shown). Moreover, all deletion strains were also verified by Southern blot analysis (data not

shown). Most zinc cluster genes could be deleted in a haploid background. However, no colonies were obtained using a PCR product aimed at deleting ORF YDR303C in the haploid strain FY73. Random spore analysis using a heterozygote strain (BY4743) carrying a deletion of ORF YDR303C revealed that all spores (about 50) were sensitive to G418 showing that YDR303C is an essential gene. Similar results were obtained with transposon insertion in the YDR303C gene (32) or deletion of the ORF (73). In addition, the zinc cluster protein Cep3p, a component of the Cbf3 kinetochore complex that binds centromeric elements (33) was shown to be encoded by an essential gene (34, 35). Deletion of the gene YHR178W encoding a putative zinc cluster protein is not lethal in the BY4742 background (Table 2); however this gene was scored as essential in the YM4587 background (36). This discrepancy may be explained by the use of different strains. For example, strain YM4587, unlike strain BY4742, carries a mutant allele of the *TYR1* gene involved in tyrosine synthesis. Thus, according to our data, only two members of the Gal4p zinc cluster family are encoded by essential genes.

Growth on non-fermentable carbon sources

Respiratory-deficient mutants are unable to grow on non-fermentable carbon sources such as glycerol and lactate (37). Thus, we tested the ability of the deletion strains to grow on non-fermentable carbon sources. Cells were serially diluted and spotted on YEP-glycerol and YEP-lactate plates and grown at 30°C for 3 days. Deletion of ORFs YER184C, YFL052W, YIL130W, YLL054C, YLR266C, YOR162C (YRR1), YOR380W and YPL133C impaired growth on both glycerol and lactate plates (Table 2) while normal growth was observed on plates containing glucose as the carbon source (Fig. 1 and data not shown). An example of data is provided in Fig. 1 with strains carrying deletions of ORFs YER184C, YFL052W, YLL054C and

YOR380W. Thus, deletion of ORFs YER184C, YFL052W, YIL130W, YLL054C, YLR266C, YOR162C (YRR1), YOR380W and YPL133C prevents growth in medium containing lactate or glycerol as the only carbon source. In addition, deletion of YOR162C (YRR1) leads to hypersensitivity to the mutagen 4-nitroquinoline *N*-oxide due to reduced expression of the ABC transporter SNQ2 (38).

Temperature Sensitivity

Growth of the deletion strains was tested at high and at low temperatures (Table 2). The strains were spotted on YPD and minimal plates and grown at 37°C or 20°C. One strain, YZS (YFL052W), did not grow in rich medium at 37 °C but grew on minimal medium at 37 °C. Since salt concentration is higher in minimal than rich medium, deletion of the YFL052W gene may render the cells sensitive to a combination of both high temperature and low salt concentration. In addition, knockout of the YFL052W gene renders the cell hypersensitive to calcofluor white (see below and Fig. 4), a phenotype associated with cell wall mutants (37). Thus, YFL052W may be involved in maintenance of cell wall integrity. Deletion of YHR178W (STB5) resulted in a cold sensitive phenotype since the strain did not grow on either YPD or SD at 20 °C. Both Stb5p and Stb4p (encoded by YMR019W) were shown to interact with Sin3p, a protein that recruits the histone deacetyltransferase Rpd3p involved in repression of transcription (39). Thus, Stb4p and Stb5p may encode transcriptional repressors.

Requirement for specific compounds

A number of zinc cluster proteins, such as Dal81p, Uga3p, Put3p and Cha4p have been shown to be involved in the use of alternate nitrogen sources (see Table 3). We tested if other genes encoding zinc cluster proteins would play a similar role by growing deletion strains on plates containing urea, GABA, serine, threonine or proline as the sole nitrogen source. Only one strain carrying a deletion of the YHR178W (STB5) gene showed reduced growth (Table 2). We also tested growth of deletion strains on minimal Halvorson medium (Table 2). Again, only deletion of the gene YHR178W (STB5) resulted in reduced growth. Moreover, besides deletion strain YHR178W, omission of folic acid, pantothenic acid or biotin in the Halvorson medium did not alter growth of any deletion strain (data not shown). As expected (40), a strain carrying a deletion of the THI2 gene (YBR240C) was auxotrophic for thiamine but not sensitive to the omission of folic acid, pantothenic acid or biotin (data not shown). In conclusion, deletion of YHR178W results in slightly slower cell growth under minimal growth conditions such as "Halvorson" medium or when nitrogen sources other than ammonium sulphate are used.

Sensitivity to caffeine

Caffeine, a purine analog, has a toxic effect on cells through inhibition of the MAP kinase pathway and phosphodiesterase of the cAMP pathway (37). Deletion strains were tested for hypersensitivity to caffeine. Although wild type strains are all derived from SC228, they showed different sensitivities to caffeine: YPH499 was more sensitive than FY73 and BY4742.

All the FY73 and BY4742-based strains grew well on caffeine plates with the exception of ΔYHR178W which was highly sensitive to caffeine (Table 2). An example of results is provided in Fig. 2. Normal growth of the strain deleted of YHR178W is seen on YPD plates but greatly reduced growth is observed if caffeine is added (Fig. 2). A strain carrying a deletion of YDR520C was slightly more sensitive to caffeine than wild type YPH499 while deletions of YKL222C, YLR228C or YLR278C resulted in moderate sensitivity (Table 2). Severe sensitivity to caffeine was observed with a deletion of ORF YMR019W as compared to wild type YPH499. In addition, deletion of the latter ORF resulted in cells clumping when grown in YPD liquid media (data not shown).

Sensitivity to calcofluor white

Calcofluor white is a compound that has high affinity for the cell wall component chitin. Hypersensitivity to that compound has been associated with cell wall mutants (37, 65). Deletion of 8 ORFS (YER184C, YFL052W, YIL130W, YLL054C, YLR266C, YOR162C (YRR1), YOR380W and YPL133C) rendered the cells hypersensitive to calcofluor white (Table 2 and Fig. 3). A strain carrying a transposon insertion in the promoter of the YLR228C has been shown to be hypersensitive to calcofluor (65). However, in our assay with a deletion strain, no effect of calcofluor was observed. In summary, 8 deletion strains are hypersensitive to calcofluor white. Interestingly, the same deletion strains were unable to grow on non-fermentable carbon sources. We do not know the relationship (if any) between these two phenotypes.

Anaerobic Conditions

Since yeast is a facultative anaerobe, we tested the effect of growing deletion strains under anaerobic conditions to identify zinc cluster genes necessary for growth under these conditions. Growth was assayed on rich plates as well as minimal plates. All the tested knockout strains were able to grow in both media under these conditions (Table 2). Thus, Hap1p is the only zinc cluster protein which may be necessary for anaerobic growth (41).

CONCLUSION

The *S. cerevisiae* sequencing project has allowed the identification of many new (putative) members of the zinc cluster protein family. Only two members (YDR303C and YMR168C) of the GAL4 family of zinc cluster proteins are essential genes. Our deletion analysis has revealed phenotypes for a number of genes encoding zinc cluster proteins (Table 2). The most prevalent class of phenotypes is the inability to grow on non-fermentable carbon sources and sensitivity to calcofluor white. Other phenotypes observed include temperature and caffeine sensitivity. A number of deletions (e.g. YFL052W, YHR178W, YMR019W) resulted in multiple phenotypes. For example, deletion of ORF YFL052W results in an inability to grow on non-fermentable carbon sources, sensitivity to high temperature and calcofluor white (Table 2). Similarly, deletion of ORF YMR019W renders the cells clumpy and sensitive to caffeine. It is difficult to establish a relationship (if any) between these various phenotypes. Since our analysis focused on a class of transcriptional regulators, deletion of their genes may have a widespread effect on gene expression and some phenotypes observed may be due to greatly altered cell physiology. For example, whole-genome analysis with DNA microarrays revealed that

expression of a large number of genes is affected by deletion of ORF YMR019W including most genes encoding ribosomal proteins (B.A. and B.T., unpublished results). Thus, multiple phenotypes may be due to indirect effects.

Although many conditions were tested in our analysis, no phenotypes could be identified for a number of GAL4 family members. These proteins may perform highly specialized functions or, alternatively, there may be redundant genes encoding zinc cluster proteins. Analysis of double deletion mutants as well as whole-genome analysis of gene expression with microarrays should help to identify functions of zinc cluster proteins.

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REFERENCES

1. Vallee, B. L., Coleman, J. E. and Auld, D. S. (1991) Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 999-1003.

2. Todd, R. B. and Andrianopoulos, A. (1997) Evolution of a fungal regulatory gene family: the Zn(II)2Cys6 binuclear cluster DNA binding motif. *Fungal Gen. Biol.*, **21,** 388-405.

3. Lohr, D., Venkov, P. and Zlatanova, J. (1995) Transcriptional regulation in the yeast GAL gene family: a complex genetic network. *FASEB J.*, **9,** 777-87.

4. Pfeifer, K., Kim, K. S., Kogan, S. and Guarente, L. (1989) Functional dissection and sequence of yeast HAP1 activator. *Cell*, **56,** 291-301.

5. Creusot, F., Verdiere, J., Gaisne, M. and Slonimski, P. P. (1988) CYP1 (HAP1) regulator of oxygen-dependent gene expression in yeast. I. Overall organization of the protein sequence displays several novel structural domains. *J. Mol. Biol.*, **204,** 263-76.

6. Koloczkowska, A. and Goffeau, A. (1999) Regulation of pleiotropic drug resistance in yeast. *Drug Resistance Updates*, **2,** 403-14.

7. Schjerling, P. and Holmberg, S. (1996) Comparative amino acid sequence analysis of the C6 zinc cluster family of transcriptional regulators. *Nucl. Acids Res.*, **24,** 4599-607.

8. Zhou, K. M. and Kohlhaw, G. B. (1990) Transcriptional activator LEU3 of yeast. Mapping of the transcriptional activation function and significance of activation domain tryptophans. *J. Biol. Chem.*, **265,** 17409-12.

9. Friden, P., Reynolds, C. and Schimmel, P. (1989) A large internal deletion converts yeast LEU3 to a constitutive transcriptional activator. *Mol. Cell. Biol.*, **9,** 4056-60.

10. Jackson, J. C. and Lopes, J. M. (1996) The yeast UME6 gene is required for both negative and positive transcriptional regulation of phospholipid biosynthetic gene expression. *Nucleic Acids Res.*, **24,** 1322-9.

11. Ozcan, S., Leong, T. and Johnston, M. (1996) Rgt1p of Saccharomyces cerevisiae, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription. *Mol. Cell. Biol.*, **16,** 6419-26.

12. Schwabe, J. W. and Rhodes, D. (1997) Linkers made to measure. *Nature Struc. Biol.*, **4,** 680-3.

13. Marmorstein, R., Carey, M., Ptashne, M. and Harrison, S. C. (1992) DNA recognition by GAL4: structure of a protein-DNA complex. *Nature*, **356,** 408-14.

14. Zhang, L. and Guarente, L. (1994) The yeast activator HAP1--a GAL4 family member-binds DNA in a directly repeated orientation. *Genes Dev.*, **8,** 2110-9.

15. Ha, N., Hellauer, K. and Turcotte, B. (1996) Mutations in target DNA elements of yeast HAP1 modulate its transcriptional activity without affecting DNA binding. *Nucl. Acids Res.*, **24,** 1453-9.

16. King, D. A., Zhang, L., Guarente, L. and Marmorstein, R. (1999) Structure of a HAP1-DNA complex reveals dramatically asymmetric DNA binding by a homodimeric protein. *Nature Struc. Biol.*, **6,** 64-71.

17. Hellauer, K., Rochon, M.-H. and Turcotte, B. (1996) A novel DNA binding motif for yeast zinc cluster proteins: the Leu3p and Pdr3p transcriptional activators recognize everted repeats. *Mol. Cell. Biol.*, **16,** 6096-6102.

18. Nikolaev, I., Lenouvel, F. and Felenbok, B. (1999) Unique DNA binding specificity of the binuclear zinc AlcR activator of the ethanol utilization pathway in Aspergillus nidulans. *J. Biol. Chem.*, **274,** 9795-802.

19. Karpichev, I. V., Luo, Y., Marians, R. C. and Small, G. M. (1997) A complex containing two transcription factors regulates peroxisome proliferation and the coordinate induction of beta-oxidation enzymes in Saccharomyces cerevisiae. *Mol. Cell.Biol.*, **17,** 69-80.

20. Rottensteiner, H., Kal, A. J., Hamilton, B., Ruis, H. and Tabak, H. F. (1997) A heterodimer of the Zn2Cys6 transcription factors Pip2p and Oaf1p controls induction of genes encoding peroxisomal proteins in Saccharomyces cerevisiae. *Eur. J. Biochem.*, **247,** 776-83.

21. Amar, N., Messenguy, F., El Bakkoury, M. and Dubois, E. (2000) ArgRII, a component of the ArgR-Mcm1 complex involved in the control of arginine metabolism in Saccharomyces cerevisiae, is the sensor of arginine. *Mol. Cell. Biol.*, **20,** 2087-97.

22. Winston, F., Dollard, C. and Ricupero-Hovasse, S. L. (1995) Construction of a set of convenient Saccharomyces cerevisiae strains that are isogenic to S288C. *Yeast*, **11,** 53-5.

23. Sikorski, R. S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics*, **122,** 19-27.

24. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P. and Boeke, J. D. (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C:

a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast*, **14,** 115-32.

25. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F. and Cullin, C. (1993) A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae. *Nucleic Acids Res.*, **21,** 3329-30.

26. Noël, J. and Turcotte, B. (1998) Zinc cluster proteins Leu3p and Uga3p recognize highly related but distinct DNA targets. *J. Biol. Chem.*, **273,** 17463-8.

27. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1989) Current protocols in molecular biology, Greene Publishing Associates and Wiley-Interscience, New York.

28. Philippsen, P., Stotz, A. and Scherf, C. (1991) DNA of Saccharomyces cerevisiae. *Meth. Enz.*, **194,** 169-82.

29. Adams, A., Gottschling, D. E. and Stearns, T. (1997) Methods in yeast genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

30. Bourot, S. and Karst, F. (1995) Isolation and characterization of the Saccharomyces cerevisiae SUT1 gene involved in sterol uptake. *Gene*, **165,** 97-102.

31. Weierstall, T., Hollenberg, C. P. and Boles, E. (1999) Cloning and characterization of three genes (SUT1-3) encoding glucose transporters of the yeast Pichia stipitis. *Mol. Microbiol*, **31,** 871-83.

32. Burns, N., Grimwade, B., Ross-Macdonald, P. B., Choi, E. Y., Finberg, K., Roeder, G. S. and Snyder, M. (1994) Large-scale analysis of gene expression, protein localization, and gene disruption in Saccharomyces cerevisiae. *Genes Dev.*, **8,** 1087-105.

33. Clarke, L. (1998) Centromeres: proteins, protein complexes, and repeated domains at centromeres of simple eukaryotes. *Curr. Opin. Genetics Dev.*, **8,** 212-8.

34. Lechner, J. (1994) A zinc finger protein, essential for chromosome segregation, constitutes a putative DNA binding subunit of the Saccharomyces cerevisiae kinetochore complex, Cbf3. *EMBO J.*, **13,** 5203-11.

35. Strunnikov, A. V., Kingsbury, J. and Koshland, D. (1995) CEP3 encodes a centromere protein of Saccharomyces cerevisiae. *J. Cell Biol.*, **128,** 749-60.

36. Niedenthal, R., Riles, L., Guldener, U., Klein, S., Johnston, M. and Hegemann, J. H. (1999) Systematic analysis of S. cerevisiae chromosome VIII genes. *Yeast*, **15,** 1775-96.

37. Hampsey, M. (1997) A review of phenotypes in Saccharomyces cerevisiae. *Yeast*, **13,** 1099-133.

38. Cui, Z., Shiraki, T., Hirata, D. and Miyakawa, T. (1998) Yeast gene YRR1, which is required for resistance to 4-nitroquinoline N- oxide, mediates transcriptional activation of the multidrug resistance transporter gene SNQ2. *Mol. Microbiol.*, **29,** 1307-15.

39. Kasten, M. M. and Stillman, D. J. (1997) Identification of the Saccharomyces cerevisiae genes STB1-STB5 encoding Sin3p binding proteins. *Mol. Gen. Genet.*, **256,** 376-86.

40. Nishimura, H., Kawasaki, Y., Kaneko, Y., Nosaka, K. and Iwashima, A. (1992) Cloning and characteristics of a positive regulatory gene, THI2 (PHO6), of thiamin biosynthesis in Saccharomyces cerevisiae. *FEBS Lett.*, **297,** 155-8.

41. Chantrel, Y., Gaisne, M., Lions, C. and Verdiere, J. (1998) The transcriptional

regulator Hap1p (Cyp1p) is essential for anaerobic or heme-deficient growth of Saccharomyces cerevisiae: Genetic and molecular characterization of an extragenic suppressor that encodes a WD repeat protein. *Genetics*, **148,** 559-69.

42. Ross-Macdonald, P., Coelho, P. S., Roemer, T., Agarwal, S., Kumar, A., Jansen, R., Cheung, K. H., Sheehan, A., Symoniatis, D., Umansky, L., Heidtman, M., Nelson, F. K., Iwasaki, H., Hager, K., Gerstein, M., Miller, P., Roeder, G. S. and Snyder, M. (1999) Large-scale analysis of the yeast genome by transposon tagging and gene disruption. *Nature*, **402,** 413-8.

43. Kumar, A., Cheung, K. H., Ross-Macdonald, P., Coelho, P. S., Miller, P. and Snyder, M. (2000) TRIPLES: a database of gene function in Saccharomyces cerevisiae. *Nucleic Acids Re.s*, **28,** 81-4.

44. Delaveau, T., Delahodde, A., Carvajal, E., Subik, J. and Jacq, C. (1994) PDR3, a new yeast regulatory gene, is homologous to PDR1 and controls the multidrug resistance phenomenon. *Mol. Gen. Genet.*, **244,** 501-11.

45. Groom, K. R., Heyman, H. C., Steffen, M. C., Hawkins, L. and Martin, N. C. (1998) Kluyveromyces lactis SEF1 and its Saccharomyces cerevisiae homologue bypass the unknown essential function, but not the mitochondrial RNase P function, of the S. cerevisiae RPM2 gene. *Yeast*, **14,** 77-87.

46. Charron, M. J., Read, E., Haut, S. R. and Michels, C. A. (1989) Molecular evolution of the telomere-associated MAL loci of Saccharomyces. *Genetics*, **122,** 307-16.

47. Needleman, R. (1991) Control of maltase synthesis in yeast. *Mo.l Microbiol.*, **5,** 2079-84.

48. André, B. (1990) The UGA3 gene regulating the GABA catabolic pathway in Saccharomyces cerevisiae codes for a putative zinc-finger protein acting on RNA amount. *Mol. Gen. Genet.*, **220,** 269-76.

49. Vissers, S., Andre, B., Muyldermans, F. and Grenson, M. (1990) Induction of the 4-aminobutyrate and urea-catabolic pathways in Saccharomyces cerevisiae. Specific and common transcriptional regulators. *Eur. J. Biochem.*, **187,** 611-6.

50. Feller, A., Dubois, E., Ramos, F. and Pierard, A. (1994) Repression of the genes for lysine biosynthesis in Saccharomyces cerevisiae is caused by limitation of Lys14-dependent transcriptional activation. *Mol. Cell. Biol.*, **14,** 6411-8.

51. Feller, A., Ramos, F., Pierard, A. and Dubois, E. (1999) In Saccharomyces cerevisae, feedback inhibition of homocitrate synthase isoenzymes by lysine modulates the activation of LYS gene expression by Lys14p. *Eur. J. Biochem.*, **261,** 163-70.

52. Strich, R., Surosky, R. T., Steber, C., Dubois, E., Messenguy, F. and Esposito, R. E. (1994) UME6 is a key regulator of nitrogen repression and meiotic development. *Genes Dev.*, **8,** 796-810.

53. Crowley, J. H., Leak, F. W., Jr., Shianna, K. V., Tove, S. and Parks, L. W. (1998) A mutation in a purported regulatory gene affects control of sterol uptake in Saccharomyces cerevisiae. *J. Bacteriol.*, **180,** 4177-83.

54. Leak, F. W., Jr., Tove, S. and Parks, L. W. (1999) In yeast, upc2-1 confers a decrease in tolerance to LiCl and NaCl, which can be suppressed by the P-type ATPase encoded by ENA2. *DNA Cell Biol*, **18,** 133-9.

55. Balzi, E., Chen, W., Ulaszewski, S., Capieaux, E. and Goffeau, A. (1987) The

multidrug resistance gene PDR1 from Saccharomyces cerevisiae. *J. Biol. Chem.*, **262,** 16871-9.

56. Bricmont, P. A., Daugherty, J. R. and Cooper, T. G. (1991) The DAL81 gene product is required for induced expression of two differently regulated nitrogen catabolic genes in Saccharomyces cerevisiae. *Mol. Cell. Biol.*, **11,** 1161-6.

57. Coornaert, D., Vissers, S. and Andre, B. (1991) The pleiotropic UGA35(DURL) regulatory gene of Saccharomyces cerevisiae: cloning, sequence and identity with the DAL81 gene. *Gene*, **97,** 163-71.

58. Lesage, P., Yang, X. and Carlson, M. (1996) Yeast SNF1 protein kinase interacts with SIP4, a C6 zinc cluster transcriptional activator: a new role for SNF1 in the glucose response. *Mol. Cell. Biol.*, **16,** 1921-8.

59. Vincent, O. and Carlson, M. (1998) Sip4, a Snf1 kinase-dependent transcriptional activator, binds to the carbon source-responsive element of gluconeogenic genes. *EMBO J.*, **17,** 7002-7008.

60. des Etages, S. A., Falvey, D. A., Reece, R. J. and Brandriss, M. C. (1996) Functional analysis of the PUT3 transcriptional activator of the proline utilization pathway in Saccharomyces cerevisiae. *Genetics*, **142,** 1069-82.

61. Siddiqui, A. H. and Brandriss, M. C. (1989) The Saccharomyces cerevisiae PUT3 activator protein associates with proline-specific upstream activation sequences. *Mol. Cell. Biol.*, **9,** 4706-12.

62. Losson, R. and Lacroute, F. (1981) Cloning of a eukaryotic regulatory gene. *Mol. Gen. Genet.*, **184,** 394-9.

63. Roy, A., Exinger, F. and Losson, R. (1990) cis- and trans-acting regulatory elements of the yeast URA3 promoter. *Mol. Cell. Biol.*, **10,** 5257-70.

64. Holmberg, S. and Schjerling, P. (1996) Cha4p of Saccharomyces cerevisiae activates transcription via serine/threonine response elements. *Genetics*, **144,** 467-78.

65. Lussier, M., White, A. M., Sheraton, J., di Paolo, T., Treadwell, J., Southard, S. B., Horenstein, C. I., Chen-Weiner, J., Ram, A. F., Kapteyn, J. C., Roemer, T. W., Vo, D. H., Bondoc, D. C., Hall, J., Zhong, W. W., Sdicu, A. M., Davies, J., Klis, F. M., Robbins, P. W. and Bussey, H. (1997) Large scale identification of genes involved in cell surface biosynthesis and architecture in Saccharomyces cerevisiae. *Genetics*, **147,** 435-50.

66. Friden, P. and Schimmel, P. (1987) LEU3 of Saccharomyces cerevisiae encodes a factor for control of RNA levels of a group of leucine-specific genes. *Mol. Cell. Biol.*, **7,** 2708-17.

67. Zhou, K. M., Bai, Y. L. and Kohlhaw, G. B. (1990) Yeast regulatory protein LEU3: a structure-function analysis. *Nucleic Acids Res.*, **18,** 291-8.

68. Qui, H. F., Dubois, E. and Messenguy, F. (1991) Dissection of the bifunctional ARGRII protein involved in the regulation of arginine anabolic and catabolic pathways. *Mol. Cell. Biol.*, **11,** 2169-79.

69. Hedges, D., Proft, M. and Entian, K. D. (1995) CAT8, a new zinc cluster-encoding gene necessary for derepression of gluconeogenic enzymes in the yeast Saccharomyces cerevisiae. *Mol. Cell. Biol.*, **15,** 1915-22.

70. Mendizabal, I., Rios, G., Mulet, J. M., Serrano, R. and de Larrinoa, I. F. (1998) Yeast putative transcription factors involved in salt tolerance. *FEBS Lett.*, **425,** 323-8.

71. Gray, W. M. and Fassler, J. S. (1996) Isolation and analysis of the yeast TEA1 gene, which encodes a zinc cluster Ty enhancer-binding protein. *Mol. Cell. Biol.*, **16,** 347-58.

72. Rottensteiner, H., Kal, A. J., Filipits, M., Binder, M., Hamilton, B., Tabak, H. F. and Ruis, H. (1996) Pip2p: a transcriptional regulator of peroxisome proliferation in the yeast Saccharomyces cerevisiae. *EMBO J.*, **15,** 2924-34.

73. Lucau-Danila, A., Wysocki, R., Roganti, T. and Foury, F. (2000) Systematic disruption of 456 ORFs in the yeast Saccharomyces cerevisiae. *Yeast*, 16, 547-52.

Syst. name	Strain name	Background	Deletion	Marker	Source
YBL066C	BE	BY4742	ORF	KAN	Res. Genetics
YBR033W	FI	FY73	a.a. 27-200	HIS3	This study
YBR150C	FA	FY73	a.a. 24-167	HIS3	This study
YBR239C	YD	YPH499	ORF	HIS3	This study
YBR240C	FB	FY73	a.a. 25-166	HIS3	This study
YCR106W	FC	FY73	a.a. 23-206	HIS3	This study
YDR213W	BZY	BY4742	ORF	KAN	Res. Genetics
YDR303C	BZX	BY4743	ORF	KAN	Res. Genetics
YDR421W	BZD	BY4742	ORF	KAN	Res. Genetics
YDR520C	YZN	YPH499	ORF	HIS3	This study
YER184C	FZT	FY73	a.a. 26-225	HIS3	This study
YFL052W	YZS	FY73	a.a. 24-173	HIS3	This study
YHR178W	BT	BY4742	ORF	KAN	Res. Genetics
YIL130W	FZG	FY73	a.a. 24-246	HIS3	This study
YJL089W	BR	BY4742	ORF	KAN	Res. Genetics
YJL103C	YZL	YPH499	ORF	HIS3	This study
YJL206C	FZQ	FY73	a.a. 28-149	HIS3	This study
YKL222C	YH	YPH499	ORF	HIS3	This study
YKR064W	YK	YPH499	ORF	HIS3	This study
YLL054C	FZI	FY73	a.a. 25-220	HIS3	This study
YLR228C	YN	YPH499	ORF	HIS3	This study
YLR266C	FS	FY73	a.a. 25-246	HIS3	This study
YLR278C	YO	YPH499	ORF	HIS3	This study
YML076C	BZM	BY4742	a.a. 41-912	KAN	Res. Genetics
YMR019W	YZE	YPH499	ORF	HIS3	This study
YNR063W	FZO	FY73	a.a. 25-166	HIS3	This study
YOL089C	FZJ	FY73	a.a. 115-319	HIS3	This study
YOR162C	FZU	FY73	a.a. 28-332	HIS3	This study
YOR172W	YZV	YPH499	ORF	HIS3	This study
YOR380W	FZP	FY73	a.a.25-196	HIS3	This study
YPL133C	FZH	FY73	a.a. 25-266	HIS3	This study
YPR094W	BZZA	BY4742	ORF	KAN	Res. Genetics
YPR196W	YZ	YPH499	ORF	HIS3	This study

Table 1

Strains used in this study

Strains used in this study are listed. The names of the targeted ORFs are given ("Syst. name") as well as the wild type strains used to perform the deletions ("background"). The entire ORFs or,

alternatively, the sequences encompassing the cysteine-rich region (putative DNA binding domain) were deleted as indicated in "deletion". Markers used to select for recombination events are also listed. For more details, see Materials and Methods.

Strain	Syst. Name	Gene	Carbon Source		Nitrogen source					Minimal (Halvorson)				Sensitivity to Temperature				Calcofluor	Caffeine	Anaerobic Conditions	
			Glycerol	Lactate	Urea	GABA	Serine	Threonine	Proline	"All"	No biotin	No Pantho. No folate		37°C YPD	37°C SD	20°C YPD	20°C SD			YPD	SD
BE	YBL066C	SEF1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FI	YBR033W	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FA	YBR150C	TBS1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
YD	YBR239C	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FB	YBR240C	THI2 (PHO6)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FC	YCR106W	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BZY	YDR213W	UPC2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BZD	YDR421W	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
YZN	YDR520C	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	X	+	+
FZT	YER184C	-	XXX	XXX	+	+	+	+	+	+	+	+	+	+	+	+	+	XXX	+	+	+
YZS	YFL052W	-	XXX	XXX	+	+	+	+	+	+	+	+	+	XXX	+	+	+	XX	+	+	+
BT	YHR178W	STB5	+	+	X	X	X	X	X	X	X	X	X	+	+	XXX	XXX	+	XXX	+	+
FZG	YIL130W	-	XXX	XXX	+	+	+	+	+	+	+	+	+	+	+	+	+	XXX	+	+	+
BR	YJL089W	SIP4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
YZL	YJL103C	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FZQ	YJL206C	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
YH	YKL222C	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	XX	+	+
YK	YKR064W	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FZI	YLL054C	-	XXX	XXX	+	+	+	+	+	+	+	+	+	+	+	+	+	XXX	+	+	+
YN	YLR228C	ECM22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	XX	+	+
FS	YLR266C	-	XXX	XXX	+	+	+	+	+	+	+	+	+	+	+	+	+	XXX	+	+	+
YO	YLR278C	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	XX	+	+
BZM	YML076C	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
YZE	YMR019W	STB4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	XXX	+	+
FZO	YNR063W	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FZJ	YOL089C	HAL9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FZU	YOR162C	YRR1	XXX	XXX	+	+	+	+	+	+	+	+	+	+	+	+	+	XXX	+	+	+
YZV	YOR172W	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FZP	YOR380W	-	XXX	XXX	+	+	+	+	+	+	+	+	+	+	+	+	+	XXX	+	+	+
FZH	YPL133C	-	XXX	XXX	+	+	+	+	+	+	+	+	+	+	+	+	+	XXX	+	+	+
BZZA	YPR094W	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
YZ	YPR196W	MAL63	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2
Phenotypes resulting from deletions of genes encoding putative zinc cluster proteins

Growth of deletion strains was assayed under various conditions as described in Materials and Methods. In the column "Nitrogen source", "GABA" is the abbreviation for γ–aminobutyric acid. In the column "Halvorson", "no pantho" refers to the lack of pantothenic acid in the Halvorson medium and "all" corresponds to Halvorson medium containing biotin, pantothenic acid and folate. In the column "Calcofluor", sensitivity of the deletion strains to the compound calcofluor white is listed. No growth (or severely impaired growth) is indicated by "XXX"; moderate growth by "XX"; slightly inhibited growth by "X"; normal growth by "+".

Systematic name	GENE	FUNCTION	PHENOTYPE	REF.
YAL051W	OAF1 (YAF1)	Activator of peroxisome proliferation along with Oaf2p	Impaired in growth of oleate as a carbon source, induction of β-oxidation enzymes is abolished	19
YBL005W	PDR3	Activator related to Pdr1p	Pleiotropic drug resistance	6, 44
YBL066C	SEF1	Suppressor of essential function	Defective sporulation; high copy number suppressor of RPM2	45
YBR033W	-	Unknown	Unknown	-
YBR150C	-	Unknown	Unknown	-
YBR239C	-	Unknown	Unknown	-
YBR240C	THI2 (PHO6)	Activator of thiamin biosynthetic genes	Thiamin auxotrophy, reduced expression of thiamin biosynthetic genes	40
YBR297W	MAL3R (MAL33)	Part of complex locus MAL3; MAL activator protein	Defective maltose fermentation	46, 47
YCR106W		Unknown	Unknown	-
YDL170W	UGA3	Activator necessary for GABA-dependant function of GABA genes	Exhibits defects in activation of UGA1 and UGA4	48, 49
YDR034C	LYS14	Transcriptional activator of lysine pathway genes	Lysine requiring	50, 51
YDR207C	UME6	Regulator of both repression and induction of early meiotic genes	Exhibits defects in IME1-dependant activation and repression	52
YDR213W	UPC2	Involved in sterol uptake	upc2-1 allele shows altered sterol uptake and increased sensitivity to NaCl and LiCl	53, 54
YDR303C	-	Unknown	Null mutant is inviable	This study, 32, 73
YDR421W	-	Unknown	Moderately sensitive to SDS and benomyl	43
YDR520C	-	Unknown	Slight caffeine sensitivity	This study
YER184C	-	Unknown	Unable to grow on non-fermentable carbon sources Sensitve to calcofluor white	This study
YFL052W	-	Unknown	Unable to grow on non-fermentable	This study

			carbon sources Sensitve to calcofluor white Heat sensitive on rich but not minimal medium	
YGL013C	PDR1 (CYH3)	General positive regulator of permeability genes	Pleiotropic drug resistance	6, 55
YGR288W	MAL13	Part of complex locus MAL1 MAL activator protein (non functional in S228C and derivatives)	Defective maltose fermentation	46, 47
YHR178W	STB5	Binds Sin3p in two-hybrid assay	Cold and caffeine sensitive	This study and 39
YIL130W	-	Unknown	Unable to grow on non-fermentable carbon sources Sensitive to calcofluor white, cyclheximide, benomyl, and MMS. Slightly sensitive to hydoxyurea	This study and 43
YIR023W	DAL81 (UGA35)	Positive regulator of multiple nitrogen catabolic genes such as allantoin and GABA catabolic genes	Unable to degrade allantoin	49, 56, 57
YJL089W	SIP4	Involved in Snf1p regulated transcriptional activation	Required for maximal expression of carbon-source responsive genes Moderate sensitivity to SDS	58, 59, 43
YJL103C	-	Unknown	Unknown	-
YJL206C	-	Unknown	Unknown	-
YKL015W	PUT3	Positive regulator of PUT (proline utilization) genes	Unable to use proline as sole nitrogen source	60, 61
YKL038W	RGT1	Transcriptional repressor and activator of genes involved in glucose metabolism	Constitutive expression of glucose-induced HXT genes Sensitive to calcofluor white Sensitive to SDS, unable to grow on non-fermentable carbon source. Slight sensitivity to MMS	11, 43
YKL222C	-	Unknown	Moderately sensitive to caffeine	This study
YKR064W	-	Unknown	Unknown	-
YLL054C	-	Unknown	Unable to grow on non-fermentable carbon sources Sensitive to calcofluor white	This study
YLR014C	PPR1	Activator of URA1 and URA3	Deficient in pyrimidine biosynthetic pathway	62, 63

YLR098C	CHA4 (SIL2)	Activator of CHA1	Unable to grow with serine or threonine as sole nitrogen source	64
YLR228C	ECM22	Unknown	Moderately sensitive to caffeine	This study and 65
YLR256W	HAP1 (CYP1)	Activator of respiration genes	Essential for anaerobic or heme deficient growth Sensitive to SDS and EGTA. Moderately sensitive to hygromycin	4, 5, 43
YLR266C		Unknown	Unable to grow on non-fermentable carbon sources Sensitive to calcofluor white	This study
YLR278C	-	Unknown	Moderately sensitive to caffeine	This study
YLR451W	LEU3	Regulates expression of genes involved in branched chain amino acid biosynthesis and ammonia assimilation	Leaky leucine auxotroph	66, 67
YML076C		Unknown	Unknown	-
YML099C	ARG81 (ARGR2)	Positive and negative regulator of many arginine-responsive genes		68
YMR019W	STB4	Binds Sin3p in two-hybrid assay	Sensitive to caffeine	This study and 39, 43
YMR168C	CEP3	Cbf3 kinetochore complex binds CDE III centromere element	Essential gene	34, 35
YMR280C	CAT8	Involved in activation of gluconeogenic genes	Unable to grow on non-fermentable carbon sources and ethanol	69
YNR063W	-	Unknown	Unknown	-
YOL089C	HAL9	Involved in salt tolerance	Exhibits decreased salt tolerance and ENA1 expression	70
YOR162C	YRR1	Activator of multi-drug resistance genes	Hypersensitive to 4-nitroquinoline oxide (4-NQO) and benomyl Unable to grow on nonfermentable carbon sources Sensitive to calcofluor white	This study and 38, 43
YOR172W	-	Unknown	Unknown	-
YOR337W	TEA1	Activator of Ty1 elements	Diminished Ty1 expression Sensitive to SDS and moderately sensitive to MMS	71, 43

YOR363C	OAF2 (PIP2)	Activator of peroxisome proliferation along with Oaf1p	Impaired in growth of oleate as a carbon source, induction of β-oxidation enzymes is abolished	19, 72
YOR380W	-	Unknown	Unable to grow on non-fermentable carbon sources Sensitive to calcofluor white	This study
YPL133C	-	Unknown	Unable to grow on non-fermentable carbon sources Sensitive to calcofluor white	This study
YPL248C	GAL4	Activator of GAL genes	Cannot utilize galactose as sole carbon source	3
YPR094W	-	Unknown	Unknown	-
YPR196W	MAL63	Activator of maltose genes	Unable to ferment maltose	46, 47

Table 3

Summary of the phenotypes and functions of zinc cluster genes.

Data are derived from this study, the large scale transposon analysis of Ross-MacDonald et al. (42, 43) as well as other sources as indicated in the table.

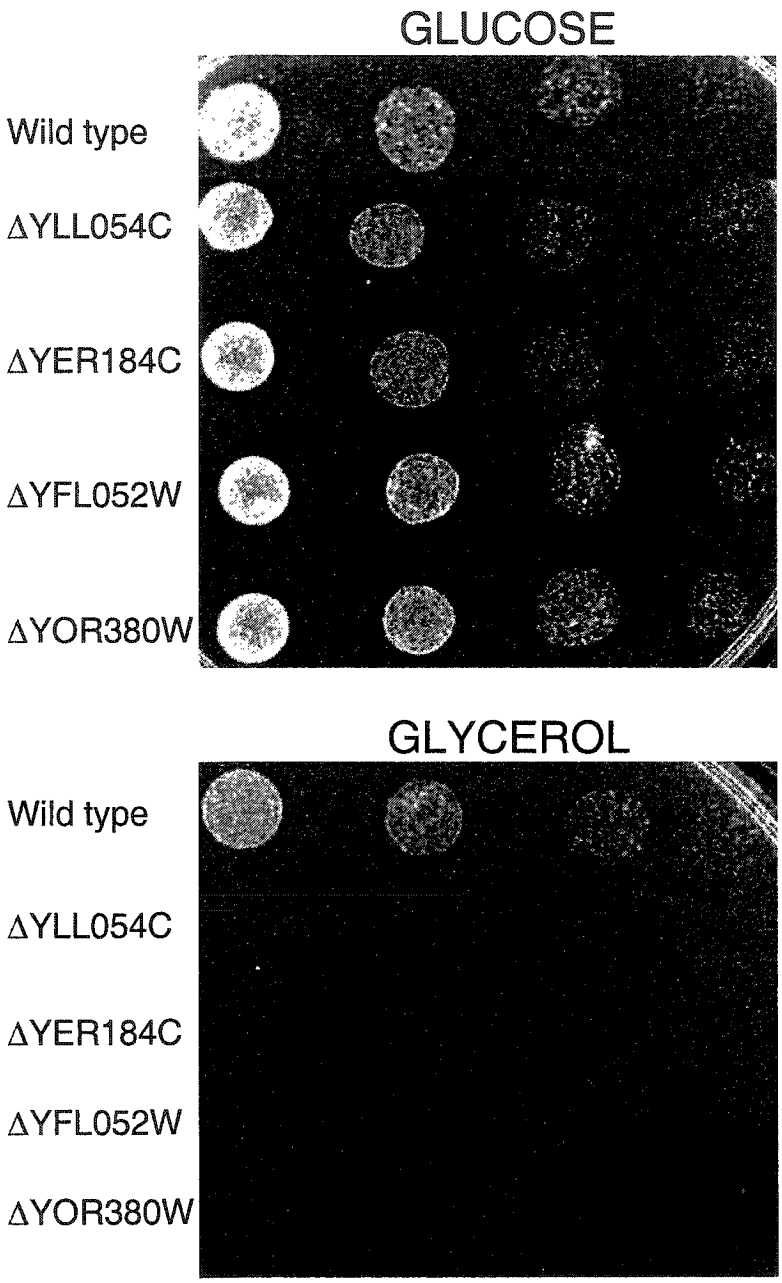

Figure 1

Growth on non-fermentable carbon source of selected strains.

Wild type and deletion strains were grown overnight in YEP-glucose, washed, serially diluted and spotted on YEP-glucose or YEP-glycerol plates, as indicated. Wild type strain is FY73. For more details, see Materials and Methods.

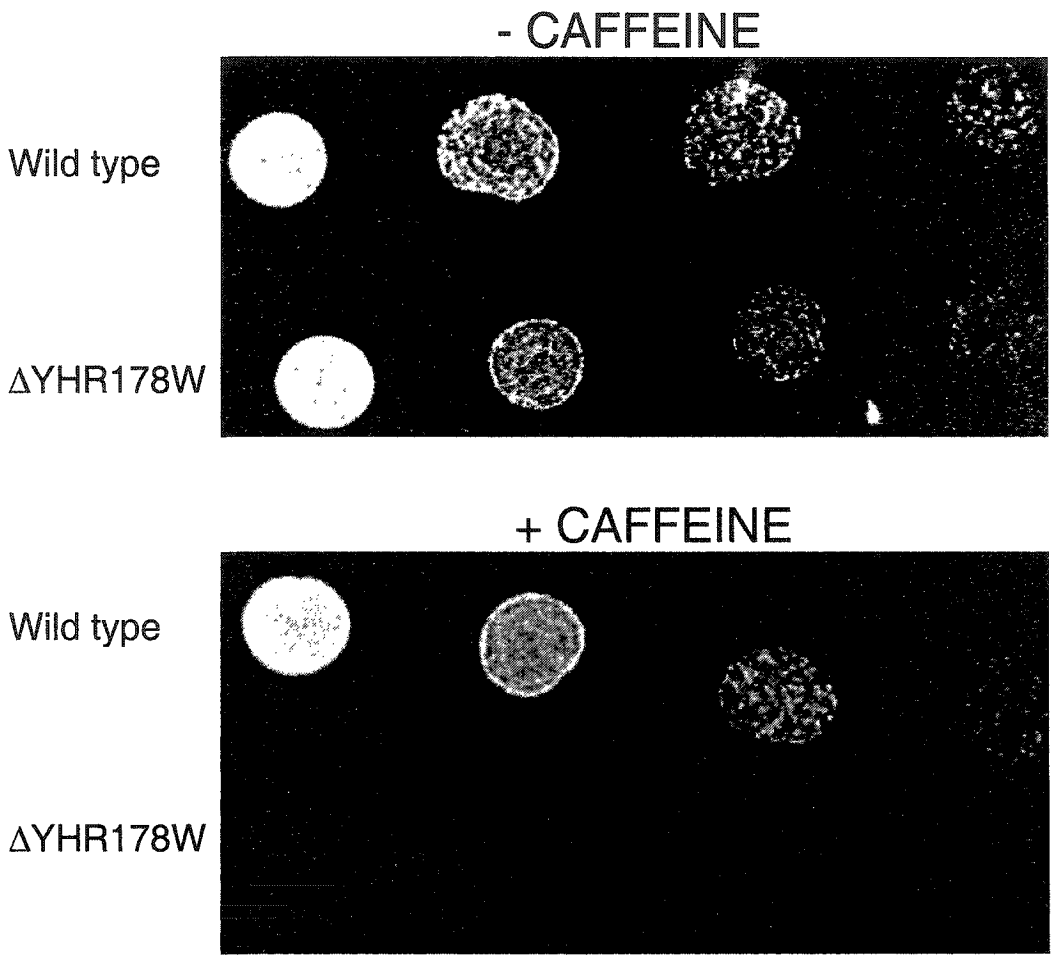

Figure 2

Caffeine sensitivity in YHR178W (STB5) deleted strain

Wild type (BY4742) and deletion strain (BT, Table 1) were grown overnight in YPD, washed, serially diluted and spotted on YPD plates containing or not 0.15% caffeine.

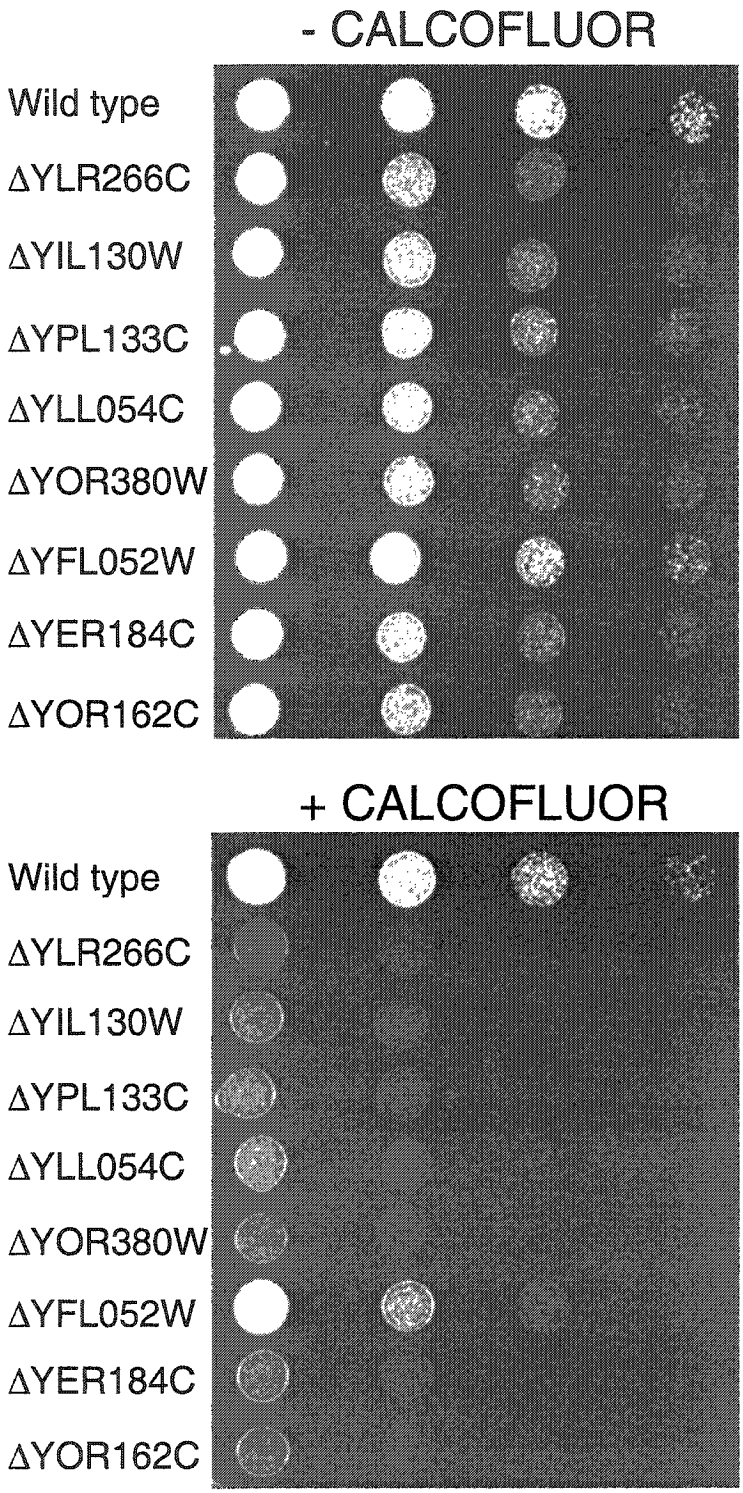

Figure 3

Calcofluor white sensitivity in deletion strains

Wild type (FY73) and deletion strains were grown overnight in YPD, washed, serially diluted and spotted on YPD plates with or without 70 μg/ ml calcofluor white. ORF deletions are indicated on left.

CONNECTING TEXT

Our phenotypic analysis assigned phenotypes to a number of zinc cluster protein deletion strains, thereby implicating them in a variety of processes within the cell. All that was known about a number of these zinc cluster proteins was their sequence, and these phenotypes were the first indication of what role these proteins may play in the cell. However, a number of zinc cluster proteins did not demonstrate any phenotype under the conditions tested. Since some zinc cluster proteins are regulators of genes involved in PDR, we decided to determine if additional zinc cluster proteins could be involved in this process. We conducted a phenotypic analysis using the same deletion strains as before, but growing them in the presence of various drugs. Each toxic compound tested targets various pathways within the cell.

SECTION 3:
NEW REGULATORS OF DRUG SENSITIVITY IN THE FAMILY OF YEAST ZINC CLUSTER PROTEINS

Running title:

Regulators of drug sensitivity

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ABSTRACT

The Gal4p family of yeast zinc cluster proteins comprises over fifty members that are putative transcriptional regulators. For example, Pdr1p and Pdr3p activate multidrug resistance genes by binding to pleiotropic drug response elements (PDREs) found in promoters of target genes such as *PDR5* encoding a drug efflux pump involved in resistance to cycloheximide. However, the role of many zinc cluster proteins is unknown. We tested a panel of strains carrying deletions of zinc cluster genes in the presence of various drugs. One deletion strain (Δrdr1) was resistant to cycloheximide while eight strains showed sensitivity to the antifungal ketoconazole or cycloheximide. Unnamed zinc cluster genes identified in our screen were called *RDS* for regulators of drug sensitivity.

RNA levels of multidrug resistance genes such as *PDR16*, *SNQ2* and *PDR5* were decreased in many deletion strains. For example, cycloheximide sensitivity of a *Δstb5* strain was correlated with decreased RNA levels and promoter activity of the *PDR5* gene. We tested if activation of *PDR5* is mediated via a PDRE by inserting this DNA element in front of a minimal promoter linked to the lacZ gene. Strikingly, activity of the reporter was decreased in a *Δstb5* strain. The purified DNA binding domain of Stb5p bound to a PDRE *in vitro*. Mutations in the PDRE known to affect binding of Pdr1p/Pdr3p showed similar effects when assayed with Stb5p. These results strongly suggest that Stb5p is a transcriptional activator of multidrug resistance genes. Thus, we have identified new regulators of drug sensitivity in the family of zinc cluster proteins.

INTRODUCTION

Multidrug or pleiotropic drug resistance (PDR)[1] is a phenomenon found in various organisms, ranging from prokaryotes to eukaryotes, such as yeast and humans. The ability of cells to become resistant to toxic compounds such as drugs is of major importance since the treatment of many diseases is hampered either by the ability of the body's own malignant cells, or of foreign pathogenic organisms to develop PDR and thereby become resistant to drugs. *Saccharomyces cerevisiae* has been widely used to study PDR, allowing us to gain insight into the mechanisms behind PDR in pathogenic fungi and in higher eukaryotes.

There are mainly three types of proteins involved in PDR: 1) ATP-Binding-Cassette (ABC) proteins, 2) Major Facilitator Superfamily (MFS) proteins, and 3) Transcription factors. ABC proteins are found in organisms ranging from bacteria to humans, and are involved in many important processes in the cell. (1,2) Most ABC proteins are ATP-powered membrane translocators, although some function as ion channels, channel regulators, receptors, proteases and sensing proteins. (3) ABC proteins are able to translocate a wide variety of compounds including ions, heavy metals, anticancer drugs, steroids, mycotoxins, antibiotics and whole proteins. (1,4-6). Two well-characterized ABC transporters, Pdr5p and Snq2p, confer PDR. They are functional homologues of mammalian P-glycoprotein. (7,8). Contrary to ABC proteins, MFS

[1] The abbreviations used are: WT, wild-type; PDR, pleiotropic drug resistance; MFS, major facilitator superfamily; ABC, ATP binding cassette; ORF, open reading frame; PCR, polymerase chain reaction; PDRE, pleiotropic drug resistance element. *RDS*, regulator of drug sensitivity; EMSA, electrophoretic

members do not use ATP. Instead, proton-motive force is used to transport substrates across the membrane. Atr1p is one member of the MFS shown to be involved in drug resistance. (9)

Various transcription factors have been shown to regulate the expression of genes encoding ABC or MFS proteins (10). There are two major families of transcription factors involved in PDR: 1) the bZip protein family (Yap family), and 2) zinc cluster proteins. Yap1p is the best characterized member of the bZip family and is an important regulator in the stress response. (11-13) Yap1p regulates the expression of the ABC transporter, Ycf1p. (14). Another class of transcription factors involved in PDR is composed of zinc cluster or binuclear zinc cluster proteins. They form a family of transcription factors found exclusively in fungi. Zinc cluster proteins are characterized by a zinc finger which contains the Zn(II)2Cys6 (or C6 zinc) binuclear cluster DNA-binding motif with the consensus sequence of $CysX_2CysX_6CysX_{5\text{-}12}CysX_2CysX_{6\text{-}8}Cys$. The cysteines mediate the binding of two zinc atoms, which are necessary for the zinc finger to bind DNA. (15,16) Many zinc cluster proteins bind DNA as homodimers to recognition sites that usually fall within three types: inverted, direct and everted repeats. (17) These proteins have been shown to be involved in various processes in the cell including regulation of primary and secondary metabolism, drug resistance and meiotic development (18). For example, Gal4p is involved in the activation of genes that encode enzymes for galactose metabolism, (19) while Hap1p activates genes involved in

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mobility shift assay; GST, glutathione S-transferase; 4-nitroquinoline *N*-oxide, 4-NQO; a.a., amino acids; DBD, DNA binding domain; bp, base pairs.

respiration. (20,21) Two zinc cluster proteins, Pdr1p and Pdr3p, have been shown to positively control the expression of genes involved in multidrug resistance. (10).

Target genes of Pdr1p and Pdr3p include *PDR5*, *SNQ2*, and *YOR1* encoding ABC transporters as well as *HXT9* and *HXT11* encoding hexose transporters which belong to the MFS family (22-25). Overexpression of the ABC transporters renders yeast resistant to drugs. However the overexpression of the hexose transporters leads to drug sensitivity. Even though Pdr1p and Pdr3p recognize the same pleiotropic drug response element (PDRE), with Pdr3p binding an everted repeat CCGCGG, they have different roles. (26,27) The *PDR3* promoter contains two PDREs, allowing for autoregulation. (26). Another zinc cluster protein, Yrr1p, is implicated in PDR. For example, Yrr1p has been shown to regulate the expression of *SNQ2* (28).

The yeast genome contains 55 genes encoding putative zinc cluster proteins (for a complete list see Akache et al. 29 and ref. 30). However, the function of many of these putative zinc cluster proteins is unknown. A phenotypic analysis was carried out on thirty-three genes encoding yeast zinc cluster proteins in order to better understand their role (29). For example, we have shown that deletion of 8 different zinc cluster genes impairs growth on non-fermentable carbon sources. In this study, we have extended our previous analysis by assaying the growth of these deletion strains in the presence of various drugs. Our results show that nine of these deletion strains are either resistant or sensitive to at least one drug.

EXPERIMENTAL PROCEDURES

Strains

The wild-type strain used was BY4742, MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* (31). The deletion strains were obtained from Research Genetics (Huntsville, Al) (32). Deletions for a number of strains were verified by Southern blot analysis (see list below). Research Genetics strain number 11677 does not carry a deletion of the ORF *YOR380W*[2]. Deletion of the *YOR380W* ORF was performed using the PCR method of Baudin et al. (33) using oligos with homology to the target gene at their 5' end and 3' sequences complementary to the KanMX ($G418^R$) selection marker. Plasmid pFA6 (34) was used as a template for PCR with the oligonucleotides TAACTTAGCGCACACTTTCCTACTTTAAGCTCACCAAATGTGGGCCACAGAAGCAACTC ACGTACGCTGCAGGTCGAC and AATTTGCTTCTCGATACACATAATCTATAATACTCTTTTATCTGGCGACGCTATGACGT ATCGATGAATTCGAGCTCG

Media and Drug Assays

Media were prepared according to Adams et al. (35). YPD contained 1% yeast extract, 2% peptone, 2% glucose. SD contained 2% glucose, 0.67% yeast nitrogen base (without amino acids) and was supplemented with adenine and appropriate amino acids at a final concentration of 0.004%. Drugs were obtained from Sigma. Stock solutions were prepared as described below and stored at –20 °C. Cycloheximide, 2 mg/ml in 100% ethanol; ketoconazole, 5 mg/ml in H_2O; chloramphenicol 34 mg/ml in 100% ethanol; 4-

nitroquinoline *N*-oxide (4-NQO), 10 mg/ml in dimethyl sulfoxide; rhodamine 6-G 10 mg/ml in 100% ethanol; oligomycin, 5 mg/ml in 100% ethanol. Cycloheximide, ketoconazole, chloramphenicol, and 4-nitroquinoline *N*-oxide assays were performed with glucose as a carbon source while rhodamine 6-G and oligomycin were tested with glycerol as a carbon source. Concentrations of drugs used for the assays are indicated in Table 2.

β-galactosidase assays

The *lacZ* reporters PDR5-lacZ and SNQ2-lacZ have been described previously (36). Briefly, the reporters are low copy plasmids (ARSCEN) containing a *URA3* marker. The PDR5 and SNQ2 reporters contain 1000 bp and 700 bp of sequences upstream of the ATG, respectively. Reporters PDRE3-lacZ, PDRE3A-lacZ and PDRE3B lacZ are high copy (2 micron) URA3 marked plasmids containing a single Pdr1/Pdr3p binding site inserted upstream of minimal CYC1 promoter driving lacZ transcription (36). β-galactosidase assays were performed as described previously (36) with permeabilized cells. Results were obtained from at least two independent transformations performed at least with duplicate samples. Variation between duplicates was typically less than 20%.

Southern and Northern Blot analysis

Northern blot analysis and probes have been described previously (36). Southern blot analysis was performed as described (37) and the probe was obtained by purifying a KAN^R fragment by digesting pFA6 (34) with ClaI. Strains *YBR033W, YBR150C,*

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YBR239C, YDR520C, YJL103C, YKR064W, YLR228C, YLR278C, YMR019W, YPR196W were verified by Southern blot analysis and strains *YBL066C, YDR213W, YDR421W, YHR178W, YJL089W, YML076C, YPR094W* had been characterized previously (29). Research Genetics deletion strain #11677 (*YOR380W*) did not give a band of the expected size with a probe corresponding to the promoter region of the *YOR380W* gene (data not shown, see also section "Strains").

Electrophoretic Mobility Shift Assay (EMSA)

A DNA fragment encoding the DNA-binding domain of Stb5p (a.a. 1- 163) was amplified by PCR using the oligos CGGGATCCATGGATGGTCCCAATTTTGC and GGAATTCCTTGGTACGTCTTGGGGCTC and genomic DNA (isolated from strain YPH499, ref. 38) as a template. The PCR product was digested with BamHI and EcoRI and subcloned into plasmid pGEX-F (27) cut with the same enzymes to give pGST-STB5. The DNA-binding domains of Stb5 and Pdr3p fused to GST were expressed in *E. coli* and purified as described (27). The GST moiety was removed by thrombin cleavage. EMSA was performed according to (27). The probes used in the EMSA correspond to site number 3 of the *PDR5* promoter (39) and span sequences -372 to -337 bp relative to the ATG. Oligos were annealed and filled-in with Klenow and dGTP, dTTP, dATP and [^{32}P]dCTP.

Oligos for PDRE3:

TCGAAAAAGAGAAATGTCTCCGCGGAACTCTTCTACGCCG and its complement

TCGACGGCGTAGAAGAGTTCCGCGGAGACATTTCTCTTTT

PDRE3A:

TCGAAAAAGAGAAATGTCTC**T**GCGGAACTCTTCTACGCCG and its complement

TCGACGGCGTAGAAGAGTTCCGC**A**GAGACATTTCTCTTTT

PDRE3B:

TCGAAAAAGAGAAATGTCTCCGC**A**GAACTCTTCTACGCCG and its complement

TCGACGGCGTAGAAGAGTTC**T**GCGGAGACATTTCTCTTTT

(mutations are in bold characters and underlined).

RESULTS

Our study focused on 32 members of the Gal4p family of yeast zinc cluster proteins (Table 1). Many members are putative proteins of unknown function. We determined if these zinc cluster genes play a role in multidrug resistance by testing the ability of strains carrying deletions of these genes to grow in the presence of 6 different drugs: cycloheximide, ketoconazole, chloramphenicol, 4-NQO, rhodamine 6-G and oligomycin. The mode of action of these drugs is listed in Table 2. Wild-type and deletion strains were serially diluted and spotted on plates containing the drugs and grown for the time indicated in Table 2. As expected (28), deletion of *YRR1* resulted in hypersensitivity to the mutagen 4-NQO (Table 3). However, none of the thirty-one other strains showed altered sensitivity to 4-NQO, oligomycin, rhodamine 6-G and chloramphenicol (data not shown).

When assayed with the antifungal ketoconazole or the translation inhibitor cycloheximide, nine strains demonstrated a clear phenotype with at least one drug (Table 3). Three of the genes deleted were not named previously. Since they potentially encode transcriptional regulators and show altered drug sensitivity, we named them *RDS1* to *3* (for regulator of drug sensitivity, see Tables 2 and 3). Two strains (Δ*upc2* and Δ*rds2*) were hypersensitive to ketoconazole (Fig. 1). Deletion of *RDS3* resulted in a slightly decreased resistance as seen from the reduced number of colonies at low cell concentration. The Δrds3 strain was also hypersensitive to cycloheximide (see below). Moreover, seven strains revealed a phenotype when grown in the presence of cycloheximide. One strain (Δrdr1) was resistant to that drug. The same phenotype was

observed when *RDR1* was deleted in the strain FY73 (36). A more detailed analysis of *RDR1* will be presented elsewhere (36). Strains carrying deletions of *YIL130W* or *YKL222C* were slightly resistant to cycloheximide (data not shown). Because of the subtle phenotype observed with these two genes, they were not scored as regulators of drug sensitivity. Six other deletion strains showed sensitivity to cycloheximide (Fig. 2). For example, deletion of *STB5* or *RDS3* abolished growth on plates containing cycloheximide while normal growth was observed in the absence of the drug when compared to the wild-type strain. Two strains showed phenotypes on more than one drug: strain: Δ*yrr1* was sensitive to 4-NQO and cycloheximide while Δrds3 was sensitive to both ketoconazole and cycloheximide. In summary, our study has assigned new drug sensitivity phenotypes for nine genes encoding zinc cluster proteins.

Deletion strains that showed a phenotype most probably lack a transcriptional regulator. Thus, we tested if these strains had altered expression of selected genes involved in multidrug resistance. RNA was isolated from the wild-type strain and the deletion strains that showed altered drug sensitivity and probed for *PDR5*, *SNQ2* and *PDR16* mRNAs (Fig. 3). As stated above, *SNQ2* and *PDR5* encode multidrug transporters. For example, Pdr5p has been shown to be a major mediator of cycloheximide resistance (40-42). As expected (28), the level of *SNQ2* mRNA was reduced in cells lacking *YRR1* (Fig. 3, lane 8). Interestingly, SNQ2 RNA was also reduced in a Δ*stb5* strain (Fig. 3, lane 11). However, actin level was also reduced with that strain. We doubled the amount of Δ*stb5* RNA and repeated the Northern blot analysis (Fig. 3, lanes 12 and 13). Clearly, the levels of SNQ2 RNA were reduced in a Δ*stb5*

strain while signals with an actin probe were similar in wild-type and deletion strains. The levels of *PDR16* mRNA were reduced in Δ*ecm22*, Δ*rds2*, Δ*hal9* and Δ*stb5* strains as compared to the wild-type strain (Fig. 3, compare lanes 4, 6, 7 and 11 with lane 1). *PDR5* mRNA levels were reduced in many strains, but the decrease was not as severe as with *PDR16* and *SNQ2*. Strains Δ*ecm22* and Δ*stb5* had the lowest amount of *PDR5* mRNA when compared to a wild-type strain, while a decrease was also observed in Δ*rds1*, Δ*rds2*, Δ*hal9*, Δ*upc2*, and Δ*rds3* strains. No major changes in *PDR5*, *PDR16* and *SNQ2* mRNAs were observed with deletion of ORFs *YKL22C* and *YIL130W*, in agreement with their slight resistance to cycloheximide. All the drug sensitive strains had lower mRNA levels for either one or more of the tested RNAs. Thus, the observed phenotypes correlate with the reduced amount of the tested mRNAs. Strikingly, a strain deleted of *STB5* is sensitive to cycloheximide and has reduced mRNA levels for *PDR5* (as well as *SNQ2* and *PDR16*). Our data strongly suggest that Stb5p is an additional regulator of genes encoding ABC transporters.

In order to determine if changes in *PDR5* and *SNQ2* mRNA levels are due to altered promoter activity, we transformed *PDR5* and *SNQ2* lacZ reporters into the wild-type and the deletion strains (Table 4). Only a slightly reduced activity of the SNQ2 reporter was observed with the Δyrr1 strain even though *SNQ2* mRNA levels were drastically reduced in the absence of Yrr1p. Similar results were obtained in another study (43). We do not know the reason for the discrepancy between the Northern blot analysis and the reporter assay. The activity of the *PDR5* reporter in strain Δ*hal9* was decreased about 2-fold while the activity of the *SNQ2* reporter was slightly decreased

(Table 4). Deletion of *STB5* decreased activity of the *PDR5* and *SNQ2* reporters 2- and 7-fold, respectively. In addition, deletion of *RDS3* decreased activity of the *SNQ2* and *PDR5* promoters 2- fold and 3-fold, respectively.

Since both *PDR5* and *SNQ2* promoters contain PDREs, known to be important in regulating transcription by binding of the transcriptional regulators Pdr1p and Pdr3p, we wanted to determine if the decrease in activity was mediated through this response element. A lacZ reporter was constructed with a PDRE (derived from the *PDR5* promoter) inserted upstream of a minimal CYC1 promoter driving lacZ transcription. Activity of that reporter was greatly increased (more than 50 fold) when compared to a similar construct lacking the PDRE (data not shown). No difference in activity of the PDRE-CYC1 reporter was observed between the wild-type and the strains deleted of *HAL9* or *RDS3* (Table 5). Therefore, the decreased activity of the *PDR5* reporter in Δ*hal9*, and Δ*rds3* strains may be due to an element other than the PDREs within the *PDR5* and *SNQ2* promoters (or indirect effects). However, deletion of *STB5* reduced activity of the PDRE-CYC1 reporter by a factor of 2.7 (Table 5). These results suggest that activation of the PDR5 and SNQ2 genes by Stb5p is mediated by PDREs. This possibility is supported by mutational analysis of the PDRE. Indeed, we tested two PDREs containing mutations located in either of the CGG triplets that are crucial for binding of Pdr3p (27). As expected, activity of the two mutants was decreased in a wild-type strain. A mutation in the first CGG triplet (mutant PDRE3A, Table 5) resulted in a modest decrease of activity in a Δ*stb5* strain as compared to the wild-type strain. However, mutating the second CGG triplet (mutant PDRE3B) reduced reporter activity

2.6 fold in cells lacking Stb5p. These results suggest that the first CGG triplet is important for maximal activation by Stb5p. In addition, our data suggest that Stb5p and Pdr1p/Pdr3p recognize highly related DNA elements.

Since our results suggest that Stb5p activates transcription through PDREs, we tested if it can bind directly to that DNA element. The putative DNA-binding domain (DBD) of Stb5p was fused to GST, expressed in bacteria, purified and the GST moiety removed by thrombin cleavage. The DBD of Stb5p was then assayed by EMSA using a Pdr1p/Pdr3 binding site (Fig. 4). In the presence of the DBD of Stb5p, two major retarded complexes were observed. It is possible that the two complexes correspond to monomeric and dimeric forms of Stb5p. Strikingly, mutations that prevent binding of the activator Pdr3p (ref. 27 and data not shown) also greatly diminished binding of Stb5p (Fig. 4, mutants PDRE3A and PDRE3B). Thus, our results strongly suggest that Stb5p activates transcription of multidrug resistance genes by binding to PDREs that are also recognized by the well-characterized activators Pdr1p and Pdr3p.

DISCUSSION

The zinc cluster proteins Pdr1p, Pdr3p and Yrr1p are well-known transcriptional activators of multidrug resistance genes (4,10,28). However, the role of many other zinc cluster proteins is unknown. We have performed a systematic phenotypic analysis of strains deleted of zinc cluster genes to determine if additional members of this family are involved in conferring multidrug resistance. Interestingly, we found that nine different strains lacking zinc cluster proteins showed a phenotype when assayed with the antifungal ketoconazole and the translation inhibitor cycloheximide (Table 3). Eight strains were sensitive to a drug, while one (Δrdr1) was resistant to cycloheximide. In another study (36), we performed whole-genome analysis of gene expression and have shown that Rdr1p is a transcriptional repressor of five genes including *PDR5*. Thus, the effect of Rdr1p is highly specific. For example, expression of SNQ2 is not affected by removal of Rdr1p while expression of *PDR5* is increased about 5-fold, in agreement with the increased cycloheximide resistance. Furthermore, we have shown that a PDRE derived from the *PDR5* promoter mediates the repression effect.

With the exception of *RDR1*, all strains were sensitive to drugs (Table 3). For example, Yrr1p was previously shown to confer 4-NQO resistance by controlling expression of *SNQ2* (28). Our results show that removal of Yrr1p also results in cycloheximide sensitivity. Similarly, Hal9p confers salt resistance (44) and our study shows that this protein is also involved in conferring resistance to cycloheximide. Upc2p and Ecm22p are activators of the sterol biosynthetic genes (45). Deletion of *UPC2* results

only in ketoconazole sensitivity while deletion of *ECM22* yields a strain sensitive to cycloheximide but not ketoconazole (Table 3, Figs 1 and 2). Moreover, *ECM22* but not *UPC2* is sensitive to caffeine (29), an inhibitor of the MAP kinase pathway and cAMP phosphodiesterase (46). Thus, even though Upc2p and Ecm22p have been shown to have overlapping functions (45), our phenotypic analysis suggests that they also have specific targets.

Other genes identified in our screen were not named previously and, because of their phenotype, they were called *RDS* for regulators of drug sensitivity. Two of these genes (*RDS1*, *RDS3*) are involved in conferring resistance to cycloheximide while the third one (*RDS2*) mediates ketoconazole resistance. Thus, we have identified additional zinc cluster proteins responsible for drug resistance. The number of strains scored with a phenotype in our screen may seem to be high when considering the numerous studies on multidrug resistance in yeast. However, one has to take into account that we have targeted the biggest family of transcriptional regulators in yeast.

Our phenotypic analysis raises the question of the mechanism of action of these zinc cluster proteins: do they play a direct role in regulating one or more genes involved in PDR, or do they have an indirect effect? To help distinguish between these two possibilities, we determined if expression of some genes implicated in multidrug resistance is affected by removal of zinc cluster proteins. Northern blot analysis showed that deletion of *STB5* greatly decreased RNA levels for *SNQ2* and *PDR16* (and to a lesser extent *PDR5*) while deletion of *YRR1* reduced *SNQ2* RNA (Fig. 3). Moreover, a strain

deleted of *RDS3* has lower *PDR5* mRNA levels. However, we did not observe significant changes in *PDR5*, *SNQ2* and *PDR16* RNA levels for many other strains that showed drug sensitivity. Multidrug resistance genes not tested in our study may be responsible for the observed phenotype. Whole-genome analysis of gene expression will be invaluable in identifying targets for these putative transcriptional regulators.

We then tested if activity of *PDR5* and *SNQ2* reporters is altered in deletion strains (Table 4). Reduced activity was observed in *Δstb5*, *Δhal9* and *Δrds3* strains in agreement with the Northern blot analysis. Similarly, a *SNQ2* reporter showed reduced activity in a *Δstb5* strain, in agreement with the reduced RNA levels. However, decreased activity of a SNQ2 reporter in a *Δrds3* strain does not correlate with the Northern blot analysis. We do not know the reason for this discrepancy.

In summary, our results show that deletion of *STB5* results in cycloheximide sensitivity and reduced *PDR5*, *SNQ2* and *PDR16* RNA levels. These observations are also correlated with decreased activity of *PDR5* and *SNQ2* reporters. Since the tested genes affected by removal of Stb5p all contain PDREs, we were interested in determining if the effect of Stb5p on gene expression is mediated by that DNA element. Strikingly, a reporter containing a PDRE inserted in front of a minimal CYC1 promoter showed decreased activity in a Δstb5 strain (Table 5). Additional support for a direct role of Stb5p in regulating transcription of PDR5 (and other genes) was provided by EMSA. Indeed, the purified DBD of Stb5p bound specifically to a PDRE (Fig. 4). Mutations known to reduce binding of Pdr3p also decreased binding of Stb5p.

Thus, our results strongly suggest that Stb5p activates multidrug resistance genes by binding directly to PDREs. The same DNA element is also recognized by the transcriptional activators Pdr1p and Pdr3p (23-25,39,47-50). Moreover, we have previously shown that another zinc cluster protein, Rdr1p, negatively regulates expression of *PDR5* by acting on a PDRE (36). Thus, the regulation of multidrug resistance genes via PDREs is more complex than initially anticipated. Even though our work strongly suggests that Stb5p is a transcriptional activator, previous studies have shown that it interacts with Sin3p in a two-hybrid assay (51). Sin3p represses gene expression by interacting with the histone deacetylase Rpd3p (52). Therefore, Stb5p may be both a positive and a negative regulator of gene expression as observed with the zinc cluster proteins Ume6p and Rgt1p (53,54).

Many questions remain to be answered. For example, does the binding affinity of Stb5p for different PDREs in the promoters of target genes differ? This would explain the differential effect of Stb5p on expression of the *SNQ2*, *PDR16* and *PDR5*. What is the mechanism of action of the zinc cluster proteins (other than Stb5p) identified in our screen? Importantly, our studies have identified new players involved in multidrug resistance. Our work also shows the power of a systematic functional genomic approach.

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REFERENCES

1. Higgins, C. F. (1992) *Annu. Rev. Cell. Biol.* **8,** 67-113

2. Michaelis, S., and Berkower, C. (1995) *Cold Spring Harb. Symp. Quant. Biol.* **60,** 291-307

3. Higgins, C. F. (1995) *Cell* **82,** 693-696

4. Bauer, B. E., Wolfger, H., and Kuchler, K. (1999) *Biochim. Biophys. Acta* **1461,** 217-36

5. Kuchler, K., and Thorner, J. (1992) *Endocrine Rev.* **13,** 499-514

6. Dean, M., and Allikmets, R. (1995) *Curr. Opin. Genet. Dev.* **5,** 779-85.

7. Balzi, E., and Goffeau, A. (1995) *J. Bioenerg. Biomembr.* **27,** 71-6.

8. Decottignies, A., and Goffeau, A. (1997) *Nature Genet.* **15,** 137-45

9. Gompel-Klein, P. (1990) *Curr. Genet.* **18,** 93-6

10. Koloczkowska, A., and Goffeau, A. (1999) *Drug Resist. Updates* **2,** 403-14

11. Wu, A., Wemmie, J. A., Edgington, N. P., Goebl, M., Guevara, J. L., and Moye-Rowley, W. S. (1993) *J. Biol. Chem.* **268,** 18850-8

12. Schnell, N., and Entian, K. D. (1991) *Eur. J. Biochem.* **200,** 487-93.

13. Gounalaki, N., and Thireos, G. (1994) *EMBO J.* **13,** 4036-41.

14. Wemmie, J. A., Szczypka, M. S., Thiele, D. J., and Moye-Rowley, W. S. (1994) *J. Biol. Chem.* **269,** 32592-7

15. Pan, T., and Coleman, J. E. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87,** 2077-81

16. Gardner, K. H., Pan, T., Narula, S., Rivera, E., and Coleman, J. E. (1991) *Biochemistry* **30,** 11292-302

17. Schwabe, J. W., and Rhodes, D. (1997) *Nat. Struct. Biol.* **4,** 680-3

18. Todd, R. B., and Andrianopoulos, A. (1997) *Fungal Genet. Biol.* **21,** 388-405

19. Lohr, D., Venkov, P., and Zlatanova, J. (1995) *FASEB J.* **9,** 777-87

20. Pfeifer, K., Kim, K. S., Kogan, S., and Guarente, L. (1989) *Cell* **56,** 291-301

21. Creusot, F., Verdiere, J., Gaisne, M., and Slonimski, P. P. (1988) *J. Mol. Biol.* **204,** 263-76

22. Decottignies, A., Lambert, L., Catty, P., Degand, H., Epping, E. A., Moye-Rowley, W. S., Balzi, E., and Goffeau, A. (1995) *J. Biol. Chem.* **270,** 18150-7

23. Katzmann, D. J., Burnett, P. E., Golin, J., Mahe, Y., and Moye-Rowley, W. S. (1994) *Mol. Cell. Biol.* **14,** 4653-61

24. Mahe, Y., Parle-McDermott, A., Nourani, A., Delahodde, A., Lamprecht, A., and Kuchler, K. (1996) *Mol. Microbiol.* **20,** 109-17

25. Nourani, A., Wesolowski-Louvel, M., Delaveau, T., Jacq, C., and Delahodde, A. (1997) *Mol. Cell. Biol.* **17,** 5453-60

26. Delahodde, A., Delaveau, T., and Jacq, C. (1995) *Mol. Cell. Biol.* **15,** 4043-50

27. Hellauer, K., Rochon, M.-H., and Turcotte, B. (1996) *Mol. Cell. Biol.* **16,** 6096-6102

28. Cui, Z., Shiraki, T., Hirata, D., and Miyakawa, T. (1998) *Mol. Microbiol.* **29,** 1307-15

29. Akache, B., Wu, K., and Turcotte, B. (2001) *Nucl. Acids Res.* **29,** 2181-90

30. Angus-Hill, M. L., Schlichter, A., Roberts, D., Erdjument-Bromage, H., Tempst, P., and Cairns, B. R. (2001) *Mol. Cell* **7,** 741-51

31. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) *Yeast* **14,** 115-32

32. Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., Chu, A. M., Connelly, C., Davis, K., Dietrich, F., Dow, S. W., M, E. L. B., Foury, F., Friend, S. H., Gentalen, E., Giaever, G., Hegemann, J. H., Jones, T., Laub, M., Liao, H., Liebundguth, N., Davis, R. W., and et al. (1999) *Science* **285,** 901-906

33. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F., and Cullin, C. (1993) *Nuc. Acids Res.* **21,** 3329-30

34. Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994) *Yeast* **10,** 1793-808

35. Adams, A., Gottschling, D. E., and Stearns, T. (1997) *Methods in yeast genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor

36. Hellauer, K., Akache, B., MacPherson, S., Sirard, E., and Turcotte, B. (2002) *J. Biol. Chem.* **277,** 17671-6.

37. Hellauer, K., Sirard, E., and Turcotte, B. (2001) *J. Biol. Chem.* **276,** 13587-92

38. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19-27

39. Katzmann, D. J., Hallstrom, T. C., Mahe, Y., and Moye-Rowley, W. S. (1996) *J. Biol. Chem.* **271,** 23049-54

40. Leppert, G., McDevitt, R., Falco, S. C., Van Dyk, T. K., Ficke, M. B., and Golin, J. (1990) *Genetics* **125,** 13-20

41. Meyers, S., Schauer, W., Balzi, E., Wagner, M., Goffeau, A., and Golin, J. (1992) *Curr. Genet.* **21,** 431-6

42. Hirata, D., Yano, K., Miyahara, K., and Miyakawa, T. (1994) *Curr. Genet.* **26,** 285-94

43. Cui, Z. F., Hirata, D., and Miyakawa, T. (1999) *Biosci. Biotechn. Biochem.* **63,** 162-167

44. Mendizabal, I., Rios, G., Mulet, J. M., Serrano, R., and de Larrinoa, I. F. (1998) *FEBS Lett.* **425,** 323-8

45. Vik, A., and Rine, J. (2000) *Mol. Cell. Biol.* **20,** 4381-92

46. Hampsey, M. (1997) *Yeast* **13,** 1099-133

47. Katzmann, D. J., Hallstrom, T. C., Voet, M., Wysock, W., Golin, J., Volckaert, G., and Moye-Rowley, W. S. (1995) *Mol. Cell. Biol.* **15,** 6875-83

48. Wolfger, H., Mahe, Y., Parle-McDermott, A., Delahodde, A., and Kuchler, K. (1997) *FEBS Lett.* **418,** 269-74

49. Hallstrom, T. C., and Moye-Rowley, W. S. (1998) *J. Biol. Chem.* **273,** 2098-104

50. Hallstrom, T. C., Lambert, L., Schorling, S., Balzi, E., Goffeau, A., and Moye-Rowley, W. S. (2001) *J. Biol. Chem.* **276,** 23674-80

51. Kasten, M. M., and Stillman, D. J. (1997) *Mol. Gen. Genet.* **256,** 376-86

52. Kadosh, D., and Struhl, K. (1997) *Cell* **89,** 365-71

53. Jackson, J. C., and Lopes, J. M. (1996) *Nucl. Acids Res.* **24,** 1322-9

54. Ozcan, S., Leong, T., and Johnston, M. (1996) *Mol. Cell. Biol.* **16,** 6419-26

55. Groom, K. R., Heyman, H. C., Steffen, M. C., Hawkins, L., and Martin, N. C. (1998) *Yeast* **14,** 77-87

56. Nishimura, H., Kawasaki, Y., Kaneko, Y., Nosaka, K., and Iwashima, A. (1992) *FEBS Lett.* **297,** 155-8

57. Crowley, J. H., Leak, F. W., Jr., Shianna, K. V., Tove, S., and Parks, L. W. (1998) *J. Bacteriol.* **180,** 4177-83

58. Iraqui, I., Vissers, S., Andre, B., and Urrestarazu, A. (1999) *Mol. Cell. Biol.* **19,** 3360-3371

59. Lesage, P., Yang, X., and Carlson, M. (1996) *Mol. Cell. Biol.* **16,** 1921-8

60. Vincent, O., and Carlson, M. (1998) *EMBO J.* **17,** 7002-7008

61. Needleman, R. (1991) *Mol. Microbiol.* **5,** 2079-84

Table 1

Syst. name	Gene	Function	Ref.
YBL066C	*SEF1*	Suppressor of essential function	(55)
YBR033W	-	Unknown	-
YBR150C	-	Unknown	-
YBR239C	-	Unknown	-
YBR240C	*THI2(PHO6)*	Activator of thiamin biosynthetic genes	(56)
YCR106W	*RDS1*	Regulator of drug sensitivity	This study
YDR213W	*UPC2*	Activator of sterol biosynthetic genes	This study, (45,57)
YDR421W	*ARO80*	Activator of the gene encoding aromatic aminotransferase	(58)
YDR520C	-	Unknown	-
YER184C	-	Unknown	-
YFL052W	-	Unknown	-
YHR178W	*STB5*	Binds Sin3p in two-hybrid assay	This study, (51)
YIL130W	-	Unknown	-
YJL089W	*SIP4*	Involved in Snf1p regulated transcriptional activation	(59,60)
YJL103C	-	Unknown	-
YJL206C	-	Unknown	-
YKL222C	-	Unknown	-
YKR064W	-	Unknown	-
YLL054C	-	Unknown	-
YLR228C	*ECM22*	Activator of sterol biosynthetic genes	This study, (45)
YLR266C	-	Unknown	-
YLR278C	-	Unknown	-
YML076C	-	Unknown	-
YMR019W	*STB4*	Binds Sin3p in two-hybrid assay	(51)
YNR063W	-	Unknown	-
YOL089C	*HAL9*	Involved in salt tolerance	This study, (44)
YOR162C	*YRR1*	Activator of multidrug resistance genes	This study, (28)
YOR172W	-	Unknown	-
YOR380W	*RDR1*	Repressor of multidrug resistance genes	(36)
YPL133C	*RDS2*	Regulator of drug sensitivity	This study
YPR094W	*RDS3*	Regulator of drug sensitivity	This study
YPR196W	*MAL63*	Activator of Maltose genes	(61)

Genes tested in this study

Genes tested in this study are listed. They all encode (putative) zinc cluster proteins. Systematic names are given on the left as well as the name of the genes. *YCR106W*, *YPL133C* and *YPR094W* were named *RDS1* to *3* respectively, since this study shows that they are regulators of drug sensitivity. For more details, see Materials and Methods.

Table 2

Drug	Target	Drug concentration used	Growth time (days)
CHLORAMPHENICOL	Inhibits DNA synthesis	3 mg/ml	2
CYCLOHEXIMIDE	Inhibits protein translation	1 μg/ml	9
KETOCONAZOLE	Antifungal. Inhibitor of the *ERG11* gene product involved in ergosterol synthesis	4 μg/ml	2
4-NQO	DNA mutagen	0.35 μg/ml	2
OLIGOMYCIN	Inhibits oxidative phosphorylation	1 μg/ml	4
RHODAMINE 6-G	Inhibits oxidative phosphorylation	5 μg/ml	4

Conditions used for drug assays

Drugs tested in this study and their targets are listed. Drug concentrations and growth times are also indicated.

Table 3

Syst. name	Gene	Phenotype of deletion strains		
		Ketoconazole	**Cycloheximide**	**4-NQO**
YCR106W	*RDS1*	-	Sensitive	-
YDR213W	*UPC2*	Sensitive	-	-
YHR178W	*STB5*	-	Sensitive	-
YIL130W	-	-	Slightly resistant	-
YKL222C	-	-	Slightly resistant	-
YLR228C	*ECM22*	-	Sensitive	-
YOL089C	*HAL9*	-	Sensitive	-
YOR162C	*YRR1*	-	Sensitive	Sensitive
YOR380W	*RDR1*	-	Resistant	-
YPL133C	*RDS2*	Sensitive	-	-
YPR094W	*RDS3*	Slightly sensitive	Sensitive	-

Summary of the drug sensitivity assays.

Zinc cluster genes whose deletion results in altered drug sensitivity are listed. Phenotypes (resistance or sensitivity to ketoconazole, cycloheximide and 4-NQO) are also indicated.

Table 4

Strain	β–galactosidase activity	
	PDR5-lacZ	SNQ2-lacZ
WT	56	13
Δrds1	62	13
Δupc2	51	12
Δstb5	23	1.7
Δecm22	50	12
Δhal9	33	10
Δyrr1	87	10
Δrds2	80	13
Δrds3	33	3.4

Activity of PDR5-lacZ or SNQ2-lacZ reporters is decreased in cells lacking Stb5p, Hal9p or Rds3p.

β-galactosidase activity was measured in wild-type and deletion strains containing reporters for *PDR5* or *SNQ2* as described in Materials and Methods.

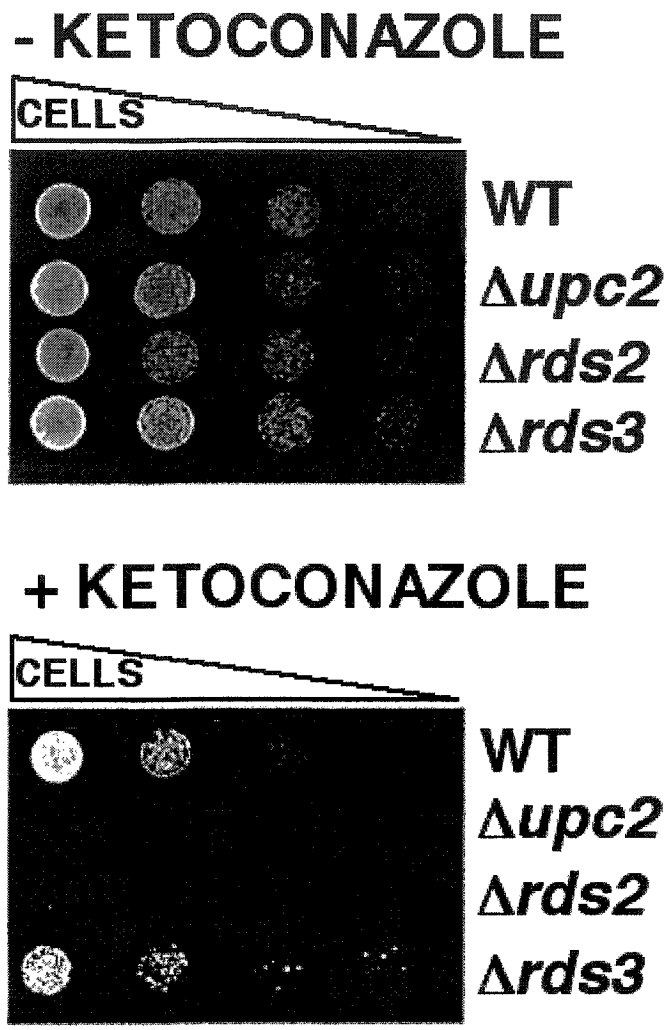
Table 5

Strain	β-galactosidase activity		
	PDRE3-CYC1-lacZ	PDRE3A-CYC1-lacZ	PDRE3B-CYC1-lacZ
WT	46	10	18
Δstb5	17	7.1	6.8
Δhal9	42	NT	NT
Δrds3	39	NT	NT

Transcriptional activation by Stb5p is mediated by a PDRE.

β-galactosidase activity was measured in wild-type and deletion strains transformed with lacZ reporters driven by a minimal CYC1 reporter or a CYC1 containing a wild-type Pdr1p/Pdr3p binding site ("PDRE3") or mutant sites ("PDRE3A" and "PDRE3B") inserted upstream of the *CYC1* promoter. The core sequence of the PDREs is shown Fig. 4. Reporters were assayed for β-galactosidase activity as described in Materials and Methods. "NT": not tested.

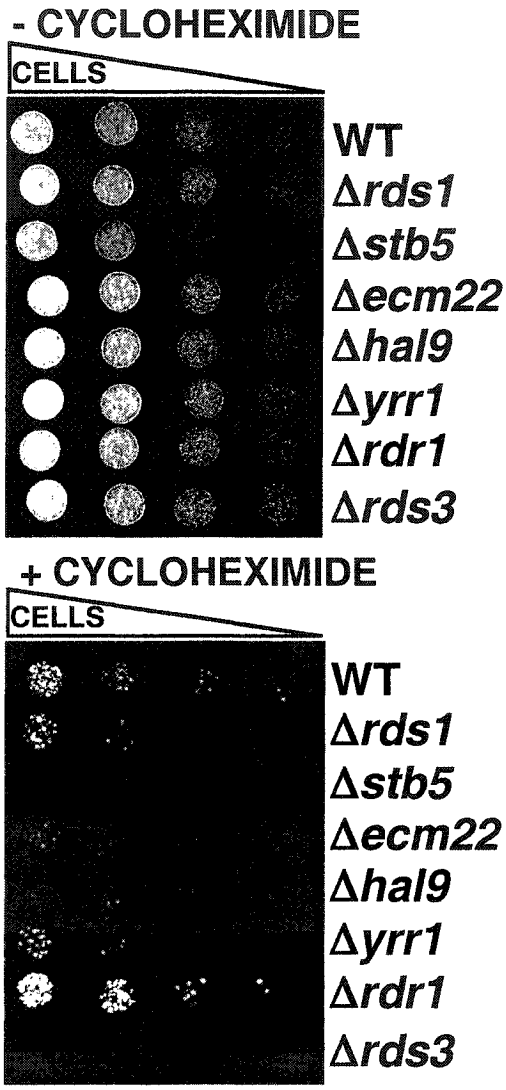
Figure 1



Deletion of the *UPC2*, *RDS2* or *RDS3* genes results in altered sensitivity to ketoconazole.

Wild-type or deletion strains were grown overnight in YPD. Cells were spun down, resuspended in water and serially diluted (left to right: approximately $1.25X10^4$, $2.5X10^3$, $5X10^2$ and $1X10^2$ cells). Cells were then spotted on YPD plates either with (lower panel) or without (upper panel) ketoconazole. Gene deletions are indicated on the right part of the figure. "WT", wild-type strain.

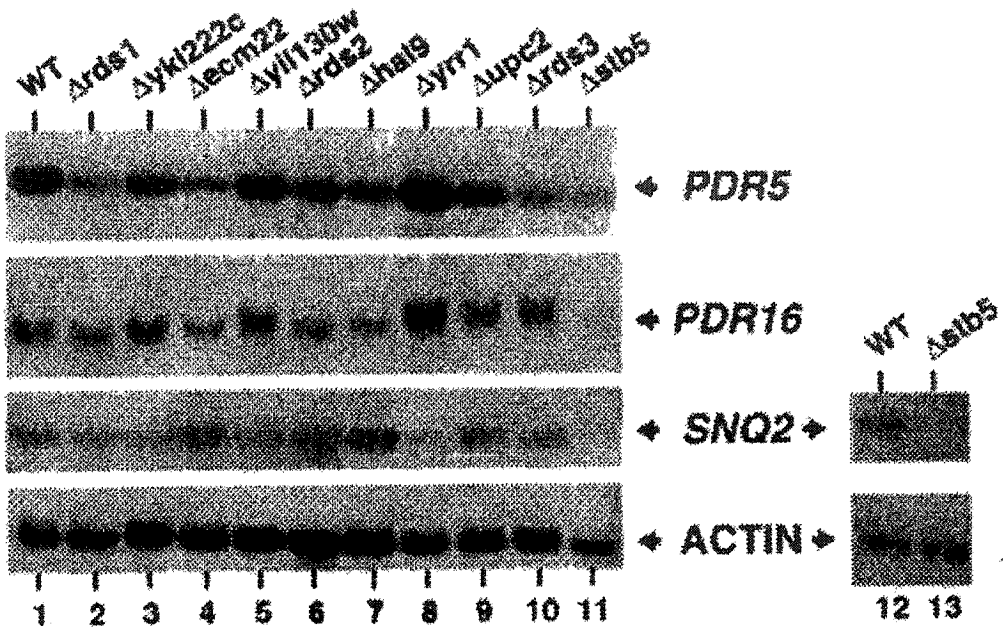
Figure 2



Deletion of various genes encoding zinc cluster proteins results in altered sensitivity to cycloheximide.

Wild-type or deletion strains were grown overnight in YPD. Cells were spun down, resuspended in water and serially diluted (left to right: approximately $1.25X10^4$, $2.5X10^3$, $5X10^2$ and $1X10^2$ cells). Cells were then spotted on YPD plates either with (lower panel) or without (upper panel) cycloheximide. Gene deletions are indicated on the right part of the figure. "WT", wild-type strain.

Figure 3



Northern blot analysis of selected genes.

Wild-type and deletion strains were grown in rich medium and RNA isolated. About twenty μg of total RNA were loaded per lane (1 to 11) for Northern blot analysis (see Materials and Methods). For the *Δstb5* strain, RNA samples were adjusted, reloaded and probed with *SNQ2* and actin (lanes 12 and 13). Probes are indicated on right of the autoradiograms and the strains on top.

Figure 4

Stb5p binds to a PDRE.

The purified DNA binding domain (a.a. 1-163) of Stb5p was used in an EMSA. Core sequences of the probes are shown at the bottom with mutations underlined. Arrows correspond to CGG triplets known to be important for binding of Pdr3p. Top, "-" no Stb5p; GST: EMSA performed with purified GST.

CONNECTING TEXT

Our phenotypic analysis revealed that in addition to Pdr1p, Pdr3p, and Yrr1p, other zinc cluster proteins appear to have a role in PDR. One of these in particular, Stb5p, appears to play a key role in regulating multidrug resistance genes, since its deletion leads to hypersensitivity to drugs and to a substantial decrease in *SNQ2* expression. Stb5p was also shown to bind a PDRE, the same element that mediates Pdr1p and Pdr3p activity. Since Pdr1p and Pdr3p are able to homo- and heterodimerize, we tested the ability of the newly identified regulator of PDR, Stb5p, to homo- and heterodimerize with Pdr1p, Pdr3p, and Yrr1p.

SECTION 4:
Complex interplay among regulators of drug resistance genes in *S. cerevisiae*

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Running title:

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ABSTRACT

The Gal4p family of yeast zinc cluster proteins comprises activators of multidrug resistance genes. For example, Pdr1p and Pdr3p bind as homo- or heterodimers to pleiotropic drug response elements (PDREs) found in promoters of target genes. Other zinc cluster activators of multidrug resistance genes include Stb5p and Yrr1p. To better understand the interplay among these regulators, we have performed native co-immunoprecipitation experiments. Interestingly, Stb5p is found predominantly as a Pdr1p heterodimer while Yrr1p dimerizes with itself and Pdr1p. Similar results were obtained using GST pull-down assays. Importantly, the purified DNA binding domains of Stb5p and Pdr1p bound to a PDRE as heterodimers *in vitro*. We assayed the contribution of these zinc cluster proteins in the activation of a *lacZ* reporter derived from the multidrug resistance gene *SNQ2*. Deletion of *STB5* or *PDR1* had the strongest effect on reporter activity. Moreover, mutational analysis showed that activation by Stb5p and Pdr1p is mediated via two PDREs found in the *SNQ2* promoter. Our results demonstrate a complex interplay among these activators and suggest that Pdr1p is a master regulator involved in recruiting other zinc cluster proteins to fine-tune the regulation of multidrug resistance genes.

INTRODUCTION

Toxic compounds such as drugs are used to treat many diseases by killing the harmful target cells, which can be either foreign pathogenic organisms or the patient's own tumor cells. However, both prokaryotic and eukaryotic cells can acquire the ability to become resistant to toxic compounds through the phenomenon of multidrug or pleiotropic drug resistance (PDR). *Saccharomyces cerevisiae* can also acquire PDR, making it a valuable tool in the study of this phenomenon so that we may gain insights into the mechanisms behind PDR in pathogenic fungi and in higher eukaryotes.

Cells that have acquired PDR have consistently shown higher levels of expression of drug efflux pumps. These pumps fall within two protein families: ATP-Binding Cassette (ABC) transporters and Major Facilitator Superfamily (MFS) transporters. Their increased expression allows expulsion of drugs from within the cell and, as a result, survival in the presence of these drugs. These higher levels of expression are often due to mutations in the transcription factors that regulate the expression of these pumps.

A complex network of various transcription factors has been shown to be involved in the regulation of the expression of genes encoding ABC or MFS proteins. There are two major families of transcription factors involved in PDR: 1) the bZip protein family (Yap family), and 2) zinc cluster proteins. Yap1p is the best characterized member of the bZip family and is an important regulator in the stress response (14, 38, 47). Yap1p regulates the expression of *YCF1* which encodes an ABC transporter (43). The other class of transcription factors involved in PDR

is composed of a subclass of zinc finger proteins, the zinc cluster or binuclear zinc cluster proteins (2, 3, 36, 40). These proteins contain a DNA binding domain (DBD) which possesses the well-conserved motif $CysX_2CysX_6CysX_{5-16}CysX_2CysX_{6-8}Cys$ with cysteines binding to two zinc atoms which coordinate folding of the domain involved in DNA recognition (41). Two highly homologous zinc cluster proteins, Pdr1p and Pdr3p, positively control the expression of genes involved in multidrug resistance (7, 23, 46). Target genes of Pdr1p and Pdr3p include the ABC transporters genes *PDR5*, *SNQ2*, and *YOR1* (11, 13, 20, 28, 47). Other targets include *HXT9* and *HXT11* which encode hexose transporters belonging to the MFS family (34). Another zinc cluster protein, Yrr1p, regulates the expression of *SNQ2* and *YOR1* (10, 26, 48).

In addition to these three zinc cluster proteins, Stb5p and Rdr1p have been recently implicated in the regulation of expression of *PDR5* and/or *SNQ2* (2, 17). Pleiotropic drug response elements (PDREs) present in the promoters of genes encoding ABC transporters, as well as in the *PDR3* promoter, have been shown to be important in the regulation of these genes. Pdr1p, Pdr3p, Stb5p and Rdr1p all act through this element, with Pdr1p, Pdr3p and Stb5p able to bind to an everted repeat CCGCGG. (2, 12, 16, 18, 20, 21, 28). Characterization of PDREs in the *PDR3* promoter indicates that autoregulation can occur at this gene (12). Even though they act through the same element, all these zinc cluster proteins may perform different functions. Pdr1p and Pdr3p have recently been shown to be able to form homo- and heterodimers (29). Different combinations of homo- and heterodimers may regulate the expression of different genes, which might help explain how these two proteins act differently.

Pdr1p, Pdr3p and Yrr1p have been extensively studied, and their roles in the cell are relatively well understood. However, with the finding that Stb5p regulates genes involved in PDR, new questions have arisen regarding the exact mechanism of regulation of these genes. The DBD of Stb5p has been shown to bind a PDRE from the *PDR5* promoter, and Stb5p is involved in the regulation of *PDR16, PDR5* and *SNQ2* expression. However, it seemed to play a greater role in the regulation of the *SNQ2* gene than of the *PDR5* gene, even though they both contain PDREs. We have tested the ability of Stb5p and other zinc cluster proteins described above to act on the different PDREs found in the *SNQ2* promoter and whether or not they act in coordination. We show that Stb5p interacts with Pdr1p (but not Pdr3p) and that Yrr1p can form homodimers or heterodimers with Pdr1p.

MATERIALS AND METHODS

Strains

Wild-type strains used were BY4741, MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0,* and BY4742, MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* (8). The deletion strains for *STB5*, *PDR1*, *PDR3* and *YRR1* were obtained from Research Genetics (Huntsville, Al) (44). The open reading frames (ORFs) of *GAL4*, *PDR1*, *PDR3*, *STB5* and *YRR1* were tagged at their natural chromosomal locations with triple HA and MYC epitopes according to Schneider et al. (37). Gal4p, Pdr1p, Stb5p and Yrr1p were N-terminally tagged. Since a N-terminally tagged Pdr3p was not functional (data not shown), it was tagged at its C-terminus. Tagging was performed by transforming the strains BY4741 and BY4742 with the PCR products generated with the following oligos (purified on a denaturing polyacrylamide gel) using p3XMYC and p3XHA as templates (37).

GAL4:
CCATCATTTTAAGAGAGGACAGAGAAGCAAGCCTCCTGAAAG**ATG**AGGGAACAAA
AGCTGGAG
and
AAGTCGGCAAATATCGCATGCTTGTTCGATAGAAGACAGTAGCTTTAGGGCGAATTG
GGTACC

PDR1:
CTCAGCCAAGAATATACAGAAAAGAATCCAAGAAACTGGAAG**ATG**AGGGAACAAA
AGCTGGAG
and
CGGACCCGTCTCAATATGTACACCGTTCTTAGGTGTCAAGCCTCGTAGGGCGAATTG
GGTACC

PDR3:
TTATATCATACTCTGTGGAATGACAATACTTCATATCCCTTCTTAAGGGAACAAAAG
CTGGAG
and
TTTACTATGGTTATGCTCTGCTTCCCTATTTCTTTTGCGTTTTCATAGGGCGAAT
TGGGTACC

STB5:
GTACAGGGCTAAAAAATTAATACAAAGGTGTAAAAGAAGGAC**ATG**AGGGA
ACAAAAGCTGGAG
and
AGTACGTTGTGATCTCCCGCCTTGATGTGCAAAATTGGGACCATCTAGGG
CGAATTGGGTACC

YRR1:
AAGTTTATTGCCCTCAGCCGTGCCAATAAGAATAGCGTCACA**ATG**AGGGAACAAAA
GCTGGAG
and
GTTGGTGGCCTGGAAACTTCCCAACAAAGCATCGCTTCTTCTTTTTAGGGCGAATTG
GGTACC

Nucleotides in bold correspond to the initiator codon. Transformants were selected on plates lacking uracil. Homologous recombinations were verified by loss of function (reduced drug resistance for *PDR1*, *PDR3* and *STB5* or absence of growth on galactose plates for *GAL4*). Cells were grown overnight in rich medium to allow internal recombination between sequences encoding epitopes and *ura3*$^-$ strains were selected on plates containing 5-fluoroorotic acid (37).

Strains of the opposite mating type were crossed to obtain diploid strains expressing combinations of tagged proteins.

Media

Media were prepared according to Adams et al. (1). YPD contained 1% yeast extract, 2% peptone, and 2% glucose. SD contained 2% glucose, 0.67% yeast nitrogen base (without amino acids) and was supplemented with adenine and appropriate amino acids at a final concentration of 0.004%.

Reporters and β-galactosidase assays

Reporter pSNQ2-lacZ is a low copy plasmid (ARSCEN) containing ~700 bp of promoter sequences upstream of the ATG codon (17). Mutations in each of the three PDREs (see Table 1) were introduced by subcloning the *Xho*I-*Bam*HI fragment (containing the *SNQ2* promoter) of pSNQ2-lacZ into pBluescriptIIKS$^-$ cut with the same enzymes. The resulting plasmid was used for site-directed mutagenesis (25) using the oligonucleotides GTAGAAATCTAGCATCTGCAGAGCTATTTTAAGTTT (PDRE#1), AGCTATTTTAAGTTTCTGCAGATGCCTTTCGATCCT (PDRE#2) and CCCAGTCGTCTGGTTCTGCAGACATATGACTAAATG (PDRE#3) (mutations are underlined). The *Bam*HI-*Xho*I fragment of pSNQ2-lacZ was then replaced with the mutated inserts to give pSNQ2ΔPDRE1-lacZ, pSNQ2ΔPDRE2-lacZ and pSNQ2ΔPDRE3-lacZ. Mutants carry a mutation in each CGG (or CGC) triplet of the core sequence of the PDREs (CCGCGG to CTGCAG). β-galactosidase assays were performed as described previously (17) with

permeabilized cells. β-galactosidase assays were performed in duplicate from at least two independent transformations.

Bacterial expression vectors

A bacterial expression vector for Yrr1p, pGST-Yrr1(1-170), was constructed by amplifying the sequences encoding the DBD of Yrr1p (a.a. 1-170) using oligos CGGGATCCATGAAAAAGAAGAAGCGATGC and ACTACGCAATTGTTAGTAGTACCGGTCGGCATATG and yeast genomic DNA (isolated from strain YPH499 as a template, ref. 39). The PCR product was cut with *Bam*HI and *Mfe*I and subcloned into pGEX-f (18) cut with *Bam*HI and *Eco*RI. Similarly, pGST-Pdr1(1-152) was constructed using oligos CGGGATCCATGCGAGGCTTGACACCTAA and GGAATTCAATCGTCGTCATTCT. The PCR product was cut with *Bam*HI and *Eco*RI and subcloned into pGEX-f cut with the same enzymes. The same DNA fragment was subcloned into pRSET-A (Invitrogen) cut with *Bam*HI and *Eco*RI to give pHis-Pdr1(1-152). Sequences encoding a triple HA epitope were amplified by PCR using oligos GAAGATCTCTGCAGATGTACCCATACGATGTTCCT and GAAGATCTAGCAGCGTAATCTGGAACG using plasmid p3XHA (37) as a template. The PCR product was cut with *Bgl*II and subcloned into the *Bam*HI site of pHis-Pdr1(1-152) to give pHis-HA-Pdr1(1-152).

Protein expression and electrophoretic mobility shift assay (EMSA)

Expression, purification of fusion proteins and EMSA were performed as described (18, 33) except that the amount of probe was five times higher (0.3 pmole per binding reaction). The

probe for EMSA was obtained by annealing oligos (TCGANx with Nx corresponding to the PDRE 1 found in the *SNQ2* promoter and its complement, see Table 1), filling-in with Klenow and dGTP, dTTP, dATP and [^{32}P]dCTP.

***In vitro* pull-down assays**

BY4741 cells containing the ORF encoding the HA-tagged Stb5p, Gal4p or Yrr1p were grown in 200 ml of YPD to an OD_{600} of around 1. Cells were spun down and washed with ice-cold water. Cells were resuspended in an equal volume of ice-cold IP-1 buffer (150 mM NaCl, 15 mM Tris-HCl pH 7.6, 1% TritonX-100, 10 mM pyrophosphate, 1mM PMSF, 2 mM DTT, 1 µg/ml pepstatin and 1 µg/ml leupeptin) as modified from Mamnun et al. (29). An equal volume of chilled glass beads was added, and the cells were vortexed three times for 1 min. The lysate was separated from the debris and unlysed cells by centrifugation. GST fusion proteins were expressed in *E. coli* and purified as described (18) using plasmids pGST-Pdr1(1-152) (see above), pGST-Stb5(1-163) (2) and pGal4(1-143) (18). Half of the GST proteins attached to glutathione sepharose beads were mixed with the yeast lysates and left overnight at 4^0 C. The beads were washed with the IP-1 buffer and the proteins were suspended in 50 µl of 1x Laemmli buffer. The proteins were resolved on 7.5% SDS-PAGE gels, and analyzed by immunoblotting with the HA antibody (12Ca5, Roche).

***In vivo* co-immunoprecipitation assays**

Diploid strains described above were grown in YPD to an OD_{600} of around 1 in a volume of 200 ml. Proteins were isolated as described above and incubated for 2 h with 4 µg of MYC antibody (9E10, Upstate). Then 20 µl of a 50% protein G sepharose slurry were added to the

lysates and incubated overnight at 4^0C. The samples were washed with the IP-1 buffer and then the proteins were dissociated from the beads by boiling the sample for 5 min in 1x Laemmli buffer. The samples were run on a 7.5% SDS-PAGE gel, and analyzed by immunoblotting with a HA antibody (12Ca5, Roche).

RESULTS

Pdr1p and Pdr3p activate transcription through PDREs by forming homo- and heterodimers (29). Since Stb5p also activates transcription by binding to PDREs (2), we tested if it could dimerize with either Pdr1p, Pdr3p or itself using co-immunoprecipitation assays. Triple MYC or HA epitopes were inserted into chromosomal DNA at the loci encoding these proteins and at the *GAL4* locus. Gal4p, another member of the family of zinc cluster proteins, activates expression of genes involved in galactose metabolism by binding to target DNA sequences as a homodimer (31 and refs. therein). Tagged Gal4p was used as a negative control since it does not play any role in conferring drug resistance and, as a result, it should not interact with Stb5p, Pdr1p, Pdr3p or Yrr1p. All tagged proteins were fully functional since the haploid strains expressing these tagged proteins had a wild-type phenotype when tested on drugs for *PDR1, PDR3, STB5* and *YRR1* and when grown on galactose for *GAL4* (data not shown). Since the tagged proteins are expressed from their natural promoters, levels of these proteins should not differ from those normally present in wild-type cells.

Diploid strains expressing different combinations of tagged Stb5p, Pdr1p, Pdr3p and Gal4p were grown in rich medium (YPD) to mid-log phase. Extracts were used for immunoprecipitation with an anti-MYC or an anti-HA antibody. Upon Western blot analysis, all

tagged proteins tested could be detected at a position expected from their predicted molecular weight (data not shown). Co-immunoprecipitation experiments were performed using an anti-MYC antibody and proteins were then run on a gel and visualized by immunoblotting with an anti-HA antibody. A strong signal was obtained with HA-Pdr1p co-immunoprecipitated with MYC-Stb5p (Fig. 1A, lane 5), while there was no signal for HA-Gal4p (Fig. 1A, lane 4). In another study (29), it has been shown that multiple bands detected with Pdr1p correspond to different phosphorylated forms of the protein. No signal was obtained when HA-Stb5p was co-immunoprecipitated with MYC-Pdr3p or MYC-Stb5p (Fig. 1A, lanes 6 and 7). However, upon overexposure, a weak signal was observed but it was at least 20 times weaker than the Pdr1p signal (data not shown). In summary, our results strongly suggest that Stb5p is primarily found as a heterodimer with Pdr1p *in vivo*.

To test if the DBD of Pdr1p is sufficient for interaction with Stb5p, *in vitro* GST pull-down assays were performed. The DBDs of Pdr1p and Gal4p fused to GST were expressed in bacteria, purified and bound to glutathione sepharose beads. Protein extracts were prepared from haploid yeast strains expressing HA-Gal4p or HA-Stb5p and added to the GST fusion proteins bound to beads. After washing, bound proteins were eluted, run on a gel and visualized by immunoblotting. GST-Pdr1p interacted with HA-Stb5p but not with HA-Gal4p (Fig. 1B). Moreover, HA-Stb5p did not interact with GST-Gal4p. These results from the GST pull-down assay are in agreement with the co-immunoprecipitation experiments.

Since Yrr1p is also involved in PDR, we were interested in determining if it could interact with itself as well as other PDR activators such as Pdr1p, Pdr3p and Stb5p. Co-

immunoprecipitation experiments similar to those described above were performed with MYC- and HA-tagged Yrr1p. Results show that Yrr1p forms homodimers *in vivo* (Fig. 2A, lane 7) and heterodimers with Pdr1p (Fig. 2A, lane 4). No interaction of Yrr1p with Gal4p, Pdr3p and Stb5p was detected in this assay (Fig. 2A, lanes 3, 5 and 6, respectively). Results were confirmed using a GST pull-down assay (Fig. 2B). HA-Yrr1p was pulled down with GST-Yrr1p and GST-Pdr1p but not GST-Gal4p. As observed with Stb5p, the DBD of Pdr1p is sufficient for interaction with Yrr1p. Moreover, the DBD of Yrr1p allows interaction with its full-length counterpart. Thus, the GST pull-down assay with Yrr1p is also in agreement with the co-immunoprecipitation experiment, as seen with Stb5p.

We wished to determine if the interaction of Stb5p with Pdr1p seen *in vivo* could be observed *in vitro* using purified components. The DBD of Pdr1p was expressed in *E. coli* as a fusion with 6XHis and a triple HA epitope (His-HA-Pdr1p) and purified on a nickel column. Similarly, GST-Stb5p was expressed in *E. coli*, purified and the GST moiety removed by thrombin cleavage. *In vitro* binding of the purified proteins was assayed by EMSA using a probe corresponding to the PDRE number 1 found in the *SNQ2* promoter (Table 1). With the DBD of Stb5p alone, two major retarded complexes of fast mobility were observed (Fig. 3, lanes 2 to 5). It is possible that these complexes correspond to Stb5p bound as a homodimer to DNA. With the DBD of Pdr1p alone, a retarded complex was observed only at high protein concentration (Fig. 3, lanes 6 to 9). Smearing suggests that the DNA-Pdr1p complex dissociated during electrophoresis. Stronger binding *in vitro* of Pdr1p was observed in another study (45). However, the GST moiety was not removed for EMSA analysis. It is possible that dimerization of GST increased binding to a DNA target. Interestingly, a complex of intermediate mobility was

observed when mixing the DBDs of Pdr1p and Stb5p, strongly suggesting the formation of a heterodimer (Fig. 3, lanes 10 to 13). Importantly, this heterodimeric complex was the predominant species (Fig. 3; lanes 12 and 13). These results suggest that the DBDs of Stb5p and Pdr1p bind *in vitro* cooperatively to a target DNA sequence.

Given the interplay among the zinc cluster proteins studied, we were interested to determine their respective roles in the activation of *SNQ2*. We first assessed the importance of the putative PDREs found in the *SNQ2* promoter. Sequences of PDREs and their positions are shown in Table 1. Each PDRE was individually mutated from CCGCGG (or CCGCGC for PDRE 3) to CTGCAG. Alteration of CGG triplets is known to prevent *in vitro* binding of Pdr1p, Pdr3p and Stb5p (2, 18, 21). Low copy SNQ2-lacZ reporters were transformed into a wild-type strain and β-galactosidase activity measured. Mutating either of the first two PDREs, but not the third one, reduced activity of the reporter (Fig. 4; lanes 1, 6, 11 and 16). The third PDRE has a CGC triplet instead of CGG (Table 1). Studies with another zinc cluster protein, Hap1p, have shown reduced transcriptional activity when assayed with target DNA sequences containing a CGC triplet (15). Thus, our results suggest that the first two PDREs are the main sites of activation of transcription.

We then assayed the contribution of Pdr1p, Pdr3p, Stb5p and Yrr1p in the activation of the SNQ2-lacZ reporter. Deletion of *PDR1* or *STB5* led to a decrease in the activity of the wild-type reporter, with a 3-fold difference in activity with the Δ*stb5* strain and a 1.5-fold difference with the Δ*pdr1* strain (Fig. 4; lanes 2 and 3). Removal of Yrr1p or Pdr3p had no or minor effects on the activity of the reporter (Fig. 4; lanes 4 and 5). When a *SNQ2* lacZ reporter bearing a

mutant PDRE 1 was assayed in *STB5* and *PDR1* deletion strains, activity was further decreased as compared to a wild-type strain (for example, compare lanes 6, 7 and 8 in Fig. 4). This effect can be explained by the fact that activation can still occur via the second PDRE in a wild-type strain. Similar results are observed when mutating PDREs 2 or 3 (Fig. 4, lanes 12, 13, 17 and 18). In summary, Stb5p and Pdr1p positively control the activity of the *SNQ2* promoter through PDREs 1 and 2.

DISCUSSION

At least four zinc cluster proteins activate transcription of multidrug resistance genes. Initially, it was shown that the expression of these genes was activated by Pdr1p, Pdr3p and Yrr1p through the PDREs found in their promoters (7, 10, 23, 26). Recently, Stb5p has been identified as an additional transcriptional activator of multidrug resistance genes (2). Stb5p activates transcription by binding to PDREs, the same elements found to be critical for Pdr1p and Pdr3p activity. Pdr1p and Pdr3p have shown to homo- and heterodimerize (29). Since Stb5p and Yrr1p have similar roles and act through the same elements as Pdr1p and Pdr3p, we tested the ability of these four proteins to dimerize with each other. Interestingly, both Stb5p and Yrr1p dimerize with Pdr1p. The interactions among these various transcriptional activators are summarized in Fig. 5.

Using native co-immunoprecipitation experiments, we determined that Stb5p was found predominantly as a heterodimer with Pdr1p (Fig. 1A). Only a small fraction (less than 5%) of Stb5p was found as a homodimer or as a Stb5p-Pdr3p heterodimer. The Stb5-Pdr1p interaction

was also observed when a GST-Pdr1p(DBD) fusion was able to specifically pull-down Stb5p (Fig. 1B), indicating that the 152 N-terminal amino acids of Pdr1p are sufficient to mediate dimerization with Stb5p. The co-immunoprecipitation experiments also showed that Yrr1p was able to homodimerize and to heterodimerize with Pdr1p. Unlike Stb5p, it appears that there are similar amounts of homo- and heterodimeric Yrr1p (Fig. 2A). In agreement with the immunoprecipitation experiments, Yrr1p was also pulled down by the Yrr1p and Pdr1p DBD-GST fusion proteins (Fig. 2B). Therefore, Yrr1p exists in two different subpopulations in the cell. Perhaps each population acts differently at various target DNA sites. Interestingly, genes induced by a chimeric activator bearing the DBD of Yrr1p have PDRE-like sequences [(T/A)CCG(C/T)(G/T)(G/T)(A/T)(A/T)] in their promoters (26). Only the first half of the site matches the nucleotides found in PDREs. In a Pdr1p-Yrr1p heterodimer, Pdr1p may bind to the 5' half of the site while Yrr1p would recognize the second half.

Many zinc cluster proteins (Gal4p, Leu3p, Hap1p, Ppr1p, Put3p etc.) initially characterized in *S. cerevisiae* were shown to bind to DNA as homodimers (see 3, 36, 40 for refs.). More recently, Oaf1p and Oaf2p (Pip2p) were shown to bind as heterodimers to target sequences of genes for peroxisome proliferation (19, 35). Moreover, the zinc cluster protein ArgRIIp heterodimerizes with members of the MADS family to activate genes for arginine metabolism (5). Our results combined with those of Mamnun et al. (29) demonstrate that Pdr1p can dimerize with itself, Pdr3p, Stb5p, and Yrr1p. In addition, we have previously shown that another zinc cluster protein, Rdr1p, represses the expression of some PDR genes such as *PDR5* and *PDR16* (17). We also demonstrated that the repressive effect of Rdr1p is mediated by PDREs. Interestingly, Rdr1p interacts *in vivo* with both Pdr1p and Pdr3p (S. MacPherson and B.

Turcotte, unpublished results). Formation of heterodimeric complexes by zinc cluster proteins may therefore be a more predominant mechanism for regulation of gene expression than initially anticipated. Regulation of a relatively simple pathway, such as the one triggered by galactose, is efficiently performed by Gal4p homodimers, while more complex processes, like PDR, must require various combinations of homo- and heterodimers to integrate different signals allowing for precise expression of target genes. Taken together, the data suggest that Pdr1p is a master PDR regulator involved in recruiting other zinc cluster proteins to fine-tune the regulation of multidrug resistance genes. This is reminiscent of the mammalian nuclear receptor RXR which forms heterodimers with the receptors for 9-*cis* retinoic acid, thyroid hormone, and vitamin D, as well as peroxisome proliferator activators to differentially regulate expression of target genes (30).

An EMSA showed that the purified DBDs of Pdr1p and Stb5p bound cooperatively to a PDRE *in vitro* (Fig. 3). Even though the proteins were individually expressed and purified, upon mixing with each other, Pdr1p was only found in the heterodimeric form. Therefore, either all of Pdr1p added was in the form of heterodimers, or the heterodimers have a much higher affinity for the PDREs than either homodimer. Either way, it appears that the Stb5p-Pdr1p heterodimer is the predominant complex that binds to DNA *in vitro*. Moreover, the N-termini of Pdr1p (a.a. 1-152) and Stb5p (a.a. 1-163) are sufficient to allow formation of heterodimers on DNA. Crystal and solution structures of the DBDs of zinc cluster proteins Gal4p, Ppr1p, Put3p and Hap1p show that these proteins homodimerize via a coiled-coil dimerization domain composed of heptad repeats located at the C-terminus of the zinc finger (6, 22, 24, 31, 32, 39, 42). However, no obvious heptad repeats are predicted to be present in the Pdr1p polypeptide used for EMSA

(36) while only one repeat is found in the DBD of Stb5p (unpublished results). Clearly, further studies will be required to define the structural basis for the multiple interactions of Pdr1p with other zinc cluster proteins.

The importance of the three PDREs in the *SNQ2* promoter was gauged by mutating each of them individually. Mutating PDRE 1 or 2 of *SNQ2* resulted in approximately a 2-fold decrease in activity in the wild-type strain (Fig. 4). Similar effects (2- to 2.5-fold) were observed when measuring the activity of the *PDR5* promoter bearing single PDRE mutants (21). In contrast, mutating PDRE 3 of *SNQ2* did not cause a significant decrease in activity (Fig. 4). This supports the EMSA results where the proteins were not able to bind this PDRE that well (data not shown). The β-galactosidase results suggest that Pdr1p, Pdr3p, Stb5p, and Yrr1p have various roles within the cell, since deletion of each protein resulted in a different effect on the activity of the *SNQ2* reporter. Deletion of Stb5p or Pdr1p resulted in reduced activity in agreement with Northern blot analyses of *SNQ2* RNA (2, 27) while the deletion of Pdr3p or Yrr1p did not cause a significant decrease. Similar results were obtained when measuring levels of *SNQ2* mRNA in a Δ*pdr3* strain (28). However, the deletion of Yrr1p results in lower levels of *SNQ2* mRNA (2, 10) and the reason for this discrepancy is not known. It may be due to a difference in promoter context, or perhaps the site of Yrr1p action is more upstream or downstream of the region included in our construct. When a lacZ reporter with the PDREs inserted in front of a minimal CYC1 promoter was assayed in the *PDR1, PDR3, STB5* and *YRR1* deletion strains, a 2-fold decrease in activity was only seen in the *Δstb5* strain with the PDRE 1 of *SNQ2* (data not shown). The β-galactosidase results indicate that there are differences among the PDREs and that

when one protein is removed, the other proteins may compensate. To summarize, deletion of Stb5p or Pdr1p had the largest effect on the activity of the *SNQ2* promoter.

As observed for *SNQ2*, zinc cluster proteins contribute differently to the regulation of specific PDR genes. For example, induced-expression of *FLR1*, a gene involved in PDR (4), is dependent on Pdr3p but not Pdr1p (9). Conversely, a deletion of Pdr1p greatly affects expression of *PDR5* while removal of Pdr3p has marginal effects (28). Moreover, deletion of *PDR1* or *PDR3* results in increased or decreased expression of *PDR15* (a homologue of *PDR5*), respectively (45). This pattern of regulation is further complicated by the fact that Pdr3p undergoes positive auto-regulation (12) while expression of *YRR1* is under the control of Pdr1p/Pdr3p and itself (48). The observation that zinc cluster proteins form various combinations of dimers will be invaluable in better understanding the complex regulation of PDR genes.

In conclusion, we have shown that the four zinc cluster protein activators of multidrug resistance genes do not act individually. Instead, they form various populations of homo- and heterodimers (Fig. 5). There may be differences in the binding specificity or activity of each of these populations, allowing for a very specific and varied expression of genes involved in PDR. Pdr1p was the only protein able to interact with the other three proteins and itself, indicating that it is similar to mammalian nuclear receptor RXR in its ability to recruit various partners. With four different zinc cluster proteins regulating the expression of PDR genes via different PDREs, the cell's ability to respond to drugs is much more adaptable and flexible.

These discoveries present many more questions regarding PDR. For example, would various environmental signals cause a shift in the balance of the various populations of homo- and heterodimers? Is the hyperactivity of the Pdr1p and Pdr3p mutants due to a change in the activity of the protein or to a change in the partner of the protein? Are other regulators of drug resistance, such as members of the Yap1p family, able to dimerize with these zinc cluster proteins?

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REFERENCES

1. **Adams, A., D. E. Gottschling, and T. Stearns.** 1997. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

2. **Akache, B., and B. Turcotte.** 2002. New regulators of drug sensitivity in the family of yeast zinc cluster proteins. Journal of Biological Chemistry **277:**21254-60.

3. **Akache, B., K. Wu, and B. Turcotte.** 2001. Phenotypic analysis of genes encoding yeast zinc cluster proteins. Nucleic Acids Research **29:**2181-90.

4. **Alarco, A. M., I. Balan, D. Talibi, N. Mainville, and M. Raymond.** 1997. AP1-mediated multidrug resistance in Saccharomyces cerevisiae requires FLR1 encoding a transporter of the major facilitator superfamily. Journal of Biological Chemistry **272:**19304-13.

5. **Amar, N., F. Messenguy, M. El Bakkoury, and E. Dubois.** 2000. ArgRII, a component of the ArgR-Mcm1 complex involved in the control of arginine metabolism in Saccharomyces cerevisiae, is the sensor of arginine. Molecular & Cellular Biology **20:**2087-97.

6. **Baleja, J. D., R. Marmorstein, S. C. Harrison, and G. Wagner.** 1992. Solution structure of the DNA-binding domain of Cd2-GAL4 from S. cerevisiae. Nature **356:**450-3.

7. **Bauer, B. E., H. Wolfger, and K. Kuchler.** 1999. Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. Biochimica et Biophysica Acta **1461:**217-36.

8. **Brachmann, C. B., A. Davies, G. J. Cost, E. Caputo, J. Li, P. Hieter, and J. D. Boeke.** 1998. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a

useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast **14:**115-32.

9. **Broco, N., S. Tenreiro, C. A. Viegas, and I. Sa-Correia.** 1999. FLR1 gene (ORF YBR008c) is required for benomyl and methotrexate resistance in Saccharomyces cerevisiae and its benomyl-induced expression is dependent on Pdr3 transcriptional regulator. Yeast **15:**1595-1608.

10. **Cui, Z., T. Shiraki, D. Hirata, and T. Miyakawa.** 1998. Yeast gene YRR1, which is required for resistance to 4-nitroquinoline N- oxide, mediates transcriptional activation of the multidrug resistance transporter gene SNQ2. Mol Microbiol **29:**1307-15.

11. **Decottignies, A., L. Lambert, P. Catty, H. Degand, E. A. Epping, W. S. Moye-Rowley, E. Balzi, and A. Goffeau.** 1995. Identification and characterization of SNQ2, a new multidrug ATP binding cassette transporter of the yeast plasma membrane. Journal of Biological Chemistry **270:**18150-7.

12. **Delahodde, A., T. Delaveau, and C. Jacq.** 1995. Positive autoregulation of the yeast transcription factor Pdr3p, which is involved in control of drug resistance. Molecular & Cellular Biology **15:**4043-50.

13. **DeRisi, J., B. van den Hazel, P. Marc, E. Balzi, P. Brown, C. Jacq, and A. Goffeau.** 2000. Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants. FEBS Letters **470:**156-60.

14. **Gounalaki, N., and G. Thireos.** 1994. Yap1p, a yeast transcriptional activator that mediates multidrug resistance, regulates the metabolic stress response. EMBO J **13:**4036-41.

15. **Ha, N., K. Hellauer, and B. Turcotte.** 1996. Mutations in target DNA elements of yeast HAP1 modulate its transcriptional activity without affecting DNA binding. Nucleic Acids Research **24:**1453-9.

16. **Hallstrom, T. C., and W. S. Moye-Rowley.** 1998. Divergent transcriptional control of multidrug resistance genes in Saccharomyces cerevisiae. Journal of Biological Chemistry **273:**2098-104.

17. **Hellauer, K., B. Akache, S. MacPherson, E. Sirard, and B. Turcotte.** 2002. Zinc cluster protein Rdr1p is a transcriptional repressor of the *PDR5* gene encoding a multidrug transporter. Journal of Biological Chemistry **277:**17671-6.

18. **Hellauer, K., M.-H. Rochon, and B. Turcotte.** 1996. A novel DNA binding motif for yeast zinc cluster proteins: the Leu3p and Pdr3p transcriptional activators recognize everted repeats. Molecular & Cellular Biology **16:**6096-6102.

19. **Karpichev, I. V., Y. Luo, R. C. Marians, and G. M. Small.** 1997. A complex containing two transcription factors regulates peroxisome proliferation and the coordinate induction of beta-oxidation enzymes in Saccharomyces cerevisiae. Molecular & Cellular Biology **17:**69-80.

20. **Katzmann, D. J., P. E. Burnett, J. Golin, Y. Mahe, and W. S. Moye-Rowley.** 1994. Transcriptional control of the yeast PDR5 gene by the PDR3 gene product. Molecular & Cellular Biology **14:**4653-61.

21. **Katzmann, D. J., T. C. Hallstrom, Y. Mahe, and W. S. Moye-Rowley.** 1996. Multiple Pdr1p/Pdr3p binding sites are essential for normal expression of the ATP binding cassette transporter protein-encoding gene PDR5. Journal of Biological Chemistry **271:**23049-54.

22. **King, D. A., L. Zhang, L. Guarente, and R. Marmorstein.** 1999. Structure of a HAP1-DNA complex reveals dramatically asymmetric DNA binding by a homodimeric protein. Nature Structural Biology **6**:64-71.

23. **Koloczkowska, A., and A. Goffeau.** 1999. Regulation of pleiotropic drug resistance in yeast. Drug Resistance Updates **2**:403-14.

24. **Kraulis, P. J., A. R. Raine, P. L. Gadhavi, and E. D. Laue.** 1992. Structure of the DNA-binding domain of zinc GAL4. Nature **356**:448-50.

25. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proceedings of the National Academy of Sciences of the United States of America **82**:488-92.

26. **Le Crom, S., F. Devaux, P. Marc, X. T. Zhang, W. S. Moye-Rowley, and C. Jacq.** 2002. New insights into the pleiotropic drug resistance network from genome-wide characterization of the YRR1 transcription factor regulation system. Molecular & Cellular Biology **22**:2642-2649.

27. **Mahe, Y., Y. Lemoine, and K. Kuchler.** 1996. The ATP binding cassette transporters Pdr5 and Snq2 of Saccharomyces cerevisiae can mediate transport of steroids in vivo. Journal of Biological Chemistry **271**:25167-72.

28. **Mahe, Y., A. Parle-McDermott, A. Nourani, A. Delahodde, A. Lamprecht, and K. Kuchler.** 1996. The ATP-binding cassette multidrug transporter Snq2 of Saccharomyces cerevisiae: a novel target for the transcription factors Pdr1 and Pdr3. Molecular Microbiology **20**:109-17.

29. **Mamnun, Y. M., R. Pandjaitan, Y. Mahe, A. Delahodde, and K. Kuchler.** 2002. The yeast zinc finger regulators Pdr1p and Pdr3p control pleiotropic drug resistance (PDR) as homo- and heterodimers in vivo. Molecular Microbiology **46:**1429-1440.

30. **Mangelsdorf, D. J., and R. M. Evans.** 1995. The RXR Heterodimers and Orphan Receptors. Cell **83:**841-850.

31. **Marmorstein, R., M. Carey, M. Ptashne, and S. C. Harrison.** 1992. DNA recognition by GAL4: structure of a protein-DNA complex. Nature **356:**408-14.

32. **Marmorstein, R., and S. C. Harrison.** 1994. Crystal structure of a PPR1-DNA complex: DNA recognition by proteins containing a Zn2Cys6 binuclear cluster. Genes & Development **8:**2504-12.

33. **Noël, J., and B. Turcotte.** 1998. Zinc cluster proteins Leu3p and Uga3p recognize highly related but distinct DNA targets. Journal of Biological Chemistry **273:**17463-8.

34. **Nourani, A., M. Wesolowski-Louvel, T. Delaveau, C. Jacq, and A. Delahodde.** 1997. Multiple-drug-resistance phenomenon in the yeast Saccharomyces cerevisiae: involvement of two hexose transporters. Molecular & Cellular Biology **17:**5453-60.

35. **Rottensteiner, H., A. J. Kal, B. Hamilton, H. Ruis, and H. F. Tabak.** 1997. A heterodimer of the Zn2Cys6 transcription factors Pip2p and Oaf1p controls induction of genes encoding peroxisomal proteins in Saccharomyces cerevisiae. Eur J Biochem **247:**776-83.

36. **Schjerling, P., and S. Holmberg.** 1996. Comparative amino acid sequence analysis of the C6 zinc cluster family of transcriptional regulators. Nucleic Acids Research **24:**4599-607.

37. **Schneider, B. L., W. Seufert, B. Steiner, Q. H. Yang, and A. B. Futcher.** 1995. Use of polymerase chain reaction epitope tagging for protein tagging in Saccharomyces cerevisiae. Yeast **11:**1265-74.

38. **Schnell, N., and K. D. Entian.** 1991. Identification and characterization of a Saccharomyces cerevisiae gene (PAR1) conferring resistance to iron chelators. Eur J Biochem **200:**487-93.

39. **Swaminathan, K., P. Flynn, R. J. Reece, and R. Marmorstein.** 1997. Crystal structure of a PUT3-DNA complex reveals a novel mechanism for DNA recognition by a protein containing a Zn2Cys6 binuclear cluster. Nature Structural Biology **4:**751-9.

40. **Todd, R. B., and A. Andrianopoulos.** 1997. Evolution of a fungal regulatory gene family: the Zn(II)2Cys6 binuclear cluster DNA binding motif. Fungal Genetics & Biology **21:**388-405.

41. **Vallee, B. L., J. E. Coleman, and D. S. Auld.** 1991. Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. Proceedings of the National Academy of Sciences of the United States of America **88:**999-1003.

42. **Walters, K. J., K. T. Dayie, R. J. Reece, M. Ptashne, and G. Wagner.** 1997. Structure and mobility of the PUT3 dimer. Nature Structural Biology **4:**744-50.

43. **Wemmie, J. A., M. S. Szczypka, D. J. Thiele, and W. S. Moye-Rowley.** 1994. Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene, YCF1. Journal of Biological Chemistry **269:**32592-7.

44. **Winzeler, E. A., D. D. Shoemaker, A. Astromoff, H. Liang, K. Anderson, B. Andre, R. Bangham, R. Benito, J. D. Boeke, H. Bussey, A. M. Chu, C. Connelly, K. Davis, F.**

Dietrich, S. W. Dow, E. L. B. M, F. Foury, S. H. Friend, E. Gentalen, G. Giaever, J. H. Hegemann, T. Jones, M. Laub, H. Liao, N. Liebundguth, R. W. Davis, and et al. 1999. Functional characterization of the S-cerevisiae genome by gene deletion and parallel analysis. Science **285:**901-906.

45. **Wolfger, H., Y. Mahe, A. Parle-McDermott, A. Delahodde, and K. Kuchler.** 1997. The yeast ATP binding cassette (ABC) protein genes PDR10 and PDR15 are novel targets for the Pdr1 and Pdr3 transcriptional regulators. FEBS Letters **418:**269-74.

46. **Wolfger, H., Y. M. Mamnun, and K. Kuchler.** 2001. Fungal ABC proteins: pleiotropic drug resistance, stress response and cellular detoxification. Research in Microbiology **152:**375-389.

47. **Wu, A., J. A. Wemmie, N. P. Edgington, M. Goebl, J. L. Guevara, and W. S. Moye-Rowley.** 1993. Yeast bZip proteins mediate pleiotropic drug and metal resistance. Journal of Biological Chemistry **268:**18850-8.

48. **Zhang, X., Z. Cui, T. Miyakawa, and W. S. Moye-Rowley.** 2001. Cross-talk between transcriptional regulators of multidrug resistance in Saccharomyces cerevisiae. Journal of Biological Chemistry **276:**8812-9.

PDRE	PDRE centered at position (bp):	SEQUENCE
SNQ2#1	-601	GTAGAAATCTAGCA**TCCGCGGA**GCTATTTTAAGTTT
SNQ2#2	-580	AGCTATTTTAAGT**TTCCGCGGA**TGCCTTTCGATCCT
SNQ2#3	-542	CCCAGTCGTCTGG**TTCCGCG**C**A**CATATGACTAAATG

Table 1

Sequences of PDREs found in the *SNQ2* promoter.

Sequences of PDREs are given with conserved nucleotides of the core sequence in bold. Positions of the PDREs relative to the ATG codon are also given.

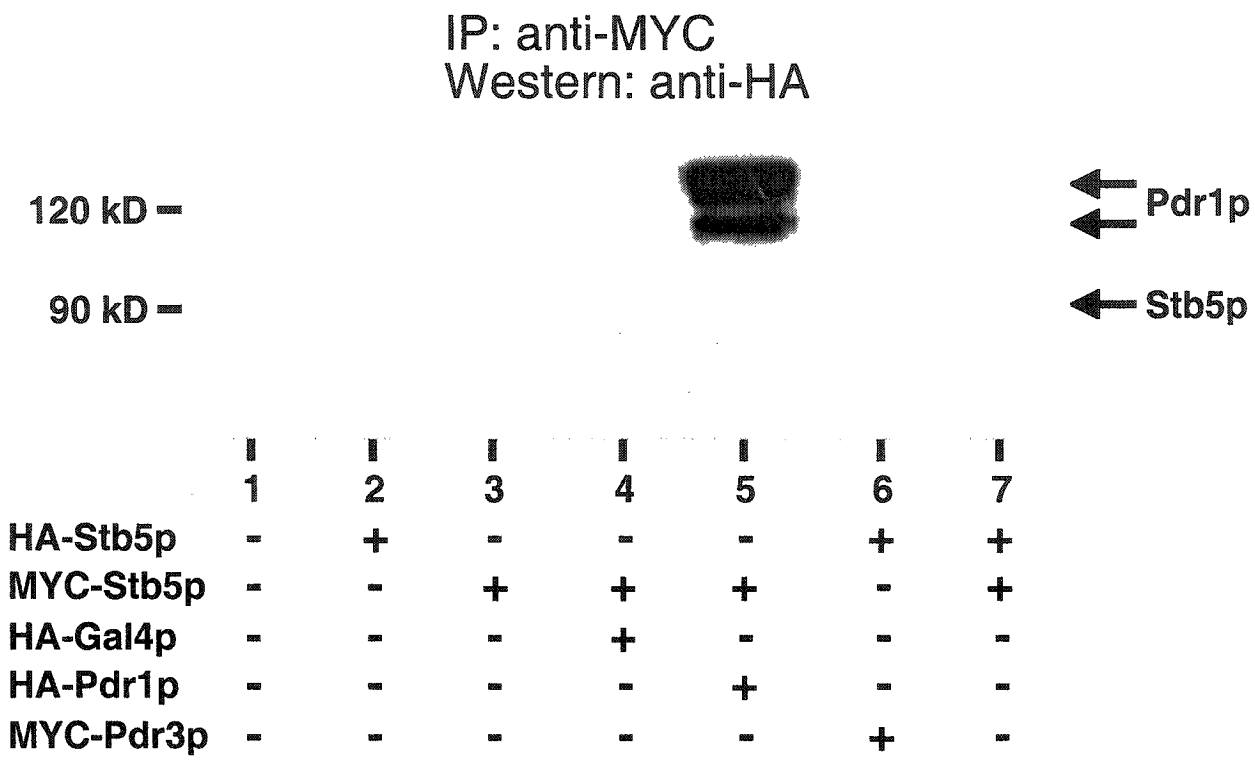
IP: anti-MYC
Western: anti-HA
120 kD –
90 kD –
Pdr1p
Stb5p
1 2 3 4 5 6 7
HA-Stb5p - + - - - + +
MYC-Stb5p - - + + + - +
HA-Gal4p - - - + - - -
HA-Pdr1p - - - - + - -
MYC-Pdr3p - - - - - + -

Fig. 1A

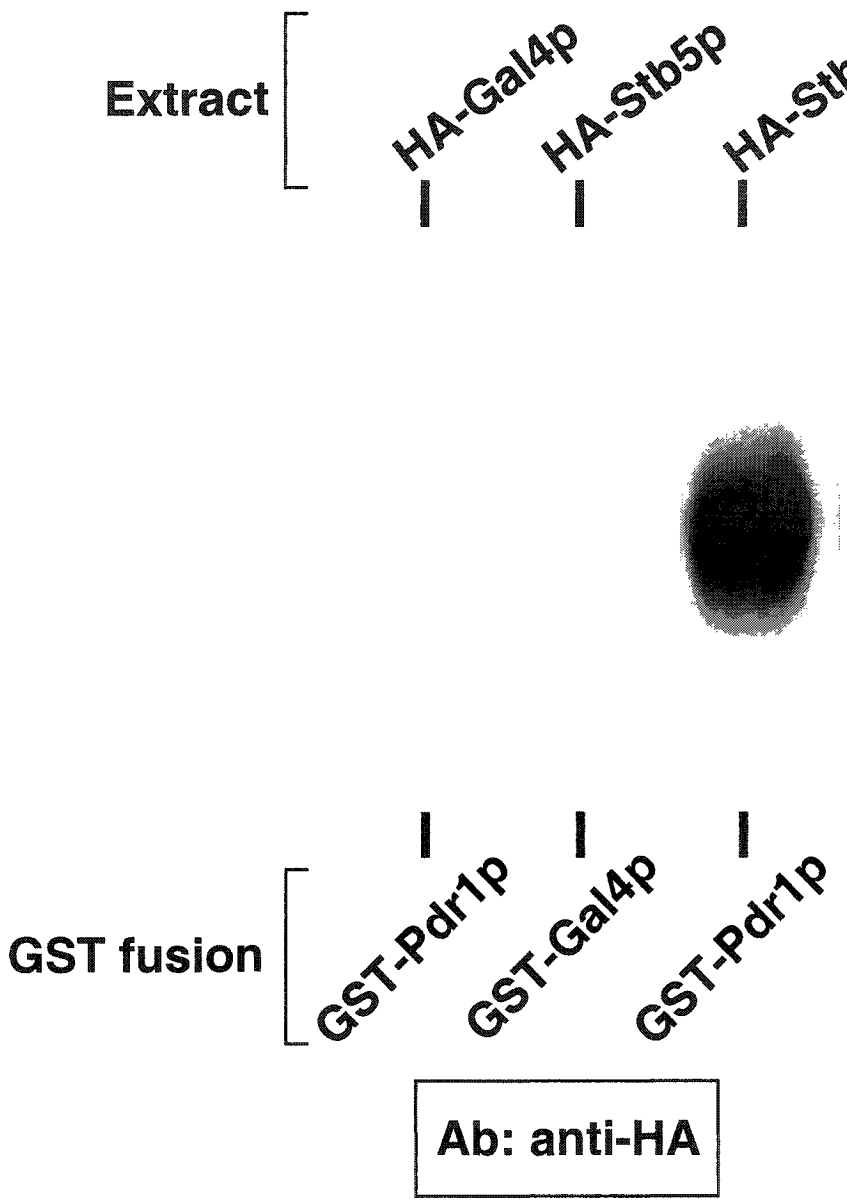

Fig. 1B

Figure 1

Stb5p interacts with Pdr1p *in vivo* and in a GST pull-down assay.

A) Strains expressing various tagged proteins (as indicated by "+" on the bottom of the figure) were used to prepare extracts for immunoprecipitation with an anti-MYC antibody.

Immmunoprecipitated proteins were then detected by Western blot analysis with an anti-HA antibody. Arrows indicate the position of Pdr1p and Stb5p (as seen upon overexposure).

B) Extracts were prepared from strains expressing HA-Gal4p or HA-Stb5p (indicated on top) and incubated with GST fusion proteins bound to beads (bottom) for pull-down assay. After washing, bound proteins were eluted and analyzed by Western blot with an anti-HA antibody.

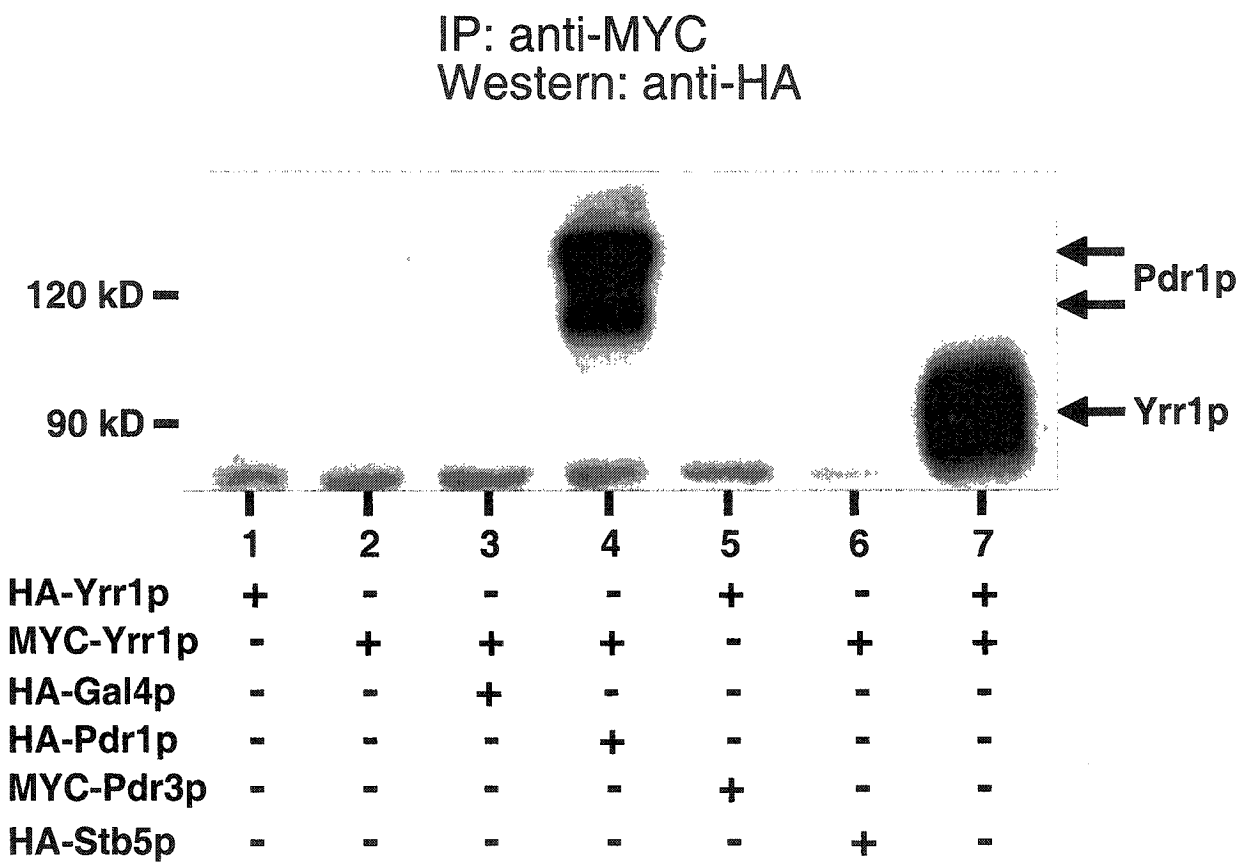
IP: anti-MYC
Western: anti-HA
120 kD
90 kD
Pdr1p
Yrr1p
1 2 3 4 5 6 7
HA-Yrr1p + - - - + - +
MYC-Yrr1p - + + + - + +
HA-Gal4p - - + - - - -
HA-Pdr1p - - - + - - -
MYC-Pdr3p - - - - + - -
HA-Stb5p - - - - - + -

Fig. 2A

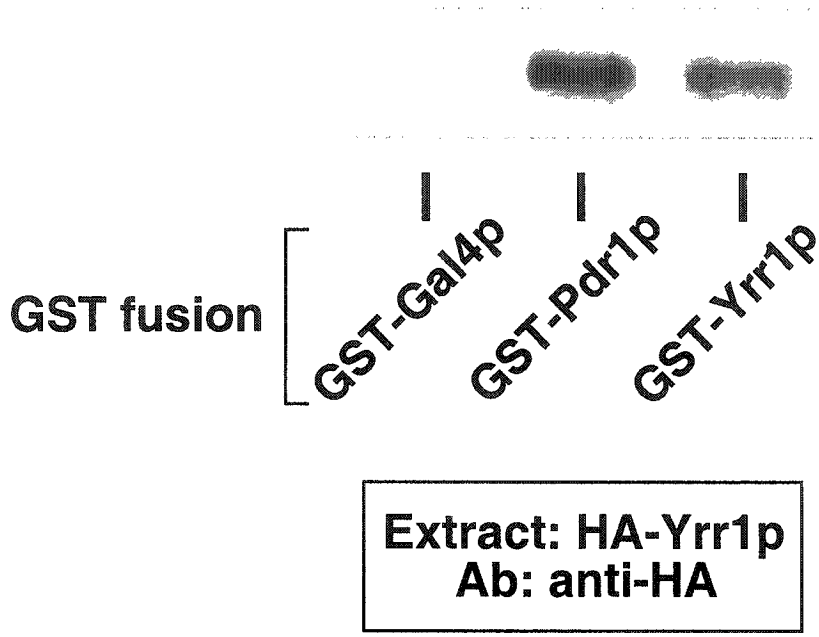

Fig. 2B

Figure 2

Yrr1p interacts with itself and Pdr1p *in vivo* and in a GST pull-down assay.

A) Strains expressing various tagged proteins (as indicated by "+" on the bottom of the figure) were used to prepare extracts for immunoprecipitation with an anti-MYC antibody. Immmunoprecipitated proteins were then detected by Western blot analysis with an anti-HA antibody. Arrows indicate the position of Pdr1p and Yrr1p.

B) An extract was prepared from a strain expressing HA-Yrr1p and incubated with GST fusion proteins (bottom) for pull-down assay. After washing, bound proteins were eluted and analyzed by Western blot with an anti-HA antibody.

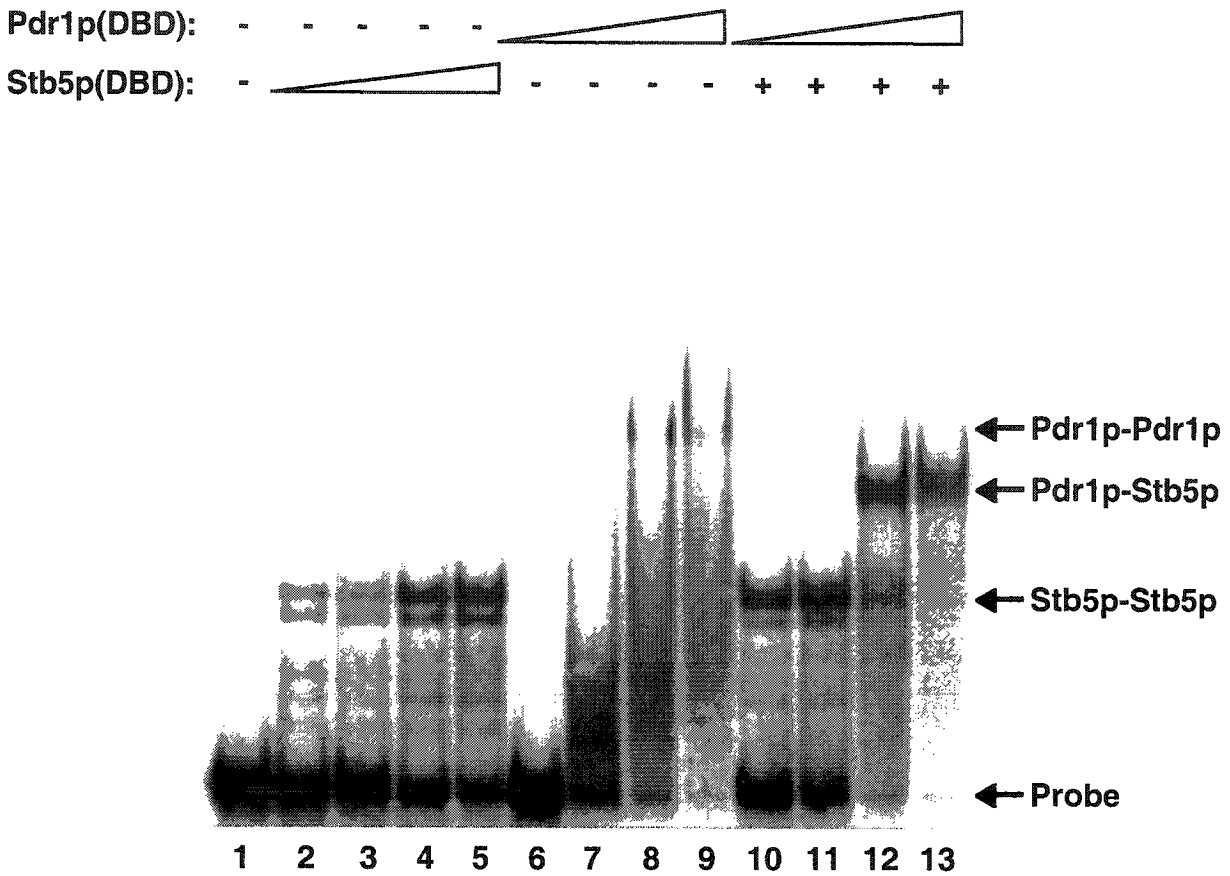

Figure 3

The DBDs of Pdr1p and Stb5p bind *in vitro* to a PDRE as heterodimers.

The purified DBD of Stb5p (1-163) and the DBD of Pdr1 (a.a. 1-152) fused to 6XHis and a triple HA epitope (see Materials and Methods) were used in an EMSA with a probe corresponding to PDRE 1 of SNQ2 (Table 1). Lane 1: probe alone. Lanes 2 to 5: EMSA performed with increasing amounts (0.5 µl, 1 µl, 2 µl and 4 µl) of the DBD of Stb5p. Lanes 6 to 9: EMSA performed with increasing amounts (0.5 µl, 1 µl, 2 µl and 4 µl) of the Pdr1p fusion protein.

Lanes 10 to 13: same as lanes 6 to 9 except that 1 µl of the DBD of Stb5p was added in each lane. Arrows on the right part of the Fig indicate positions of the various complexes.

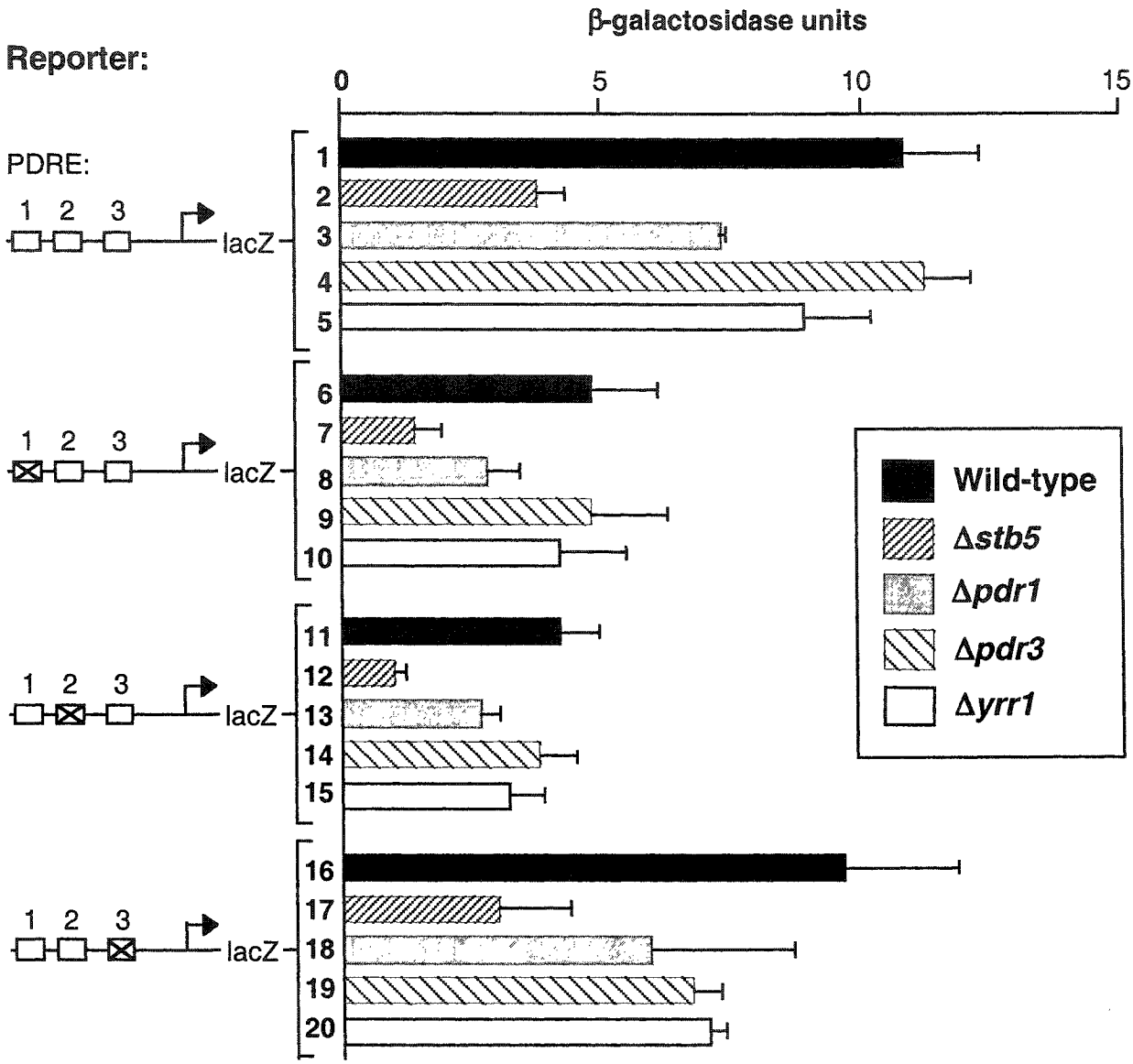

Fig. 4

Activity of SNQ2-lacZ reporters in strains lacking various zinc cluster proteins.

Reporters pSNQ2-lacZ (lanes 1 to 5), pSNQ2ΔPDRE1-lacZ (lanes 6 to 10), pSNQ2ΔPDRE2-lacZ (lanes 11 to 15) and pSNQ2ΔPDRE3-lacZ (lanes 16 to 20) were transformed into various strains as indicated in the right part of the figure. Reporters are schematically shown on the left part of the figure. Boxes correspond to PDREs found in the *SNQ2* promoter. Boxes containing a

"X" correspond to mutated PDREs. β-galactosidase activity was determined as described in Materials and Methods. Standard deviations are also shown.

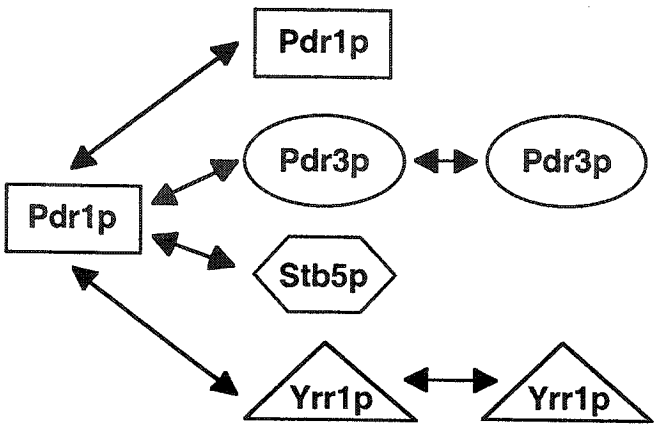

Figure 5

Summary of interactions among zinc cluster proteins Pdr1p, Pdr3p, Stb5p and Yrr1p.

Arrows indicate zinc cluster protein interactions identified in this report and by Mamnun et al. (29). Weak Stb5p-Stb5p and Stb5p-Pdr3p interactions were also detected (see Results).

SECTION 5: DISCUSSION AND CONCLUSION

The sequencing of the *S. cerevisiae* genome has led to the identification of many new putative members of the zinc cluster protein family. In an effort to obtain a better understanding of their roles in the cell, a systematic phenotypic analysis of deletion strains for thirty-two of these zinc cluster proteins was performed. This analysis revealed phenotypes for a number of genes encoding zinc cluster proteins. It identified an additional zinc cluster protein, YDR303C (*RSC3*), as an essential gene. In all, three members of the zinc cluster protein family are essential for cell viability: 1) YMR168C (*CEP3*), 2) YDR303C (*RSC3*), and 3) YPR094W (*RDS3*). Cep3p forms part of the kinetochore and is essential for chromosomal segregation, while Rsc3p is a component of the essential yeast chromatin remodeler complex RSC (6, 86, 144). A homologue of Rds3p in humans has been shown to form part of a spliceosome (163).

The most prevalent class of phenotypes was the inability to grow on non-fermentable carbon sources, and sensitivity to the compound calcofluor white. Respiratory-deficient mutants are unable to grow on non-fermentable carbon sources such as glycerol and lactate. Calcofluor white is a compound that has high affinity for the cell wall component chitin, and sensitivity to this compound is associated with cell wall mutants. Interestingly, the eight different deletion strains that were unable to utilize non-fermentable carbon sources were also sensitive to calcofluor white. The relationship between these two phenotypes is not known.

Growth of the deletion strains at high and at low temperatures, on both rich and poor media was also tested. One strain, *Δyfl052w*, did not grow at high temperatures on rich media, while its growth on poor media was not impaired at the same temperature. Since the minimal media has a higher salt concentration than the rich media, deletion of this gene may render the cells sensitive to a combination of both high temperature and low salt concentration. Another strain, *Δstb5*, was sensitive to cold temperature. Its growth was impaired on both rich and poor media when grown at 20 °C, so the low temperature alone is sufficient for this strain to display a phenotype.

A number of zinc cluster proteins, such as Dal81p, and Uga3p, are involved in the use of alternate nitrogen sources (5, 20). In order to determine if additional zinc cluster proteins could play a similar role, we tested the ability of the zinc cluster protein deletion strains to grow using γ-aminobutyric acid (GABA), serine, threonine, or proline as the sole nitrogen source. Only the deletion of *STB5* resulted in a phenotype; the *Δstb5* strain's growth was slightly impaired as compared to the wild-type strain when GABA, serine, threonine, or proline was the only source of nitrogen. We also tested if growth of the strains on minimal Halvorson media required the presence of folic acid, pantothenic acid or biotin. Again, *Δstb5* was the only strain to display a phenotype, where a slight reduction in growth was observed on Halvorson media even when folic acid, pantothenic acid, and biotin were all present. It appears that deletion of *STB5* results in slightly slower cell growth under minimal growth conditions such as minimal 'Halvorson' medium or when nitrogen sources other than ammonium sulphate are used. However,

none of the other zinc cluster proteins tested displayed a requirement for any of the specific compounds tested.

Since yeast is a facultative anaerobe, we tested the growth of the deletion strains in the absence of oxygen to identify zinc cluster proteins that would be necessary for growth under anaerobic conditions. All of the tested deletion strains were able to grow under anaerobic conditions. So it appears that the only zinc cluster protein required for anaerobic growth is Hap1p (23).

Caffeine, a purine analog, has a toxic effect on cells by inhibiting the MAP kinase pathway and phospodiesterase of the cAMP pathway (54). Deletion strains were tested for hypersensitivity to caffeine. Although the three wild-type strains used in the construction of the deletion strains in this experiment are all derived from the SC288 strain, they showed different sensitivities to caffeine. The YPH499 wild-type strain was not only more sensitive than the other two wild-type strains, FY73 and BY4742, but a higher percentage of deletion strains in YPH499 demonstrated a caffeine-sensitive phenotype. All the FY73 and BY4742-based deletion strains did not display a phenotype when grown on caffeine, except for the *Δstb5* strain. It was highly sensitive to the compound. Five deletion strains in the YPH499 background showed some sensitivity to caffeine. *Δydr520c* was slightly sensitive to caffeine, while *Δykl222c, Δylr228c* (*Δecm22*), and *Δylr278c* showed a moderate sensitivity to the compound. *Δymr019w*, also known as *Δstb4*, was severely sensitive to caffeine. Interestingly, the only two deletion strains that demonstrated a severe sensitivity to caffeine were for Stb4p and

Stb5p; two zinc cluster proteins that have been shown to interact with the transcriptional repressor, Sin3p (67). Therefore, this may indicate that Sin3p has a role in either the cAMP or MAP kinase pathways.

The deletion analysis has revealed various phenotypes for a number of genes encoding zinc cluster proteins, and this may help us to better understand their function in the cell. Some deletion strains even demonstrated multiple phenotypes. For example, deletion of the ORF YFL052W results in an inability to grow on non-fermentable carbon sources, sensitivity to high temperature and calcofluor white. Moreover, *Δstb5* was sensitive to cold temperature and caffeine, and its growth was impaired on minimal media. It is difficult to establish a relationship (if any) between these various phenotypes and some phenotypes may be due to indirect effects. However, some strains did not demonstrate any phenotype under the many different conditions tested. These proteins may perform highly specialized functions, or alternatively, there may be redundant genes encoding zinc cluster proteins. In addition, screening the growth of these deletion mutants under other conditions may reveal additional phenotypes and roles for these proteins.

The zinc cluster proteins Pdr1p, Pdr3p, and Yrr1p are well-known transcriptional activators of multidrug resistance genes (166). To determine if additional zinc cluster proteins are also involved in multidrug resistance, a systematic phenotypic analysis of zinc cluster deletion strains was performed again. However, in this case, the growth of the strains was assayed in the presence of various drugs, each of which has a different

mode of action. Interestingly, we identified eight different deletion strains that showed a phenotype when grown in the presence of the antifungal ketoconazole or the translation inhibitor cycloheximide. Seven strains were sensitive to one or both of these drugs, while one, *Δrdr1*, was resistant to cycloheximide. In another study, we demonstrated that Rdr1p is a transcriptional repressor of *PDR5*. The deletion of the *RDR1* gene causes a 5-fold increase in *PDR5* expression, leading to cycloheximide resistance. It was also shown that the repression by Rdr1p is mediated through a PDRE in the *PDR5* promoter, the same element responsible for the activation of expression by Pdr1p and Pdr3p (56).

The other seven strains identified in the screen were sensitive to drugs. Phenotypes for some of these strains had been previously identified. For example, Yrr1p was previously shown to confer resistance to the DNA mutagen 4-NQO by controlling the expression of *SNQ2* (31). Our screen has shown that the deletion of *YRR1* leads to sensitivity to cycloheximide as well. In addition, Hal9p has been previously shown to confer salt resistance, and our study shows that it also confers resistance to cycloheximide (102). Two zinc cluster proteins, Upc2p and Ecm22p, are activators of sterol biosynthetic genes (157, 158). Deletion of *UPC2* results in sensitivity to ketoconazole, while the cells become sensitive to cycloheximide upon deletion of *ECM22*. Therefore, even though these zinc cluster proteins have similar roles in the sterol biosynthetic pathway, our phenotypic analysis indicates they may have different and specific targets.

Three genes identified in our screen were not named previously, and due to their phenotypes, we called them RDS for regulators of drug sensitivity. *RDS1* is involved in conferring resistance to cycloheximide, while deletion of *RDS2* leads to sensitivity to ketoconazole. The mechanism of action of these newly identified zinc cluster proteins responsible for drug resistance is still undetermined. The zinc cluster proteins may play a direct role in regulating genes involved in PDR, or the phenotype may be due to indirect effects. To help distinguish between these two possibilities, the expression levels of certain genes implicated in multidrug resistance were determined in strains lacking the zinc cluster proteins and compared to the wild-type strain. Northern blot analysis revealed that the deletion of *STB5* greatly decreased RNA levels of *SNQ2* and *PDR16*. Furthermore, the RNA levels of *SNQ2* were greatly reduced in the *Δyrr1* strain. However, many other strains identified in our screen did not show a significant change in *PDR5, SNQ2*, or *PDR16* RNA. Their phenotype may be due to the effects of multidrug resistance genes not tested in our study.

To determine if the differences in RNA levels were due to changes in gene transcription or in RNA stability, we tested whether the activity of *PDR5* and *SNQ2* reporters were altered in the deletion strains. Reduced activity with the *PDR5* reporter was observed in three strains, *Δstb5, and Δhal9*, in agreement with the Northern blot analysis. The *SNQ2* reporter showed reduced activity in the *Δstb5* strain, in agreement with the Northern blot.

In summary, deletion of *STB5* results in cycloheximide sensitivity and reduced *PDR5, SNQ2*, and *PDR16* RNA levels. The decrease in RNA levels correlated with a reduced activity of *PDR5* and *SNQ2* reporters. Since the tested genes all contain PDREs in their promoters, we determined whether the effect of Stb5p on gene expression is mediated through this DNA element. A reporter containing a PDRE inserted in front of a minimal *CYC1* promoter showed decreased activity in a *Δstb5* strain. In addition, the purified DBD of Stb5p was shown to bind specifically to the PDRE in an EMSA. Mutations known to reduce binding of Pdr3p also decreased binding of Stb5p. This suggests that Stb5p acts directly by binding to the PDREs in the *PDR5, SNQ2*, and *PDR16* promoters to activate transcription. This same element is recognized by the transcriptional activators, Pdr1p and Pdr3p (52, 53, 68-70, 92, 114, 165). Yrr1p also appears to activate transcription of *SNQ2* through PDREs (85, 171). In addition, the transcriptional repressor, Rdr1p, has also been shown to act through a PDRE to repress *PDR5* transcription (56). Therefore, the regulation of multidrug resistance genes through PDREs involves many transcriptional regulators and is more complex than initially anticipated. Even though Stb5p appears to be a transcriptional activator of genes involved in PDR, its interaction with Sin3p indicates that it may be both a positive and a negative regulator of gene expression as observed with the zinc cluster proteins Ume6p and Rgt1p (61, 67, 117).

The identification of Stb5p as an important activator of multidrug resistance genes has raised questions regarding its mechanism of activation. Stb5p binds and activates PDREs, the same elements found to be critical for Pdr1p, Pdr3p, and Yrr1p activity (3).

Pdr1p and Pdr3p are able to form homo- and heterodimers (95). Since Stb5p and Yrr1p have similar roles and act through the same element as Pdr1p and Pdr3p, we tested the ability of these four proteins to dimerize with each other. Interestingly, both Stb5p and Yrr1p were able to dimerize with Pdr1p.

Using native co-immunoprecipitation experiments, we determined that Stb5p was found predominantly as a heterodimer with Pdr1p, with a much smaller amount of Stb5p found as a homodimer, and as a Stb5p-Pdr3p heterodimer. In addition, a GST-Pdr1p DBD fusion was able to pull-down Stb5p specifically, indicating that the 152 N-terminal amino acids of Pdr1p are sufficient to mediate dimerization with Stb5p. The native co-immunoprecipitaion experiment also showed that Yrr1p was also able to homodimerize and to heterodimerize with Pdr1p. But unlike Stb5p, it appears that there are similar amounts of homo- and heterodimeric Yrr1p. In agreement with the immunoprecipitation experiments, Yrr1p was pulled down specifically by the GST-Yrr1p DBD and GST-Pdr1p DBD fusion proteins. Therefore, Yrr1p exists in two different subpopulations in the cell. Perhaps each population acts differently at various target DNA sites. We have previously shown that another zinc cluster protein, Rdr1p, represses the expression of some PDR genes such as *PDR5* and *PDR16* through PDREs found in their promoters (56). Interestingly, Rdr1p interacts *in vivo* with both Pdr1p and Pdr3p (S. MacPherson and B. Turcotte, unpublished results). In summary, Pdr1p interacts with Pdr3p, Stb5p, Yrr1p, and Rdr1p (Fig. 1). It appears that Pdr1p is a master regulator involved in recruiting other zinc cluster proteins to fine-tune the regulation of various genes involved in the complex process of PDR. These observations also suggest that the formation of

heterodimeric complexes by zinc cluster proteins may be more common than initially anticipated.

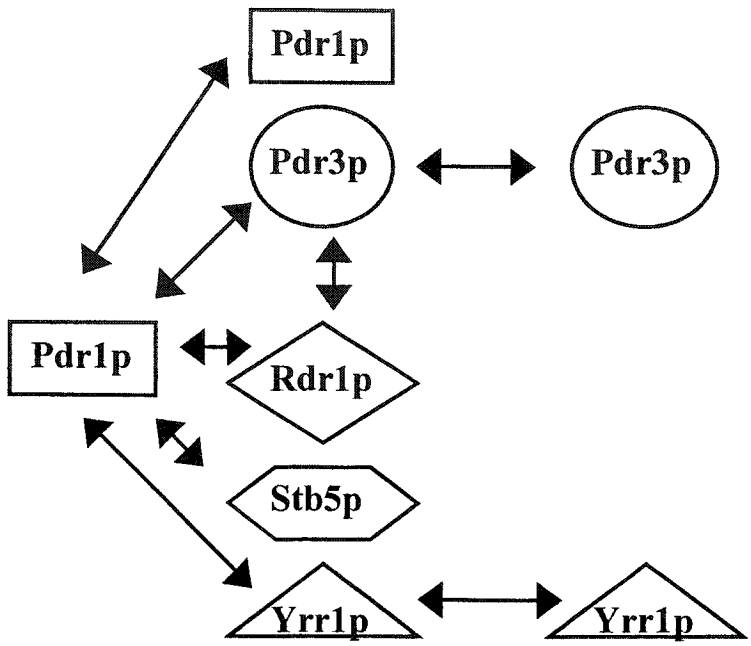

***Figure 1**: Summary of interactions among Pdr1p, Pdr3p, Rdr1p, Stb5p, and Yrr1p.*

An EMSA showed that the purified DBDs of Pdr1p and Stb5p bound cooperatively to a PDRE *in vitro*. Even though the proteins were individually expressed and purified, upon mixing with each other, a stable heterodimeric form bound to DNA was observed. It appears that the Stb5p-Pdr1p heterodimer is the predominant complex that binds to DNA *in vitro*. In addition, the N-termini of Pdr1p (a.a. 1-152) and Stb5p (a.a. 1-163) are sufficient for the formation of heterodimers on DNA. Pdr3p also interacts with the N-terminus of Pdr1p (95). It would be interesting to determine whether both Pdr3p and Stb5p interact with the same domain of Pdr1p.

The importance of the three PDREs in the *SNQ2* promoter was assessed by mutating each of them individually. Mutating PDRE 1 or 2 of *SNQ2* resulted in approximately a 3-fold decrease in activity in the wild-type strain. In contrast, mutating PDRE 3 of *SNQ2* did not cause a significant decrease in activity. This supports the EMSA results where the proteins were not able to bind this PDRE that well (data not shown). The β-galactosidase assay also suggests that Pdr1p, Pdr3p, Stb5p, and Yrr1p have various roles within the cell, since deletion of each of these proteins resulted in a different effect on the activity of the wild-type *SNQ2* reporter. The deletion of Stb5p or Pdr1p resulted in the greatest reduction of activity, 3-fold and 1.5-fold, respectively. While the deletion of Pdr3p or Yrr1p did not cause a significant decrease. However, the deletion of Yrr1p results in lower levels of *SNQ2* mRNA (3, 92); the reason for this discrepancy is not known. It may be due to a difference in promoter context or perhaps the site of Yrr1p action is more upstream or downstream of the region included in our construct.

As observed for *SNQ2*, zinc cluster proteins contribute differently to the regulation of specific PDR genes. For example, a deletion of Pdr1p greatly affects expression of *PDR5* while the removal of Pdr3p has marginal effects (92). The observation that zinc cluster proteins form various combinations of dimers will be invaluable in order to better understand the complex regulation of PDR genes. There may be differences in the binding specificity or activity of each of these populations, allowing for a very specific and varied expression of genes involved in PDR. In addition,

differences also exist amongst the PDREs. Since many zinc cluster proteins, each with a different role, regulate the expression of PDR genes through many different PDREs, the cell's ability to respond to drugs is a lot more adaptable and flexible.

This study has associated some zinc cluster proteins to various processes within the cell. For example, the observation that some strains lacking certain zinc cluster proteins are sensitive to calcofluor white indicates that those zinc cluster proteins may play a role in cell wall biogenesis. In addition, a mutant strain displaying sensitivity to caffeine may reflect the presence of a defective MAP kinase signaling pathway. Cold-sensitive mutants such as *Δstb5* usually contain a general protein defect due to a problem in assembly of a multi-subunit complex (54). We have also identified multiple zinc cluster proteins as potential regulators of drug resistance genes, with Stb5p appearing to play a major role in regulating the transcription of the ABC transporter *SNQ2*. The genes identified in our phenotypic analysis can now be subjected to a focused study of their functions using techniques specific to the associated phenotype.

Since the complete sequencing and systematic deletion of the yeast genome was achieved, many large scale projects involving the study of thousands of yeast genes have been performed (79, 130). For example, transposons containing triple HA tags and a lacZ reporter gene were inserted at random into yeast chromosomal DNA (131). The monitoring of the expression of the lacZ reporter gene allows for the identification of new ORFs, while the HA tags allow for localization of the proteins within the cell. In another study, 5800 of the 6200 yeast ORFs were cloned and overexpressed as GST fusion

proteins. The purified proteins were spotted onto a yeast proteome microarray, which was used to screen the ability of the proteins to interact with specific molecules (173). In addition, in an attempt to identify the transcriptional regulatory networks in yeast, most of the identified putative transcription factors were tagged and expressed in yeast. Chromatin immunoprecipitation (ChIP) was used to isolate the specific DNA that each protein bound to, and the immunoprecipitated DNA was then hybridized to a microarray containing all the intergenic regions of the yeast genome (87). This allowed for the identification of potential target promoters for each transcription factor. These large-scale screens provide a wealth of information concerning the possible function of many uncharacterized proteins, including some of the zinc cluster proteins included in our study. However, these experiments are not conclusive, since many false-positives can be identified in such a large-scale experiment. In addition, previously identified interactions can be missed; as in the project by Lee et al., where the binding of Pdr1p to the *PDR5* promoter was not detected by the intergenic microarray (87). In contrast, the systematic analysis performed in this thesis focused on one family of transcriptional regulators. As opposed to the large-scale approaches mentioned above, our approach involves a smaller number of genes, allowing for a more detailed analysis with more accurate results.

This thesis has assigned a number of phenotypes to many previously uncharacterized zinc cluster proteins. However, a number of zinc cluster protein deletion strains did not demonstrate any phenotype under the numerous conditions we tested. One possible explanation for the lack of phenotype is that some zinc cluster proteins may perform redundant functions. Different approaches have to be taken in order to elucidate

their function. One such approach is the use of double knockouts, which could help assign phenotypes to these zinc cluster proteins. All non-essential zinc cluster proteins (52 in total) could be knocked out in tandem to generate strains carrying all possible combinations of zinc cluster protein double knockouts. This would result in around 1300 strains, with each one containing a different combination of two different zinc cluster proteins deleted. Some of these double mutant strains would be non-viable, and result in synthetic lethality. In this case, synthetic lethality would indicate that the two deleted zinc cluster proteins are redundant for a function essential for viability. Thousands of double deletion strains were tested for synthetic lethality by Tong et al., using robots after sporulation to select for the haploid double mutants. Using this approach, they implicated a number of proteins in cytoskeletal organization, and in DNA synthesis and repair (150). The viable double deletion strains can be subjected to the same phenotypic analysis used previously with the single deletion strains. The identification of a phenotype for any double deletion will imply that the two zinc cluster proteins deleted in that strain perform similar functions related to the condition resulting in the phenotype.

Since these putative zinc cluster proteins are probably transcriptional regulators, the identification of their target genes would be invaluable in understanding their functions. A general strategy for identifying the target genes of zinc cluster proteins has been developed and used to identify the targets of proteins such as Yrr1p and Pdr8p (36, 58, 85). This involves the generation of a chimeric protein consisting of the activation domain of Gal4p fused to the DNA-binding domain of the zinc cluster protein of interest. To render these chimeric proteins constitutively active, these proteins lack the zinc

cluster proteins' middle homology regions, which have been shown to inhibit the regulator's activity. The expression of these proteins is under the control of an inducible promoter such as the GAL1-10 promoter. This system is advantageous since some zinc cluster proteins are only active under certain conditions, such as Gal4p which is only active in the presence of galactose (91). With this strategy, the expression of the chimeric protein and its activation can be controlled through the presence of galactose. A yeast strain, which has had the zinc cluster protein of interest deleted, is transformed with the plasmid encoding the chimeric protein, which contains the DNA-binding domain of the deleted zinc cluster protein. The cells are grown in the presence and absence of galactose, the RNA is then isolated, and used for microarray analysis. The genes with increased expression in the presence of galactose are classified as potential target genes of the zinc cluster protein of interest. However, since the DNA-binding domain of a transcriptional repressor would be fused to an activation domain, this experiment can not differentiate between target genes that would be repressed or activated by the wild-type zinc cluster protein. The information from this analysis combined with the phenotypic analysis would give a more complete picture regarding a protein's role in the cell.

Our phenotypic analysis did indicate that eight zinc cluster proteins might play a role in drug resistance. However, the exact role of six of these proteins in multidrug resistance (if any) remains to be elucidated. The target gene strategy described above can prove very useful to better understand their role in the cell and in identifying the specific multidrug resistance genes responsible for the phenotype. The other two identified proteins, Rdr1p and Stb5p, have been shown to play an important role in multidrug

resistance. Some of their target genes have been identified and their mechanism of action is somewhat understood. Rdr1p is a repressor of multidrug resistance genes, such as *PDR5* and *PDR16*, and has been shown to act through a PDRE found in the *PDR5* promoter (56). However, its exact mechanism of action is not clear. It may repress transcription by interacting with activators of PDR genes, sequestering them, and thereby preventing their binding of DNA to activate the target genes. Another possibility is that it binds to the promoter of the gene, and recruits co-repressors. This mechanism is seen with the zinc cluster protein Ume6p, which represses transcription by recruiting the Isw2 chromatin remodeler complex and/or the histone deacetylase Rpd3p- Sin3p complex to target promoters to which it is bound (46, 62). Our lab has found that the addition of the HDAC inhibitor, Trichostatin A (TSA), upregulates expression of the *PDR5* gene in a wild type strain, but not in a *Δrdr1* deletion strain (S. MacPherson and B. Turcotte, unpublished results). In addition, we have shown that Rdr1p interacts with Pdr1p and Pdr3p, but not with itself *in vivo*, while the purified DNA-binding domains of Rdr1p and Pdr1p can cooperatively bind PDREs *in vitro* (S. MacPherson and B. Turcotte, unpublished results). These results imply that perhaps Rdr1p/Pdr1p or Rdr1p/Pdr3p heterodimers bind PDREs in the promoters of the target genes and repress their transcription by recruiting histone deacetylases.

As mentioned previously, regulators of PDR genes are able to heterodimerize, leading to the presence of various sub-populations of regulators: some containing only transcriptional activators and others that contain the repressor, Rdr1p. Since most characterized zinc cluster proteins such as Gal4p and Hap1p bind DNA as homodimers

(148), other transcriptional regulators were assumed to also act solely as homodimers. In addition to the PDR heterodimers, other zinc cluster proteins such as Oaf1p and Pip2p have been recently shown to act as heterodimers (66, 132). Therefore, the existence of zinc cluster proteins acting as heterodimers may be more widespread than previously thought; it might even be the prevalent mode of regulation by zinc cluster proteins. Certain zinc cluster proteins have similar roles to each other, and have the same target genes. For example, Ecm22p and Upc2p are both involved in sterol biosynthesis. They both activate transcription of *ERG2* by binding sterol regulatory elements (SREs) found in its promoter (158). As in the case with Pdr1p, Pdr3p and Stb5p, Ecm22p and Upc2p bind the same element to activate the transcription of the same gene, but still have different roles within the cell. A strain lacking Ecm22p is sensitive to cycloheximide, but not to ketoconazole, while the inverse is true for *Δupc2* (3). Therefore, different sub-populations of Upc2p and Ecm22p homo- and heterodimers may exist, allowing these proteins to simultaneously have some overlapping and some different functions within the cell. Other examples of different zinc cluster proteins with similar roles are also found in yeast. Dal81p and Uga3p are both activators of nitrogen catabolic genes (159), while the zinc cluster proteins, Mal13p, Mal33p, and Mal63p, are all activators of genes necessary to ferment maltose (24, 106). Moreover, one zinc cluster protein, ArgRIIp, heterodimerizes with members of another family of transcriptional regulators, the MADS family, to activate genes involved in arginine metabolism (4). Since, Pdr1p, Pdr3p and members of the Yap family regulate some of the same multidrug resistance genes, it is possible that members of these two families of regulators can act as heterodimers. In fact, it has been demonstrated that Pdr1p and Yap5p bind to many of the same promoters

in a study identifying transcriptional regulatory networks in *Saccharomyces cerevisiae* (87).

The different sub-populations of homo- and heterodimeric Stb5p, Pdr1p, Pdr3p, and Yrr1p were detected in cells grown in rich medium during log phase, but the proportions of these sub-populations might shift under different conditions. The presence of stress or drugs might induce one protein to associate preferentially with a different partner, allowing the cell to respond rapidly. Since different phospho-forms of Pdr1p and Pdr3p are present simultaneously in the cell (95), it is possible that the phosphorylation state of the protein would play a role in determining its dimerization partner. Different environmental stimuli could lead to the phosphorylation or dephosphorylation of Pdr1p or Pdr3p through various signaling pathways. In mammalian cells, a nuclear receptor's dimerization partner and its ability to bind to DNA are regulated by the presence of the receptor's ligand. For example, the presence of thyroid hormone promotes the formation of heterodimeric thyroid hormone receptor complexes on DNA (127). A ligand or another protein could also interact with zinc cluster proteins leading to a change in their dimerization state. Native co-immunoprecipitation experiments conducted on strains after exposure to various drugs and substrates could help elucidate if yeast responds to various stimuli by altering the dimerization state of its regulators of PDR genes.

Many hyperactive Pdr1p and Pdr3p mutants have been identified in yeast (21, 92, 113, 126, 162). The mutations responsible for the hyperactivity lie generally in two regions of both proteins. They are either found in the C-terminal activation domain, or in

the region found between the middle homology region and the zinc cluster motif (166). Some mutations in Pdr3p are in the vicinity of the predicted coiled-coil motifs thought to be responsible for protein-protein interactions (Fig. 2) (113). The mechanism behind the hyperactivity has not been determined. One possible explanation is that the identified mutations affect the protein's ability to dimerize with certain partners. There may be differences in the binding specificity or activity of each type of dimers. The mutations might also abolish the interaction of Pdr1p and Pdr3p with Rdr1p, alleviating Rdr1p's repression. However, in some cases the presence of a hyperactive Pdr1p mutant may lead to an 80-fold increase in *PDR5* expression, while the expression of *PDR5* is increased by around 5-fold in a *Δrdr1* strain (21, 56). Therefore the inhibition of Pdr1p-Rdr1p interaction would probably not account for the entire hyperactivity of some Pdr1p mutants. Native co-immunoprecipitation experiments using the hyperactive mutant Pdr1p and Pdr3p proteins will prove useful in determining if changes in dimerization are responsible for the increased activity. Identification of the regions responsible for a protein's dimerization with each partner will 1) help determine if a change in dimerization is responsible for the hyperactivity of the mutants, and 2) clarify the mechanism of dimerization itself.

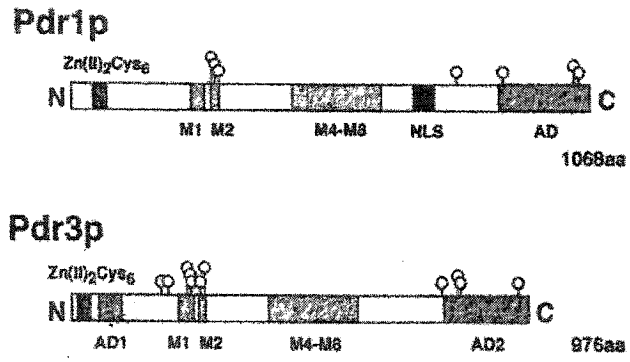

Figure 2: *The location of the gain of function mutations found within Pdr1p and Pdr3p (mutations indicated with a circle) (166).*

Crystal and solution structures of the DBDs of zinc cluster proteins Gal4p, Ppr1p, Put3p and Hap1p show that these proteins homodimerize via a coiled-coil dimerization domain composed of heptad repeats located at the C-terminus of the zinc finger (10, 71, 75, 96, 97, 145, 160). Similar motifs have been identified in Pdr1p, Pdr3p, Stb5p, and Yrr1p (137). Yrr1p has only one predicted coiled-coil domain in its DNA-binding domain, while Pdr1p has two, and Pdr3p has three. However, it has not been determined whether any of these domains do indeed mediate dimerization. Since many of the zinc cluster regulators of PDR interact with multiple partners, different domains within each protein might mediate the association with different partners. Comparison of the sequences of the predicted coil-coil domains in Pdr3p, Stb5p and Yrr1p, shows that certain residues are conserved. They all contain valine and leucine residues in the first heptad of the coiled-coil domain (Fig. 3). Since, they all interact with Pdr1p, it is not surprising that similarities might exist in their predicted coiled-coil domains. However, mutational analysis is needed to verify whether these domains are indeed responsible for the dimerization of these zinc cluster proteins. It will help to determine which residues

within each zinc cluster protein are important for its ability to dimerize with a specific partner. The number of dimerization domains within each protein, and the identity of the specific domains through which each protein interacts must be determined in order to define the structural basis for the multiple interactions of Pdr1p with other zinc cluster proteins.

Pdr3p:	a.a.77-90	**V**	QH	**L**	DTA:	**I**KLDNQY
Stb5p:	a.a.142-155	**V**	SS	**L**	ISV:	**L**TS**L**NDN
Yrr1p:	a.a.110-123	**V**	EE	**L**	ENK:	**I**R**I**L**E**AE

***Figure 3**: Sequence comparison of the heptad repeat motifs of Pdr3p, Stb5p, and Yrr1p.*

Candida albicans is an opportunistic pathogenic fungus that causes severe infections in immuno-compromised patients (121). Resistance to current anti-fungal drugs such as ketoconazole and fluconazole has become more common (108). Therefore, a better understanding of the mechanism of multidrug resistance in this fungus is needed. The lessons gained from the study of PDR in *S. cerevisiae* can also be applied to *C. albicans*. Upregulation of two ABC drug efflux pumps, Cdr1p and Cdr2p, are responsible for the development of PDR in *Candida* (122, 135, 136). The promoters of these transporters contain drug response elements (DREs) through which the expression of the genes is activated, but the transcription factor responsible for this activation has not yet been identified (32). Zinc cluster proteins are found in *C. albicans* as well, and some homologues have been shown to perform similar functions in both *S. cerevisiae* and *C.*

albicans. However, a search of the *C. albicans* genome sequence reveals no clear homologues for the PDR activators, Pdr1p and Pdr3p. One zinc cluster protein, Fcr1p, has been implicated in PDR in *C. albicans*, but its targets have not been identified (146). After we conducted the phenotypic analysis of zinc cluster protein deletion strains with a number of drugs, we searched the *Candida* genome sequence for homologues of the zinc cluster proteins we identified as potential players in PDR in *S. cerevisiae*. Clear homologues were found for many of them including Stb5p and Upc2p (Fig. 4). The purified DNA-binding domains of CaUpc2p (Ca indicates the *C. albicans* homologue) and Fcr1p have been shown to bind a DRE in an EMSA, suggesting that they regulate the expression of Cdr1p and/or Cdr2p (S. MacPherson and B. Turcotte, unpublished results). Increased expression of CaUpc2p also confers drug resistance *in vivo*, while the overexpression of CaStb5p *in vivo* results in hypersensitivity to drugs (S. MacPherson, N. Soonturngun, and B. Turcotte, unpublished results). These results indicate that CaStb5p and CaUpc2p might be major regulators of multidrug resistance genes in *C. albicans*, just like their *S. cerevisiae* homologues. These zinc cluster proteins could be promising potential targets for new therapies against *Candida* in immuno-compromised patients, especially since there are no zinc cluster proteins in human cells.

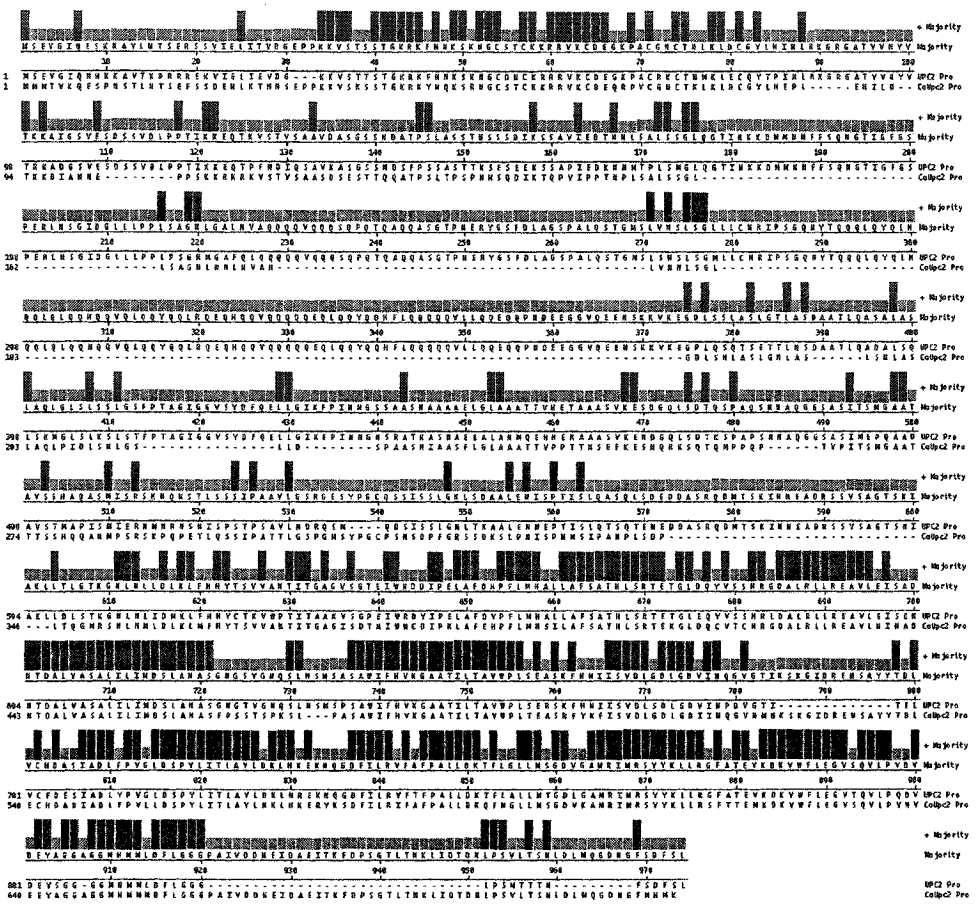

Figure 4: *Similarity between S. cerevisiae Upc2p and the gene product of CA3878 (also namedIPF7289) present in the genome of Candida albicans (higher bars indicate homology). Due to the high degree of similarity, we refer to CA3878 as CaUPC2.*

In conclusion, this study has allowed for a better understanding of the roles of many zinc cluster proteins through their association with a phenotype. It has revealed the presence of new major regulators of drug resistance. One of these newly identified regulators was shown to interact with other transcriptional activators of drug resistance genes, to form different sub-populations of regulators able to control a complex network of PDR genes. This information will prove valuable in understanding the complex

transcriptional regulatory networks in yeast and in understanding the mechanism of PDR in other pathogenic fungal species.

REFERENCES

1. **Adams, M. D., S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne, P. G. Amanatides, S. E. Scherer, P. W. Li, R. A. Hoskins, R. F. Galle, R. A. George, S. E. Lewis, S. Richards, M. Ashburner, S. N. Henderson, G. G. Sutton, J. R. Wortman, M. D. Yandell, Q. Zhang, L. X. Chen, R. C. Brandon, Y. H. C. Rogers, R. G. Blazej, M. Champe, B. D. Pfeiffer, and et al.** 2000. The genome sequence of *Drosophila melanogaster*. Science **287**:2185-2195.
2. **Agalioti, T., S. Lomvardas, B. Parekh, J. M. Yie, T. Maniatis, and D. Thanos.** 2000. Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. Cell **103**:667-678.
3. **Akache, B., and B. Turcotte.** 2002. New regulators of drug sensitivity in the family of yeast zinc cluster proteins. Journal of Biological Chemistry **277**:21254-21260.
4. **Amar, N., F. Messenguy, M. El Bakkoury, and E. Dubois.** 2000. ArgRII, a component of the ArgR-Mcm1 complex involved in the control of arginine metabolism in *Saccharomyces cerevisiae*, is the sensor of arginine. Molecular & Cellular Biology **20**:2087-97.
5. **Andre, B.** 1990. The UGA3 gene regulating the GABA catabolic pathway in *Saccharomyces cerevisiae* codes for a putative zinc-finger protein acting on RNA amount. Molecular & General Genetics **220**:269-76.
6. **Angus-Hill, M. L., A. Schlichter, D. Roberts, H. Erdjument-Bromage, P. Tempst, and B. R. Cairns.** 2001. A Rsc3/Rsc30 zinc cluster dimer reveals novel roles for the chromatin remodeler RSC in gene expression and cell cycle control. Molecular Cell **7**:741-751.
7. **Aranda, A., and A. Pascual.** 2001. Nuclear hormone receptors and gene expression. Physiological Reviews **81**:1269-1304.
8. **Ayer, D. E.** 1999. Histone deacetylases: transcriptional repression with SINers and NuRDs. Trends in Cell Biology **9**:193-198.
9. **Bachman, K. E., M. R. Rountree, and S. B. Baylin.** 2001. Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin. Journal of Biological Chemistry **276**:32282-32287.
10. **Baleja, J. D., R. Marmorstein, S. C. Harrison, and G. Wagner.** 1992. Solution structure of the DNA-binding domain of Cd2-GAL4 from *Saccharomyces cerevisiae*. Nature **356**:450-453.
11. **Balzi, E., and A. Goffeau.** 1994. Genetics and biochemistry of Yeast multidrug resistance. Biochimica et Biophysica Acta - Bioenergetics **1187**:152-162.
12. **Balzi, E., M. Wang, S. Leterme, L. Vandyck, and A. Goffeau.** 1994. Pdr5, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator Pdr1. Journal of Biological Chemistry **269**:2206-2214.
13. **Bannister, A. J., and T. Kouzarides.** 1996. The CBP co-activator is a Histone Acetyltransferase. Nature **384**:641-643.

14. **Bannister, A. J., P. Zegerman, J. F. Partridge, E. A. Miska, J. O. Thomas, R. C. Allshire, and T. Kouzarides.** 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature **410:**120-124.
15. **Bauer, B. E., H. Wolfger, and K. Kuchler.** 1999. Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. Biochimica et Biophysica Acta - Biomembranes **1461:**217-236.
16. **Beato, M., and A. Sanchez-Pacheco.** 1996. Interaction of steroid hormone receptors with the transcription initiaion complex. Endocrine Reviews **17:**587-609.
17. **Berger, S. L.** 2002. Histone modifications in transcriptional regulation. Current Opinion in Genetics & Development **12:**142-148.
18. **Bhaumik, S. R., and M. R. Green.** 2001. SAGA is an essential in vivo target of the yeast acidic activator Gal4p. Genes & Development **15:**1935-1945.
19. **Breunig, K. D.** 2000. Regulation of transcription activation by Gal4p. Food Technology & Biotechnology **38:**287-293.
20. **Bricmont, P. A., J. R. Daugherty, and T. G. Cooper.** 1991. The DAL81 gene product is required for induced expression of two differently regulated nitrogen catabolic genes in *Saccharomyces cerevisiae*. Molecular & Cellular Biology **11:**1161-6.
21. **Carvajal, E., H. B. Vandenhazel, A. Cybularzkolaczkowska, E. Balzi, and A. Goffeau.** 1997. Molecular and phenotypic characterization of Yeast Pdr1 mutants that show hyperactive transcription of various ABC multidrug transporter genes. Molecular & General Genetics **256:**406-415.
22. **Chang, W. H., and R. D. Kornberg.** 2000. Electron crystal structure of the transcription factor and DNA repair complex, core TFIIH. Cell **102:**609-613.
23. **Chantrel, Y., M. Gaisne, C. Lions, and J. Verdiere.** 1998. The transcriptional regulator Hap1p (Cyp1p) is essential for anaerobic or Heme-deficient growth of *Saccharomyces Cerevisiae* - Genetic and molecular characterization of an extragenic suppressor that encodes a Wd repeat protein. Genetics **148:**559-569.
24. **Charron, M. J., E. Read, S. R. Haut, and C. A. Michels.** 1989. Molecular evolution of the telomere-associated MAL loci of Saccharomyces. Genetics **122:**307-16.
25. **Chen, H. W., R. J. Lin, R. L. Schiltz, D. Chakravarti, A. Nash, L. Nagy, M. L. Privalsky, Y. Nakatani, and R. M. Evans.** 1997. Nuclear receptor coactivator ACTR is a novel Histone Acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/P300. Cell **90:**569-580.
26. **Chen, H. W., M. Tini, and R. M. Evans.** 2001. HATs on and beyond chromatin. Current Opinion in Cell Biology **13:**218-224.
27. **Cheung, P., C. D. Allis, and P. Sassone-Corsi.** 2000. Signaling to chromatin through histone modifications. Cell **103:**263-271.
28. **Coleman, S. T., E. Tseng, and W. S. Moye-Rowley.** 1997. *Saccharomyces cerevisiae* basic region-leucine zipper protein regulatory networks converge at the Atr1 structural gene. Journal of Biological Chemistry **272:**23224-23230.
29. **Consortium, C. e. S.** 1998. Genome sequence of the nematode *C. elegans*: A platform for investigating biology. Item Corporate Author C elegans Sequencing Consortium **282:**2012-2018.

30. **Cosma, M. P., T. Tanaka, and K. Nasmyth.** 1999. Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. Cell **97**:299-311.
31. **Cui, Z., T. Shiraki, D. Hirata, and T. Miyakawa.** 1998. Yeast gene YRR1, which is required for resistance to 4-nitroquinoline N-oxide, mediates transcriptional activation of the multidrug resistance transporter gene SNQ2. Molecular Microbiology **29**:1307-15.
32. **de Micheli, M., J. Bille, C. Schueller, and D. Sanglard.** 2002. A common drug-responsive element mediates the upregulation of the *Candida albicans* ABC transporters CDR1 and CDR2, two genes involved in antifungal drug resistance. Molecular Microbiology **43**:1197-1214.
33. **Decottignies, A., L. Lambert, P. Catty, H. Degand, E. A. Epping, W. S. Moye-Rowley, E. Balzi, and A. Goffeau.** 1995. Identification and Characterization of Snq2, a New Multidrug Atp Binding Cassette Transporter of the Yeast Plasma Membrane. Journal of Biological Chemistry **270**:18150-18157.
34. **Defranoux, N., M. Gaisne, and J. Verdiere.** 1994. Functional analysis of the zinc cluster domain of the CYP1 (HAP1) complex regulator in heme-sufficient and heme-deficient yeast cells. Molecular & General Genetics **242**:699-707.
35. **Delahodde, A., T. Delaveau, and C. Jacq.** 1995. Positive autoregulation of the Yeast transcription factor Pdr3p, which is involved in control of drug resistance. Molecular & Cellular Biology **15**:4043-4051.
36. **Devaux, F., P. Marc, C. Bouchoux, T. Delaveau, I. Hikkel, M. C. Potier, and C. Jacq.** 2001. An artificial transcription activator mimics the genome-wide properties of the yeast Pdr1 transcription factor. EMBO Reports **2**:493-498.
37. **Dujon, B.** 1996. The yeast genome project - what did we learn? Trends in Genetics **12**:263-270.
38. **Durand, B., M. Saunders, C. Gaudon, B. Roy, R. Losson, and P. Chambon.** 1994. Activation Function 2 (Af-2) of Retinoic Acid Receptor and 9-Cis Retinoic Acid Receptor - Presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on Af-2 activity. EMBO Journal **13**:5370-5382.
39. **Dvir, A., J. W. Conaway, and R. C. Conaway.** 2001. Mechanism of transcription initiation and promoter escape by RNA polymerase II. Current Opinion in Genetics & Development **11**:209-214.
40. **Featherstone, M.** 2002. Coactivators in transcription initiation: here are your orders. Current Opinion in Genetics & Development **12**:149-155.
41. **Fernandes, L., C. Rodrigues-Pousada, and K. Struhl.** 1997. Yap, a Novel Family of eight bZip proteins in *Saccharomyces cerevisiae* with distinct biological functions. Molecular & Cellular Biology **17**:6982-6993.
42. **Friden, P., C. Reynolds, and P. Schimmel.** 1989. A large internal deletion converts yeast LEU3 to a constitutive transcriptional activator. Molecular & Cellular Biology **9**:4056-60.
43. **Geiger, J. H., S. Hahn, S. Lee, and P. B. Sigler.** 1996. Crystal structure of the Yeast TFIIA/TBP/DNA complex. Science **272**:830-836.
44. **Goffeau, A., B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes,**

Y. Murakami, P. Philippsen, H. Tettelin, and S. G. Oliver. 1996. Life with 6000 genes. Science **274:**546 ff.

45. **Goffeau, A., J. Park, I. T. Paulsen, J. L. Jonniaux, T. Dinh, P. Mordant, and M. H. Saier.** 1997. Multidrug-resistant transport proteins in yeast - Complete inventory and phylogenetic characterization of yeast open reading frames within the Major Facilitator Superfamily. Yeast **13:**43-54.
46. **Goldmark, J. P., T. G. Fazzio, P. W. Estep, G. M. Church, and T. Tsukiyama.** 2000. The Isw2 chromatin remodeling complex represses early meiotic genes upon recruitment by Ume6p. Cell **103:**423-433.
47. **Gompel-Klein, P., and M. Brendel.** 1990. Allelism of *SNQ1* and *ATR1*, genes of the yeast *Sacchromyces cerevisiae* required for controlling sensitivity to 4-nitroquinoline N-oxide and aminotriazole. Current Genetics **18:**93-96.
48. **Gounalaki, N., and G. Thireos.** 1994. Yap1p, a yeast transcriptional activator that mediates multidrug resistance, Regulates the Metabolic Stress Response. EMBO Journal **13:**4036-4041.
49. **Grant, P. A., L. Duggan, J. Cote, S. M. Roberts, J. E. Brownell, R. Candau, R. Ohba, T. Owenhughes, C. D. Allis, F. Winston, S. L. Berger, and J. L. Workman.** 1997. Yeast Gcn5 Functions in Two Multisubunit Complexes to Acetylate Nucleosomal Histones - Characterization of an Ada Complex and the Saga (Spt/Ada) Complex. Genes & Development **11:**1640-1650.
50. **Guarente, L.** 1995. Transcriptional coactivators in yeast and beyond. Trends in Biochemical Sciences **20:**517-521.
51. **Ha, N., K. Hellauer, and B. Turcotte.** 1996. Mutations in target DNA elements of yeast HAP1 modulate its transcriptional activity without affecting DNA binding. Nucleic Acids Research **24:**1453-9.
52. **Hallstrom, T. C., and W. S. Moye-Rowley.** 1998. Divergent transcriptional control of multidrug resistance genes in *Saccharomyces cerevisiae*. Journal of Biological Chemistry **273:**2098-104.
53. **Hallstrom, T. C., and W. S. Moye-Rowley.** 2000. Hyperactive forms of the Pdr1p transcription factor fail to respond to positive regulation by the Hsp70 protein Pdr13p. Molecular Microbiology **36:**402-413.
54. **Hampsey, M.** 1997. A review of phenotypes in *Saccharomyces cerevisiae*. Yeast **13:**1099-1133.
55. **Heinzel, T., R. M. Lavinsky, T. M. Mullen, M. Soderstrom, C. D. Laherty, J. Torchia, W. M. Yang, G. Brard, S. D. Ngo, J. R. Davie, E. Seto, R. N. Eisenman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld.** 1997. A complex containing NCOR, mSin3 and histone deacetylase mediates transcriptional repression. Nature **387:**43-48.
56. **Hellauer, K., B. Akache, S. MacPherson, E. Sirard, and B. Turcotte.** 2002. Zinc cluster protein Rdr1p is a transcriptional repressor of the PDR5 gene encoding a multidrug transporter. Journal of Biological Chemistry **277:**17671-17676.
57. **Hellauer, K., M. H. Rochon, and B. Turcotte.** 1996. A novel DNA binding motif for yeast zinc cluster proteins: the Leu3p and Pdr3p transcriptional activators recognize everted repeats. Molecular & Cellular Biology **16:**6096-102.

58. **Hikkel, I., A. Lucau-Danila, T. Delaveau, P. Marc, F. Devaux, and C. Jacq.** 2003. A general strategy to uncover transcription factor properties identifies a new regulator of drug resistance in yeast. Journal of Biological Chemistry **278:**11427-11432.
59. **Hoeijmakers, J. H. J., J. M. Egly, and W. Vermeulen.** 1996. TFIIH - a key component in multiple DNA transactions. Current Opinion in Genetics & Development **6:**26-33.
60. **Huang, E. Y., J. S. Zhang, E. A. Miska, M. G. Guenther, T. Kouzarides, and M. A. Lazar.** 2000. Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. Genes & Development **14:**45-54.
61. **Jackson, J. C., and J. M. Lopes.** 1996. The yeast UME6 gene is required for both negative and positive transcriptional regulation of phospholipid biosynthetic gene expression. Nucleic Acids Research **24:**1322-9.
62. **Kadosh, D., and K. Struhl.** 1997. Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. Cell **89:**365-371.
63. **Kamei, Y., L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S. C. Lin, R. A. Heyman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld.** 1996. A CBP integrator complex mediates transcriptional activation and Ap-1 inhibition by nuclear receptors. Cell **85:**403-414.
64. **Kanazawa, S., M. Driscoll, and K. Struhl.** 1988. ATR1, a *Sacchromyces cerevisiae* gene encoding a transmembrane protein required for aminotriazole resistance. Molecular & Cellular Biology **8:**664-673.
65. **Kao, H. Y., M. Downes, P. Ordentlich, and R. M. Evans.** 2000. Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. Genes & Development **14:**55-66.
66. **Karpichev, I. V., Y. Luo, R. C. Marians, and G. M. Small.** 1997. A complex containing two transcription factors regulates peroxisome proliferation and the coordinate induction of beta-oxidation enzymes in *Saccharomyces cerevisiae.* Molecular & Cellular Biology **17:**69-80.
67. **Kasten, M. M., and D. J. Stillman.** 1997. Identification of the *Saccharomyces cerevisiae* genes STB1-STB5 encoding Sin3p binding proteins. Molecular & General Genetics **256:**376-86.
68. **Katzmann, D. J., P. E. Burnett, J. Golin, Y. Mahe, and W. S. Moye-Rowley.** 1994. Transcriptional control of the Yeast Pdr5 gene by the Pdr3 gene product. Molecular & Cellular Biology **14:**4653-4661.
69. **Katzmann, D. J., T. C. Hallstrom, Y. Mahe, and W. S. Moye-Rowley.** 1996. Multiple Pdr1p/Pdr3p binding sites are essential for normal expression of the ATP Binding Cassette transporter protein-encoding gene Pdr5. Journal of Biological Chemistry **271:**23049-23054.
70. **Katzmann, D. J., T. C. Hallstrom, M. Voet, W. Wysock, J. Golin, G. Volckaert, and W. S. Moylerowley.** 1995. Expression of an ATP-Binding Cassette transporter-encoding gene (Yor1) is required for Oligomycin resistance in *Saccharomyces cerevisiae.* Molecular & Cellular Biology **15:**6875-6883.

71. **King, D. A., L. Zhang, L. Guarente, and R. Marmorstein.** 1999. Structure of a HAP1-DNA complex reveals dramatically asymmetric DNA binding by a homodimeric protein. Nature Structural Biology **6**:64-71.
72. **Knoepfler, P. S., and R. N. Eisenman.** 1999. Sin meets NuRD and other tails of repression. Cell **99**:447-450.
73. **Kolaczkowska, A., and A. Goffeau.** 1999. Regulation of pleiotropic drug resistance in yeast. Drug Resistance Updates **2**:403-414.
74. **Kouzarides, T.** 2002. Histone methylation in transcriptional control. Current Opinion in Genetics & Development **12**:198-209.
75. **Kraulis, P. J., A. R. Raine, P. L. Gadhavi, and E. D. Laue.** 1992. Structure of the DNA-binding domain of zinc GAL4. Nature **356**:448-50.
76. **Krebs, J. E., C. J. Fry, M. L. Samuels, and C. L. Peterson.** 2000. Global role for chromatin remodeling enzymes in mitotic gene expression. Cell **102**:587-598.
77. **Kren, A., Y. M. Mamnun, B. E. Bauer, C. Schuller, H. Wolfger, K. Hatzixanthis, M. Mollapour, C. Gregori, P. Piper, and K. Kuchler.** 2003. War1p, a novel transcription factor controlling weak acid stress response in yeast. Molecular and Cellular Biology **23**:1775-1785.
78. **Kuge, S., N. Jones, and A. Nomoto.** 1997. Regulation of Yap-1 nuclear localization in response to oxidative stress. EMBO Journal **16**:1710-1720.
79. **Kumar, A., and M. Snyder.** 2001. Emerging technologies in yeast genomics. Nature Reviews Genetics **2**:302-312.
80. **Kwok, R. P. S., J. R. Lundblad, J. C. Chrivia, J. P. Richards, H. P. Bachinger, R. G. Brennan, S. G. E. Roberts, M. R. Green, and R. H. Goodman.** 1994. Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature **370**:223-226.
81. **Lachner, M., N. O'Carroll, S. Rea, K. Mechtler, and T. Jenuwein.** 2001. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature **410**:116-120.
82. **Lander, E. S., L. M. Linton, B. Birren, C. Nusbaum, M. C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczky, R. LeVine, P. McEwan, K. McKernan, J. Meldrim, J. P. Mesirov, C. Miranda, W. Morris, J. Naylor, and et al.** 2001. Initial sequencing and analysis of the human genome. Item Corporate Author Int Human Genome Sequencing Conso **409**:860-921.
83. **Lanz, R. B., N. J. McKenna, S. A. Onate, U. Albrecht, J. M. Wong, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley.** 1999. A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex. Cell **97**:17-27.
84. **Larschan, E., and F. Winston.** 2001. The *S. cerevisiae* SAGA complex functions in vivo as a coactivator for transcriptional activation by Gal4. Genes & Development **15**:1946-1956.
85. **Le Crom, S., F. Devaux, P. Marc, X. T. Zhang, W. S. Moye-Rowley, and C. Jacq.** 2002. New insights into the pleiotropic drug resistance network from genome-wide characterization of the YRR1 transcription factor regulation system. Molecular & Cellular Biology **22**:2642-2649.

86. **Lechner, J.** 1994. A zinc finger protein, essential for chromosome segregation, constitutes a putative DNA binding subunit of the *Saccharomyces cerevisiae* kinetochore complex, Cbf3. Embo J **13:**5203-11.
87. **Lee, T. I., N. J. Rinaldi, F. Robert, D. T. Odom, Z. Bar-Joseph, G. K. Gerber, N. M. Hannett, C. T. Harbison, C. M. Thompson, I. Simon, J. Zeitlinger, E. G. Jennings, H. L. Murray, D. B. Gordon, B. Ren, J. J. Wyrick, J. B. Tagne, T. L. Volkert, E. Fraenkel, D. K. Gifford, and R. A. Young.** 2002. Transcriptional regulatory networks in *Saccharomyces cerevisiae*. Science **298:**799-804.
88. **Lewin, B.** 1994. Chapter 29: Building the transcription complex: promoters, factors, and RNA polymerases, GENES V. Oxford University Press, Oxford.
89. **Litt, M. D., M. Simpson, M. Gaszner, C. D. Allis, and G. Felsenfeld.** 2001. Correlation between histone lysine methylation and developmental changes at the chicken beta-globin locus. Science **293:**2453-2455.
90. **Lo, W. S., L. Duggan, N. C. T. Emre, R. Belotserkovskya, W. S. Lane, R. Shiekhattar, and S. L. Berger.** 2001. Snf1 - a histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription. Science **293:**1142-1146.
91. **Lohr, D., P. Venkov, and J. Zlatanova.** 1995. Transcriptional regulation in the yeast Gal gene family - A complex genetic network. FASEB Journal **9:**777-787.
92. **Mahe, Y., A. Parlemcdermott, A. Nourani, A. Delahodde, A. Lamprecht, and K. Kuchler.** 1996. The ATP-Binding Cassette multidrug transporter Snq2 of *Saccharomyces cerevisiae* - a novel target for the transcription factors Pdr1 and Pdr3. Molecular Microbiology **20:**109-117.
93. **Malik, S., and R. G. Roeder.** 2000. Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells. Trends in Biochemical Sciences **25:**277-283.
94. **Mamane, Y., K. Hellauer, M. H. Rochon, and B. Turcotte.** 1998. A linker region of the yeast zinc cluster protein leu3p specifies binding to everted repeat DNA. Journal of Biological Chemistry **273:**18556-61.
95. **Mamnun, Y. M., R. Pandjaitan, Y. Mahe, A. Delahodde, and K. Kuchler.** 2002. The yeast zinc finger regulators Pdr1p and Pdr3p control pleiotropic drug resistance (PDR) as homo- and heterodimers in vivo. Molecular Microbiology **46:**1429-1440.
96. **Marmorstein, R., M. Carey, M. Ptashne, and S. C. Harrison.** 1992. DNA recognition by GAL4: structure of a protein-DNA complex. Nature **356:**408-414.
97. **Marmorstein, R., and S. C. Harrison.** 1994. Crystal structure of a PPR1-DNA complex: DNA recognition by proteins containing a Zn2Cys6 binuclear cluster. Genes & Development **8:**2504-12.
98. **Martens, J. A., J. Genereaux, A. Saleh, and C. J. Brandl.** 1996. Transcriptional activation by Yeast Pdr1p is inhibited by its association with Ngg1p/Ada3p. Journal of Biological Chemistry **271:**15884-15890.
99. **Martinez, E.** 2002. Multi-protein complexes in eukaryotic gene transcription. Plant Molecular Biology **50:**925-947.

100. **Martinez, E., C. M. Chiang, H. Ge, and R. G. Roeder.** 1994. TATA-binding protein-associated factor(s) in TFIID function through the initiator to direct basal transcription from a TATA-less class II promoter. EMBO Journal **13**:3115-3126.
101. **Martinez, E., H. Ge, Y. Tao, C. X. Yuan, V. Palhan, and R. G. Roeder.** 1998. Novel cofactors and TFIIA mediate functional core promoter selectivity by the human TAF(II)150-containing TFIID complex. Molecular & Cellular Biology **18**:6571-6583.
102. **Mendizabal, I., G. Rios, J. M. Mulet, R. Serrano, and I. F. de Larrinoa.** 1998. Yeast putative transcription factors involved in salt tolerance. FEBS Letters **425**:323-8.
103. **Mewes, H. W., K. Albermann, M. Bahr, D. Frishman, A. Gleissner, J. Hani, K. Heumann, K. Kleine, A. Maierl, S. G. Oliver, F. Pfeiffer, and A. Zollner.** 1997. Overview of the yeast genome (Vol 387, Pg 9, 1997). Nature **387**:737.
104. **Moye-Rowley, W. S., K. D. Harshman, and C. S. Parker.** 1989. Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins. Genes & Development **3**:283-292.
105. **Nagy, L., H. Y. Kao, D. Chakravarti, R. J. Lin, C. A. Hassig, D. E. Ayer, S. L. Schreiber, and R. M. Evans.** 1997. Nuclear receptor repression mediated by a complex containing SMRT, mSin3a, and Histone Deacetylase. Cell **89**:373-380.
106. **Needleman, R.** 1991. Control of maltase synthesis in yeast. Molecular Microbiology **5**:2079-2084.
107. **Ng, H. H., and A. Bird.** 1999. DNA methylation and chromatin modification. Current Opinion in Genetics & Development **9**:158-163.
108. **Nguyen, M. H., J. E. Peacock, A. J. Morris, D. C. Tanner, M. L. Nguyen, D. R. Snydman, M. M. Wagener, M. G. Rinaldi, and V. L. Yu.** 1996. The changing face of Candidemia - Emergence of non-*Candida albicans* species and antifungal resistance. American Journal of Medicine **100**:617-623.
109. **Nielsen, S. J., R. Schneider, U. M. Bauer, A. J. Bannister, A. Morrison, D. O'Carroll, R. Firestein, M. Cleary, T. Jenuwein, R. E. Herrera, and T. Kouzarides.** 2001. Rb targets histone H3 methylation and HP1 to promoters. Nature **412**:561-565.
110. **Nikolaev, I., F. Lenouvel, and B. Felenbok.** 1999. Unique DNA binding specificity of the binuclear zinc AlcR activator of the ethanol utilization pathway in *Aspergillus nidulans*. Journal of Biological Chemistry **274**:9795-9802.
111. **Nikolov, D. B., H. Chen, E. D. Halay, A. A. Usheva, K. Hisatake, D. K. Lee, R. G. Roeder, and S. K. Burley.** 1995. Crystal structure of a TFIIB-TBP-TATA-element ternary complex. Nature **377**:119-128.
112. **Noma, K., C. D. Allis, and S. I. S. Grewal.** 2001. Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. Science **293**:1150-1155.
113. **Nourani, A., D. Papajova, A. Delahodde, C. Jacq, and J. Subik.** 1997. Clustered amino acid substitutions in the yeast transcription regulator Pdr3p increase pleiotropic drug resistance and identify a new central regulatory domain. Molecular & General Genetics **256**:397-405.
114. **Nourani, A., M. Wesolowski-Louvel, T. Delaveau, C. Jacq, and A. Delahodde.** 1997. Multiple-drug-resistance phenomenon in the yeast

Saccharomyces cerevisiae: involvement of two hexose transporters. Molecular & Cellular Biology **17:**5453-60.

115. **Ogryzko, V. V., R. L. Schiltz, V. Russanova, B. H. Howard, and Y. Nakatani.** 1996. The transcriptional coactivators P300 and CBP Are Histone Acetyltransferases. Cell **87:**953-959.
116. **Orphanides, G., T. Lagrange, and D. Reinberg.** 1996. The general transcription factors of RNA Polymerase II. Genes & Development **10:**2657-2683.
117. **Ozcan, S., T. Leong, and M. Johnston.** 1996. Rgt1p of *Saccharomyces cerevisiae*, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription. Molecular & Cellular Biology **16:**6419-26.
118. **Peterson, C. L., and J. L. Workman.** 2000. Promoter targeting and chromatin remodeling by the SWI/SNF complex. Current Opinion in Genetics & Development **10:**187-192.
119. **Pfeifer, K., K. S. Kim, S. Kogan, and L. Guarente.** 1989. Functional dissection and sequence of yeast HAP1 activator. Cell **56:**291-301.
120. **Pham, A. D., and F. Sauer.** 2000. Ubiquitin-activating/conjugating activity of TAF(II)250, a mediator of activation of gene expression in *Drosophila*. Science **289:**2357-2360.
121. **Pla, J., C. Gil, L. Monteoliva, F. Navarrogarcia, M. Sanchez, and C. Nombela.** 1996. Understanding *Candida albicans* at the molecular level. Yeast **12:**1677-1702.
122. **Prasad, R., P. Dewergifosse, A. Goffeau, and E. Balzi.** 1995. Molecular cloning and characterization of a novel gene of *Candida albicans*, Cdr1, conferring multiple resistance to drugs and antifungals. Current Genetics **27:**320-329.
123. **Puigserver, P., C. Adelmant, Z. D. Wu, M. Fan, J. M. Xu, B. O'Malley, and B. M. Spiegelman.** 1999. Activation of PPAR gamma coactivator-1 through transcription factor docking. Science **286:**1368-1371.
124. **Rea, S., F. Eisenhaber, N. O'Carroll, B. D. Strahl, Z. W. Sun, M. Schmid, S. Opravil, K. Mechtler, C. P. Ponting, C. D. Allis, and T. Jenuwein.** 2000. Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature **406:**593-599.
125. **Reece, R. J., and M. Ptashne.** 1993. Determinants of binding-site specificity among Yeast C6 zinc cluster proteins. Science **261:**909-911.
126. **Reid, R. J. D., E. A. Kauh, and M. A. Bjornsti.** 1997. Camptothecin sensitivity is mediated by the pleiotropic drug resistance network in Yeast. Journal of Biological Chemistry **272:**12091-12099.
127. **Ribeiro, R. C., P. J. Kushner, J. W. Apriletti, B. L. West, and J. D. Baxter.** 1992. Thyroid hormone alters *in vitro* DNA binding of monomers and dimers of thyroid hormone receptors. Molecular Endocrinology **6:**1142-1152.
128. **Robzyk, K., L. Recht, and M. A. Osley.** 2000. Rad6-dependent ubiquitination of histone H2B in yeast. Science **287:**501-504.
129. **Roeder, R. G.** 1996. The role of general initiation factors in transcription by RNA polymerase II. Trends in Biochemical Sciences **21:**327-335.
130. **Ross-MacDonald, P.** 2000. Functional analysis of the yeast genome. Functional & Integrative Genomics **1:**99-113.

131. **Ross-Macdonald, P., P. S. Coelho, T. Roemer, S. Agarwal, A. Kumar, R. Jansen, K. H. Cheung, A. Sheehan, D. Symoniatis, L. Umansky, M. Heidtman, F. K. Nelson, H. Iwasaki, K. Hager, M. Gerstein, P. Miller, G. S. Roeder, and M. Snyder.** 1999. Large-scale analysis of the yeast genome by transposon tagging and gene disruption. Nature **402:**413-8.

132. **Rottensteiner, H., A. J. Kal, B. Hamilton, H. Ruis, and H. F. Tabak.** 1997. A heterodimer of the Zn2Cys6 transcription factors Pip2p and Oaf1p controls induction of genes encoding peroxisomal proteins in *Saccharomyces cerevisiae*. European Journal of Biochemistry **247:**776-83.

133. **Sanchezpacheco, A., T. Palomino, and A. Aranda.** 1995. Negative regulation of expression of the pituitary-specific transcription factor Ghf-1/Pit-1 by thyroid hormones through interference with promoter enhancer elements. Molecular & Cellular Biology **15:**6322-6330.

134. **Sande, S., and M. L. Privalsky.** 1996. Identification of Tracs (T-3 Receptor-Associating Cofactors), a family of cofactors that associate with, and modulate the activity of nuclear hormone receptors. Molecular Endocrinology **10:**813-825.

135. **Sanglard, D., F. Ischer, M. Monod, and J. Bille.** 1997. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents - Characterization of Cdr2, a new multidrug ABC transporter gene. Microbiology **143:**405-416.

136. **Sanglard, D., K. Kuchler, F. Ischer, J. L. Pagani, M. Monod, and J. Bille.** 1995. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrobial Agents & Chemotherapy **39:**2378-2386.

137. **Schjerling, P., and S. Holmberg.** 1996. Comparative amino acid sequence analysis of the C6 zinc cluster family of transcriptional regulators. Nucleic Acids Research **24:**4599-607.

138. **Schultz, P., S. Fribourg, A. Poterszman, V. Mallouh, D. Moras, and J. M. Egly.** 2000. Molecular structure of human TFIIH. Cell **102:**599-607.

139. **Schwabe, J. W. R., and D. Rhodes.** 1997. Linkers made to measure. Nature Structural Biology **4:**680-683.

140. **Shikama, N., J. Lyon, and N. B. Lathangue.** 1997. The P300/CBP family - integrating signals with transcription factors and chromatin. Trends in Cell Biology **7:**230-236.

141. **Sil, A. K., S. Alam, P. Xin, L. Y. Ma, M. Morgan, C. M. Lebo, M. P. Woods, and J. E. Hopper.** 1999. The Gal3p-Gal80p-Gal4p transcription switch of yeast: Gal3p destabilizes the Gal80p-Gal4p complex in response to galactose and ATP. Molecular & Cellular Biology **19:**7828-7840.

142. **Spencer, T. E., G. Jenster, M. M. Burcin, C. D. Allis, J. X. Zhou, C. A. Mizzen, N. J. McKenna, S. A. Onate, S. Y. Tsai, M. J. Tsai, and B. W. Omalley.** 1997. Steroid Receptor Coactivator-1 is a Histone Acetyltransferase. Nature **389:**194-198.

143. **Strich, R., R. T. Surosky, C. Steber, E. Dubois, F. Messenguy, and R. E. Esposito.** 1994. *UME6* is a key regulator of nitrogen repression and meiotic development. Genes & Development **8:**796-810.

144. **Strunnikov, A. V., J. Kingsbury, and D. Koshland.** 1995. Cep3 encodes a centromere protein of *Saccharomyces Cerevisiae*. Journal of Cell Biology **128:**749-760.
145. **Swaminathan, K., P. Flynn, R. J. Reece, and R. Marmorstein.** 1997. Crystal Structure of a Put3-DNA complex reveals a novel mechanism for DNA recognition by a protein containing a Zn(2)Cys(6) binuclear cluster. Nature Structural Biology **4:**751-759.
146. **Talibi, D., and M. Raymond.** 1999. Isolation of a putative *Candida albicans* transcriptional regulator involved in pleiotropic drug resistance by functional complementation of a pdr1 pdr3 mutation in *Saccharomyces cerevisiae*. Journal of Bacteriology **181:**231-240.
147. **Tan, S., Y. Hunziker, D. F. Sargent, and T. J. Richmond.** 1996. Crystal structure of a yeast TFIIA/TBP/DNA Complex. Nature **381:**127-134.
148. **Todd, R. B., and A. Andrianopoulos.** 1997. Evolution of a fungal regulatory gene family: the Zn(II)2Cys6 binuclear cluster DNA binding motif. Fungal Genetics & Biology **21:**388-405.
149. **Todd, R. B., R. L. Murphy, H. M. Martin, J. A. Sharp, M. A. Davis, M. E. Katz, and M. J. Hynes.** 1997. The acetate regulatory gene Facb of *Aspergillus nidulans* encodes a Zn(II)2cys6 transcriptional activator. Molecular & General Genetics **254:**495-504.
150. **Tong, A. H. Y., M. Evangelista, A. B. Parsons, H. Xu, G. D. Bader, N. Page, M. Robinson, S. Raghibizadeh, C. W. V. Hogue, H. Bussey, B. Andrews, M. Tyers, and C. Boone.** 2001. Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science **294:**2364-2368.
151. **Torchia, J., D. W. Rose, J. Inostroza, Y. Kamei, S. Westin, C. K. Glass, and M. G. Rosenfeld.** 1997. The transcriptional co-activator P/CIP binds CBP and mediates nuclear-receptor function. Nature **387:**677-684.
152. **Tsai, F. T. F., and P. B. Sigler.** 2000. Structural basis of preinitiation complex assembly on human Pol II promoters. EMBO Journal **19:**25-36.
153. **Vallee, B. L., J. E. Coleman, and D. S. Auld.** 1991. Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. Proceedings of the National Academy of Sciences of the United States of America **88:**999-1003.
154. **Vandel, L., E. Nicolas, O. Vaute, R. Ferreira, S. Ait-Si-Ali, and D. Trouche.** 2001. Transcriptional repression by the retinoblastoma protein through the recruitment of a histone methyltransferase. Molecular & Cellular Biology **21:**6484-6494.
155. **Venter, J. C.** 2001. The sequence of the human genome. Science **292:**1838.
156. **Vignali, M., A. H. Hassan, K. E. Neely, and J. L. Workman.** 2000. ATP-dependent chromatin-remodeling complexes. Molecular & Cellular Biology **20:**1899-1910.
157. **Vik, A., and J. Rine.** 2000. Membrane biology: Membrane-regulated transcription. Current Biology **10:**R869-R871.
158. **Vik, A., and J. Rine.** 2001. Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*. Molecular & Cellular Biology **21:**6395-6405.

159. **Vissers, S., B. Andre, F. Muyldermans, and M. Grenson.** 1990. Induction of the 4-aminobutyrate and urea-catabolic pathways in *Saccharomyces cerevisiae*. Specific and common transcriptional regulators. European Journal of Biochemistry **187:**611-616.

160. **Walters, K. J., K. T. Dayie, R. J. Reece, M. Ptashne, and G. Wagner.** 1997. Structure and mobility of the Put3 dimer. Nature Structural Biology **4:**744-750.

161. **Wemmie, A., A. L. Wu, K. D. Harshman, C. S. Parker, and W. S. Moye-Rowley.** 1994. Transcriptional activation mediated by the Yeast Ap-1 protein is required for normal cadmium tolerance. Journal of Biological Chemistry **269:**14690-14697.

162. **Wendler, F., H. Bergler, K. Prutej, H. Jungwirth, G. Zisser, K. Kuchler, and G. Hogenauer.** 1997. Diazaborine resistance in the yeast *Saccharomyces Cerevisiae* reveals a link between Yap1 and the pleiotropic drug resistance genes Pdr1 and Pdr3. Journal of Biological Chemistry **272:**27091-27098.

163. **Will, C. L., H. Urlaub, T. Achsel, M. Gentzel, M. Wilm, and R. Luhrmann.** 2002. Characterization of novel SF3b and 17S U2 snRNP proteins, including a human Prp5p homologue and an SF3b DEAD-box protein. EMBO Journal **21:**4978-4988.

164. **Winzeler, E. A., D. D. Shoemaker, A. Astromoff, H. Liang, K. Anderson, B. Andre, R. Bangham, R. Benito, J. D. Boeke, H. Bussey, A. M. Chu, C. Connelly, K. Davis, F. Dietrich, S. W. Dow, E. L. B. M, F. Foury, S. H. Friend, E. Gentalen, G. Giaever, J. H. Hegemann, T. Jones, M. Laub, H. Liao, N. Liebundguth, R. W. Davis, and et al.** 1999. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science **285:**901-906.

165. **Wolfger, H., Y. Mahe, A. Parle-Mcdermott, A. Delahodde, and K. Kuchler.** 1997. The Yeast ATP Binding Cassette (ABC) protein genes Pdr10 and Pdr15 are novel targets for the Pdr1 and Pdr3 transcriptional regulators. FEBS Letters **418:**269-274.

166. **Wolfger, H., Y. M. Mamnun, and K. Kuchler.** 2001. Fungal ABC proteins: pleiotropic drug resistance, stress response and cellular detoxification. Research in Microbiology **152:**375-389.

167. **Wu, A., J. A. Wemmie, N. P. Edgington, M. Goebl, J. L. Guevara, and W. S. Moye-Rowley.** 1993. Yeast bZip proteins mediate pleiotropic drug and metal resistance. Journal of Biological Chemistry **268:**18850-18858.

168. **Yudkovsky, N., J. A. Ranish, and S. Hahn.** 2000. A transcription reinitiation intermediate that is stabilized by activator. Nature **408:**225-229.

169. **Zawel, L., K. P. Kumar, and D. Reinberg.** 1995. Recycling of the general transcription factors during RNA Polymerase II transcription. Genes & Development **9:**1479-1490.

170. **Zhang, L., and L. Guarente.** 1994. The yeast activator Hap1 - a Gal4 family member - binds DNA in a directly repeated orientation. Genes & Development **8:**2110-2119.

171. **Zhang, X. T., Z. F. Cui, T. Miyakawa, and W. S. Moye-Rowley.** 2001. Cross-talk between transcriptional regulators of multidrug resistance in *Saccharomyces cerevisiae*. Journal of Biological Chemistry **276:**8812-8819.

172. **Zhou, K. M., and G. B. Kohlhaw.** 1990. Transcriptional activator LEU3 of yeast. Mapping of the transcriptional activation function and significance of the activation domain tryptophans. Journal of Biological Chemistry **265:**17409-12.

173. **Zhu, H., M. Bilgin, R. Bangham, D. Hall, A. Casamayor, P. Bertone, N. Lan, R. Jansen, S. Bidlingmaier, T. Houfek, T. Mitchell, P. Miller, R. A. Dean, M. Gerstein, and M. Snyder.** 2001. Global analysis of protein activities using proteome chips. Science **293:**2101-2105.