Functional analysis of zinc cluster proteins in *Saccharomyces cerevisiae*

Carol Wai
Department of Biochemistry
McGill University, Montréal
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TABLE OF CONTENTS

Abstract

In *Saccharomyces cerevisiae*, zinc cluster proteins form a major family of transcriptional regulators for a variety of processes, yet many putative zinc cluster proteins have unknown functions. Previous studies assigned phenotypes to some of these proteins, a few of which showed previously unknown functions in pleiotropic drug resistance (PDR). The first study presented here focuses on a phenotypic analysis of double deletion mutants to further our understanding on functional relationships among zinc cluster proteins that mediate PDR. The second study focuses on a newly characterized zinc cluster protein, Asg1p, and its functional role in regulating stress response genes. In both studies, we found that the relationship among zinc cluster proteins is highly complex and tightly regulated.

Résumé

Chez *Saccharomyces cerevisiae*, les protéines de type $Zn(II)_2Cys_6$ sont une famille majeure de régulateurs transcriptionnels. Néanmoins, le rôle pour un grand nombre d'entre elles est encore inconnu. Nos études ont permis d'attribuer des phénotypes, à quelques une de ces protéines, parmi lesquels on trouve la résistance aux multiples drogues (PDR). Dans le but de comprendre les phénotypes des protéines inconnues de la famille $Zn(II)_2Cys_6$, nous avons dans une première partie analysé les différents phénotypes de protéines possédant une double délétion dans la résistance aux multiples drogues. Dans une seconde partie, nous avons caractérisé le phénotype d'une nouvelle protéine de la famille $Zn(II)_2Cys_6$: Asg1p. Celle-ci est impliquée dans la régulation des gènes de réponse au stress. L'ensemble de nos résultats indique que les relations à travers les protéines de la famille $Zn(II)_2Cys_6$ est extrêmement complexe et hautement régulée.

Section 1:

Literature Review

1.1 Transcription

RNA is the only macromolecule known to function both in the storage and transmission of genetic information and in catalysis, leading to its possible role as an essential chemical intermediate in the development of life (111). The expression of information in a gene normally involves a process known as transcription, the production of a RNA molecule from a DNA template. Transcription occurs in response to a number of conditions including cell growth, organism development, environmental changes, and disease. All eukaryotic organisms have similar transcriptional regulatory mechanisms. Proper transcriptional control is fundamental for life and the transcriptional machinery involved in this process is highly complex and tightly regulated. This section discusses important elements of transcription in yeast and other eukaryotic systems.

1.1.1 Basal Transcription Factors

During transcription, three major kinds of RNA are produced. Messenger RNA (mRNA) encodes the amino acid sequence of one or more polypeptides specified by a gene or set of genes. Transfer RNA (tRNA) reads the information encoded in the mRNA and transfers the appropriate amino acid to a growing polypeptide chain during protein synthesis. Ribosomal RNA (rRNA) makes up ribosomes, which are enzymes involved in protein synthesis. Eukaryotes have three RNA polymerases, each synthesizing specific RNA molecules. RNA polymerase I controls the synthesis of pre-rRNA, which contains precursors for the 18S, 5.8S, and 28S rRNAs. RNA polymerase II is responsible for the synthesis of mRNAs and some specialized RNAs. RNA polymerase III makes tRNAs, 5S rRNA and some other small specialized RNAs (111). Although all RNA polymerases have important functions, only RNA polymerase II will be discussed in detail.

Common abbreviations used in this study: 4-NQO (4-nitroquinoline *N*-oxide), ABC (ATP-binding cassette), CTD (C-terminal domain), DBD (DNA-binding domain), EMSA (electrophoretic mobility shift assay), HAT (histone acetyltransferase), HDAC (histone deacetylase), HSE (heat shock element), MFS (Major Facilitator Superfamily), PDR (pleiotropic drug resistance), PDRE (pleiotropic drug response element), Pol II (RNA polymerase II), SGA (synthetic genetic array), STRE (stress response element), TBP (TATA-binding protein), UAS (upstream activator sequence), URS (upstream repressor sequence).

The yeast RNA polymerase II (Pol II) enzyme consists of 12 subunits and requires other transcription factors to initiate transcription at specific sites (147). In the step-wise assembly model, formation of an active complex begins when the TATA-binding protein (TBP) binds to the TATA element containing the consensus sequence TATAAA. In yeast, TATA elements are usually located 40 to 120 bp upstream of the mRNA initiation site; however, not all promoter regions contain TATA elements. TBP is the most highly conserved eukaryotic transcription factor, with its carboxyl terminal domain (CTD) of 180 amino acids showing greater than 80% sequence identity in a wide variety of species (62, 146). TBP is part of a larger complex known as transcription factor (TF) IID that also includes 10 or more TBP-associated factors (TAFs), depending on the organism (125). TFIID functions as the initial scaffold upon which the pre-initiation complex of transcription factors can assemble (67). TAFs are thought to stabilize TFIID-promoter binding by making specific DNA contacts at elements located near the transcription start site. These TAF-DNA interactions may be especially important at promoters lacking TATA sequences (23, 110). Following TFIID/TBP-binding, TFIIA then binds to stabilize the TFIID complex. Although not always essential, TFIIA can strengthen binding at non-consensus promoters where TBP binding is relatively weak (111). TFIIB binds to TBP and to DNA on either side of TBP. The TFIIB-TBP complex is next bound by TFIIF and Pol II. TFIIF targets Pol II to its promoters by interacting with TFIIB and reducing non-specific binding. Finally, TFIIE and TFIIH bind to create a closed complex. TFIIH has both DNA helicase activity to promote unwinding of DNA and kinase activity to phosphorylate Pol II. Pol II phosphorylation occurs at several places in the CTD of its largest subunit and causes a conformation change that activates transcription (111).

Once the complex is activated, TFIIE and TFIIH are released during the initial synthesis of 60 to 70 RNA nucleotides. TFIIF remains associated with Pol II throughout elongation and the activity of Pol II is greatly enhanced by elongation factors such as TFIIS. TFIIS, encoded by the *DST1* (DNA Strand Transfer) gene in yeast, stimulates transcript cleavage in arrested Pol II to backtrack elongation complexes that are out of register with the transcript 3' end, thus allowing Pol II to resume transcription (46). After

completion of an RNA transcript, transcription terminates by mechanisms not yet well understood. Pol II is dephosphorylated, recycled, and ready to initiate another transcript.

1.1.2 Transcriptional Activation

High levels of transcriptional activity require other promoter-specific DNA sequences called enhancer elements or upstream activator sequences (UASs) in yeast. UASs are usually 10 to 30 bp long and are typically located 50 to 500 bp upstream of the initiation site. UASs function bidirectionally at variable distances upstream of the initiation site but usually do not activate transcription when located downstream.

In general, UASs are recognized by DNA-binding proteins called transcriptional activators that determine the particular regulatory properties of a given promoter (147). The conformation and size of the DNA-binding domain (DBD), as well as the protein's ability to form homodimers or oligomeric interactions with other DNA binding partners, dictate the effective length of an UAS. The portion of the DNA contacted by a single DBD typically spans 4 to 10 bp (48). One well-studied transcriptional activator in yeast is Gal4p. It belongs to the zinc cluster family of transcriptional regulators, which will be discussed in more detail later, and activates transcription of various galactose-inducible genes (59). Gal4p binds as a homodimer to its target genes and recognizes UASs containing the sequence CGG-N_{11}-CCG, where N represents any nucleotide (13, 80, 100). Furthermore, Gal4p itself is activated by another protein known as Gal3p (124).

1.1.3 Transcriptional Repression

Contrary to transcriptional activation, inhibition of transcription occurs when transcriptional repressors bind to repressive DNA elements and prevent the basal machinery from transcribing a gene. One form of gene-specific repression involves the binding of repressor proteins to upstream repressor sequences (URSs) found in relevant yeast promoters (147). URSs are generally most efficient when located between UASs and the TATA element (147). Another form of gene-specific repression includes protein-

protein interactions that block the function of activators, such as the case of Gal4p repression by Gal80p (93). By functionally blocking the Gal4p activation domain, Gal80p represses transcription of Gal4p target genes. Aside from basal repression due to chromatin packaging (which will be discussed in more detail later), one form of global repression involves proteins that interfere directly with components of basic transcriptional machinery (147). For example, Mot1p (Modifier of Transcription) inhibits TBP binding to the TATA element in an ATP-dependent manner and affects the transcription of many, but not all, genes (11).

1.1.4 Transcriptional Co-activators and Co-repressors

In addition to basal transcriptional machinery and DNA-binding factors, transcription also requires co-activators and co-repressors. Co-activators and co-repressors are intermediary proteins that interact with activators and repressors to facilitate sequence-specific transcriptional regulation (110). These co-factors can also harbor chromatin-directed activities. When DNA is condensed within chromatin, the transcription of eukaryotic genes is strongly repressed. Therefore, activation of transcription requires changes in chromatin structure (111). Current studies suggest several mechanisms for chromatin remodeling that include proteins that use ATP to alter histone-DNA interactions, factors that chemically modify histone proteins, and the incorporation of histone variants to increase chromatin accessibility for transcription. Therefore, co-activators and co-repressors can be classified into two broad categories: 1) those that connect sequence-specific DNA binding regulators to the general transcriptional machinery, and 2) those that contain chromatin remodeling capabilities. Here we describe several important co-factors that belong to both categories, but it should be noted that these classes overlap considerably because of the multifunctional nature of large co-activator/repressor complexes.

Mediator Complex

Mediator complexes were originally purified from yeast as activities that helped stimulate activator-dependent transcriptional activity in reconstituted transcription

reactions (26). This provided evidence that at least some DNA sequence-specific activators work by recruiting Pol II to the promoter region of their target genes (110). The yeast mediator complex binds tightly to the CTD of the largest subunit of Pol II and stimulates TFIIH-dependent phosphorylation of the CTD (109). Components of the yeast mediator complex include a subset of SRB polypeptides (first identified in a genetic screen as suppressors of CTD truncations) and several previously uncharacterized proteins, Med1p through Med7p, thought to be primarily responsible for transcriptional activation (109). The yeast mediator complex was found to co-purify with a subcomplex containing Srb8p, Srb9p, Srb10p/CDK8, and Srb11p/cyclin C polypeptides, suggesting its involvement in negative regulation of gene activity (109, 142).

SWI/SNF Complex

All of the ATP-dependent chromatin-remodeling complexes contain an ATPase subunit that belongs to the *SWI2/SNF2* superfamily of proteins (17, 152). The prototypical member of this family is the SWI/SNF complex. In yeast, this complex contains 11 subunits, including Swi2p/Snf2p. Several of the subunits were initially identified as gene products involved in the regulation of the *HO* endonuclease gene and the *SUC2* invertase gene, from which SWI/SNF obtains its name; *HO* is required for mating type switching while *SUC2* mutants are classified as sucrose non-fermenters. Of the known functional subunits, Snf2p has ATPase activity, Swi1p contains an AT-rich interaction domain that allows for nonspecific DNA binding, and Snf5p is involved in assembly and has catalytic functions (49, 79, 152). SWI/SNF complexes also contain the actin-related proteins Arp7p and Arp9p, suggesting their involvement in nuclear structures such as the nuclear matrix (120).

RSC Complex

A SWI/SNF-related chromatin remodeling complex in yeast, the RSC (Remodels Structure of Chromatin) complex, was discovered on the basis of its homology to SWI/SNF. Many of its subunits are homologous to those in SWI/SNF, but unlike SWI/SNF genes, most genes in the RSC complex are essential, such as the ATPase-

encoding gene, *STH1* (SNF2 (Two) Homolog). The target genes of the RSC complex encode ribosomal and cell wall proteins (9, 25).

Histone Acetyltransferases and Histone Deacetylases

Actively transcribed genes correlate with increased histone acetylation, whereas silenced genes located in heterochromatin generally associate with hypoacetylated histones (5). Histone acetylation occurs on highly conserved lysine residues in the N-terminal tails of core histones. This modification affects the transcriptional capacity of chromatin by interfering with nucleosome assembly, higher order packing of chromatin, and interactions between histone and non-histone proteins (55). Many transcriptional co-activators and co-repressors are found in complexes with subunits that possess histone acetyltransferase (HAT) or histone deacetylase (HDAC) activities (110). Two examples of subunits with HAT activity include the yeast TAF130/145 subunit of TFIID (88) and the Gcn5p subunit of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) and ADA complexes (56).

Histone Variants

During S phase of the cell cycle, new histones are produced in abundance and this process is tightly regulated (2). However, some orphan genes escape S phase synthesis and become specialized chromatin components known as histone variants (2). Histone variants have been described for many classes of histones, but the best studied example is the Z variant of H2A (1). Recent experiments carried out in *Saccharomyces cerevisiae* show the importance of H2A.Z in both positive and negative gene transcription (1, 84, 103, 131). H2A.Z recruits both Pol II and TBP to the *GAL1-10* promoters and performs redundant functions with both the SWI/SNF and SAGA chromatin remodeling complexes (1, 131). Further studies show that the transcriptional function of H2A.Z depends on its C-terminal activation domain (84).

1.2 Zinc Finger Proteins

As mentioned in the previous section, transcriptional activators and repressors generally bind to specific DNA sequences and have discrete DBDs. These DBDs usually include one or more characteristic structural motifs (111). One such motif is the zinc finger. The term zinc finger applies to a diverse set of protein motifs that have in common the property of binding zinc ions in order to stabilize the structure of small, independently folded protein domains (75). Zinc finger proteins are a major class of transcriptional regulators in eukaryotes and can be divided into at least 3 types: the classical Cys-Cys-His-His (C_2H_2) family, the zinc twist Cys-Cys-Cys-Cys (C_4) family, and the binuclear zinc cluster family (151).

1.2.1 Classical C_2H_2 Family

In 1985, Klug and colleagues first discovered zinc fingers in the *Xenopus laevis* transcription factor IIIA as zinc-binding minidomains used to grip the regulatory region of the 5S RNA gene (105). This transcription factor had repeating zinc-binding motifs with conserved cysteine and histidine ligands which are now known to be widespread in all eukaryotic binding proteins. In fact, since this discovery, it is estimated that approximately 1% of the human genome (between 300 and 700 human genes) encodes zinc finger-containing proteins (66). Each classical zinc finger has the motif Phe/Tyr-X-Cys-X_{2-5}-Cys-X_3-Phe/Tyr-X_5-Ψ-X_2-His-X_{3-5}-His, where X represents any amino acid and Ψ represents a hydrophobic residue (158). The three-dimensional structure of each zinc finger has a $\beta\beta\alpha$-type conformation. One zinc atom tetrahedrally coordinates itself between two cysteine residues at one end of the 2-stranded anti-parallel β-sheet and two histidine residues at the C-terminal portion of the α-helix (87, 115, 117). A well-characterized zinc finger protein in yeast is the transcriptional regulator encoded by the gene *ADR1* (Alcohol Dehydrogenase Regulator) (19, 41). Adr1p contains two zinc finger DBDs that are essential for its role in transcriptional activation of the alcohol dehydrogenase gene, *ADH2*, through recognition of a six bp UAS (TTGGAG) in the *ADH2* promoter (19, 28, 41).

The functions of classical C_2H_2 zinc fingers go beyond transcriptional regulation and protein-DNA binding. They can also act in protein-protein interactions (95) and in protein-RNA binding (45). Recent studies show that zinc fingers can also act as zinc sensors. Zap1p, a metal-sensing transcriptional activator which contains seven C_2H_2 zinc fingers, responds to zinc deficiency in yeast (169). It recognizes an 11 bp zinc-responsive element in the promoters of genes encoding uptake transporters (*ZRT1*, *ZRT2*, and *FET4*), and genes encoding transporters involved in vacuolar zinc storage (*ZRC1* and *ZRT3*) (18, 94, 106, 154, 167, 168).

1.2.2 Zinc Twist C_4 Family

This family of zinc proteins includes nuclear hormone receptors, GATA proteins, and LIM proteins. The first member identified was the glucocorticoid receptor (65). It contains a highly conserved DBD with four tetrahedrally-arranged cysteine residues and requires two zinc atoms for DNA-binding activity (83). The DNA-binding motif of the glucocorticoid receptor and other members of this family appears helical and is therefore described as a twist (151). The crystal structure of this receptor shows each monomer having two zinc fingers and the consensus amino acid sequence Cys-X_2-Cys-X_n-Cys-X_2-Cys-X_n-Cys-X_2-Cys-X_n-Cys-X_2-Cys (92). Most zinc twist proteins recognize one of two consensus half-sites (AGGTCA or AGAACA) but with a variety of orientations and spacings and often as heterodimers with other receptors (75). The GATA proteins usually contain one zinc finger, while LIM proteins have a second and specific zinc finger containing three cysteines and one histidine (29). Although the LIM domain is found in more than 20 proteins, some are not DNA-binding transcription factors and may be involved in protein-protein interactions instead (75, 130).

1.2.3 Binuclear Zinc Cluster Family

The binuclear zinc cluster family is uniquely fungal. In addition to *Saccharomyces cerevisiae*, these proteins also exist in other fungal species including

Schizosaccharomyces pombe and the pathogenic species *Candida albicans* and *Aspergillus nidulans* (133, 150). All members of this family contain the well-conserved motif Cys-X_2-Cys-X_6-Cys-X_{5-16}-Cys-X_2-Cys-X_{6-8}-Cys, in which six cysteines bind two zinc atoms to coordinate proper folding of the zinc cluster domain (133, 151). Most members of this family are activators, but some proteins act as repressors, such as Rdr1p which represses expression of some genes involved in pleiotropic drug resistance (60). Furthermore, zinc cluster proteins such as Arg81p (Arginine-requiring), Rgt1p (Restores Glucose Transport), and Ume6p (Unscheduled Meiotic gene Expression), can mediate both repression and activation (21, 68, 104, 114, 145).

Like many transcriptional regulators, zinc cluster proteins have separate DNA-binding and activation domains (150). The $Zn(II)_2Cys_6$ cluster motif of the DBD is usually at the N-terminus. However, some exceptions, such as Ume6p, have C-terminal zinc cluster motifs (145, 156). Mutagenesis studies show that cysteines are required for DNA binding (35, 145, 150) and that zinc cluster motifs often bind the trinucleotide sequence CGG (150). Other studies demonstrate that binding specificity is not affected by exchanging zinc cluster motifs from one protein to another, but is affected if the entire DBD is replaced by another (97, 126).

Many zinc cluster proteins contain leucine zipper-like heptad repeat motifs located at the C-terminal end of the zinc cluster (150). These motifs form coiled-coil structures involved in protein-protein interactions. Crystal structures of the DBDs of Gal4p and Ppr1p (Pyrimidine Pathway Regulation) show that these proteins form homodimers and that heptad repeat regions of these proteins mediate dimerization (100, 101). Some zinc cluster proteins lack an obvious heptad repeat motif and therefore may act as monomers, like Ume6p (8). Other proteins, such as Oaf1p (Oleate-Activated transcription Factor) and Pip2p (Peroxisome Induction Pathway), form heterodimers to regulate the activity of genes involved in peroxisome proliferation (71, 128). Pdr1p and Pdr3p, activators involved in mediating pleiotropic drug resistance, can form both homo- and heterodimers (98). Zinc cluster proteins can also dimerize with transcriptional regulators from other families. One such example is Arg80p, which heterodimerizes with ArgRIp and Mcm1p,

members of the MADS (MCM1, AG, DEFAm, SRF) family, to activate arginine metabolism genes (6).

The region between the zinc cluster and heptad repeats is termed the linker region. Within this region, no conservation exists among members of the zinc cluster family, yet it is important in determining DNA binding specificity (97, 126). The tertiary structure of the linker determines the distance between the zinc cluster region of each subunit of the dimer (150). Therefore, variability of this region ensures that proteins bind to different sequences in order to fulfill their specific functions.

The activation domain is generally found in the C-terminal end of the protein and is usually acidic. Between the DNA-binding and the activation domains lies a region of weak homology, termed the middle homology region (133). This region of approximately 80 amino acids may have a role in transcriptional regulation of zinc cluster proteins as deletion of this region renders some zinc cluster proteins constitutively active (121, 170).

As mentioned previously, zinc clusters recognize CGG triplets and the linker region between the zinc cluster and dimerization domain is a major determinant of DNA binding specificity. Since many zinc cluster motifs of different proteins have been shown to bind CGG trinucleotides, transcriptional regulators use two strategies to increase their repertoire of specific binding sites. The first strategy involves the relative orientation of the CGG trinucleotides with respect to each other. These can be oriented as inverted, direct, or everted repeats (137). The second strategy involves the spacing between the triplets as determined by the linker region (97). For example, Gal4p binds as a homodimer to inverted CGG repeats spaced by 11 bp (i.e. CGG-N_{11}-CCG), causing the two zinc clusters to have a head-to-head conformation (100).

Zinc cluster proteins have a wide range of functions. As mentioned previously, Gal4p activates genes involved in galactose metabolism (59). Hap1p (Heme Activator Protein) activates genes involved in cellular respiration and is positively controlled by heme which acts as an oxygen sensor (121, 165). Put3p is a zinc cluster protein constitutively bound to

promoters of genes involved in proline utilization as a nitrogen source and is positively controlled by direct binding of proline (12, 138). Pdr1p and its homologue Pdr3p are zinc cluster activators that mediate pleiotropic drug resistance (PDR; as discussed later) (14, 38, 148). With the exceptions of Cep3p (which functions in chromosome segregation) and Rsc3p (a subunit of the chromatin remodeling complex RSC), most zinc cluster genes are not essential (9, 86).

As mentioned at the beginning of this thesis, transcription occurs in response to different environmental conditions, cell requirements, etc. Here, we take a closer look at the transcriptional mechanisms involved in mediating PDR and in response to stress factors including heat shock, oxidative, and weak acid stress. Furthermore, we outline the key players involved in these mechanisms with an emphasis on those belonging to the zinc cluster protein family.

1.3 Pleiotropic Drug Resistance

PDR describes the ability of an organism to become resistant to a wide range of structurally and functionally unrelated cytotoxic compounds (15). Although advantageous to the organism, this phenomenon creates major medical problems when treating bacterial and fungal infections, immunodeficiency diseases like AIDS, and cancer (15). Therefore, a broader knowledge in this area will help us to discover other ways of treating illness.

1.3.1 Multi-drug Transport Proteins

Cells that have acquired PDR consistently show higher levels of expression of drug efflux pumps (78, 160). These pumps fall into two categories: the Major Facilitator Superfamily (MFS) and the ATP-binding cassette (ABC) family of transporters. When expression of these membrane transporters increases, cells are able to pump out drugs more efficiently and thus can survive in the presence of these drugs. Higher levels of expression often result from mutations in the transcription factors that regulate the expression of these pumps.

Major Facilitator Superfamily

The MFS consists of more than 50 transporters that are present in all organisms. These transporters catalyze specific uni-, sym-, and antiport of sugars, organic acids, or drugs and are energized by proton-motive force (15, 50). Of the known MFS members, 28 of them transport multiple drugs to mediate PDR in yeast, but with different specificities (50, 116). One example in *S. cerevisiae* is the multi-drug transporter encoded by *ATR1*. In multicopy, this transporter confers resistance to aminotriazole, an inhibitor of the histidine biosynthetic pathway, and 4-nitroquinoline *N*-oxide, a DNA-damaging agent (51, 70).

ATP-Binding Cassette Transporters

The family of ABC transporters is the largest known to date with more than 1000 members (64). As their name suggest, most are purely ATP-driven membrane

translocators, but some function as ion channels, channel regulators, receptors, proteases, and sensing proteins (63). ABC transporters mediate the translocation of ions, heavy metals, antibiotics, anticancer drugs, amino acids, and steroids, to name a few (16). All ABC transporters share a similar structure that includes at least one ATP-binding cassette, or nucleotide binding domain, as well as several transmembrane segments (16). The most well-characterized yeast examples are Snq2p and Pdr5p, which, upon amplification, confer resistance to 4-nitroquinoline *N*-oxide and cycloheximide (a protein synthesis inhibitor), respectively (57, 89, 139). The promoters of both *SNQ2* and *PDR5* contain the pleiotropic drug response element (PDRE) CCGCGG, which is recognized by the zinc cluster transcriptional regulators Pdr1p, Pdr3p, Stb5p, and Rdr1p (4, 36, 58, 61, 73, 74, 96).

1.3.2 Zinc Cluster Proteins as Major Players of Pleiotropic Drug Resistance

Pdr1p, Pdr3p

Several members of the zinc cluster protein family regulate the expression of genes encoding ABC or MFS proteins to mediate PDR. The two major players of PDR are Pdr1p and its homologue Pdr3p (14, 38, 148). Both Pdr1p and Pdr3p positively control expression of the ABC transporter genes *PDR5*, *SNQ2*, and *YOR1* (which is involved in oligomycin and reveromycin A resistance upon amplification) (16, 34, 39, 73, 78, 96, 160). Pdr1p and Pdr3p are constitutively phosphorylated and localize to the nucleus (98). The N-terminus of Pdr1p contains a nuclear localization signal which binds importin Pse1p, but this signal is not present in Pdr3p (37). Both Pdr1p and Pdr3p have acidic activation domains at their C-termini as well as middle regions that contain inhibitory motifs (38, 113). Pdr1p and Pdr3p activate genes by binding to PDREs present in promoter regions of PDR target genes (36, 39, 61, 74, 96, 159). The *PDR3* promoter also contains 2 PDREs and evidence shows that it is under autoregulation by Pdr3p (36).

Yrr1p

Yrr1p is another zinc cluster protein that regulates expression of *SNQ2* and *YOR1* (31, 85, 166). A *YRR1* deletion strain also shows sensitivity to cycloheximide (4). The *YRR1*

promoter itself contains PDREs and is regulated by Pdr1p and Yrr1p (85, 166). A recent study characterized the Yrr1p homologue, Yrm1p, which acts as a transcription factor and interacts with the promoters of target genes only in the absence of Yrr1p (91). The sets of target genes directly regulated by Yrr1p or Yrm1p are similar (and include *SNQ2* and *YOR1*), but not identical.

Stb5p

The zinc cluster protein Stb5p positively regulates *SNQ2* and *PDR5* through recognition of PDREs in their promoters (4). However, previous studies indicate that Stb5p may be both a positive and negative regulator of gene expression. By a two-hybrid assay, Stb5p was shown to interact with Sin3p, a repressor of gene expression that acts through the Rpd3p HDAC (69, 72).

Newly Identified Players

A recent analysis of zinc cluster proteins revealed other players that may be involved in PDR. Strains lacking Ecm22p or Hal9p showed sensitivity to cycloheximide, while strains lacking Rds2p or Upc2p showed sensitivity to ketoconazole (an anti-fungal drug) (4). Further studies identified Rdr1p as a transcriptional repressor of PDR that regulates *PDR5* through PDREs (60). Aside from new players that may be involved in PDR, further studies show that known regulators have complex interactions among themselves. Pdr1p forms homo- and heterodimers with Pdr3p and Stb5p while Yrr1p homodimerizes (3, 98). Taken together, these data suggest that the mechanisms behind PDR are highly complicated and have yet to be determined in detail.

1.4 The Stress Response

In nature, threatening conditions including high temperature, nutrient depletion, oxidative stress, and osmotic stress continuously challenge all organisms ranging from bacteria to humans. In order for an organism to survive, it must adapt to changes in the intra- and extracellular environment. The cellular response to these and other stresses includes several lines of defense. The first line of defense consists of low molecular weight components and small proteins that function as chaperones and in repair systems necessary for immediate survival. The rapid primary response also activates signal transduction systems, which then trigger the second line of defense. The second line of defense induces transcription of genes encoding factors with protective functions such as membrane transporters and proteins involved in nutrient metabolism and detoxification pathways (102, 127). Well-characterized transcription factors that respond to various stresses include Hsf1p, Msn2p/4p, Yap1p, and War1p.

1.4.1 Hsf1p, the Heat Shock Response Factor

One of the most well-studied stress responses is the adaptation of an organism to rapid increases in temperature, which is termed the heat shock response. In response to high temperature, organisms synthesize heat shock proteins that act as chaperones involved in protein folding, trafficking, maturation, and degradation (43, 90). In yeast, many heat shock proteins contain a *cis*-regulatory element designated the heat shock element (HSE) which is recognized by the essential heat shock transcription factor, Hsf1p (90, 107, 143, 157). Hsf1p contains four clearly defined domains: a trimerization domain consisting of a three-stranded coiled-coil (119), a DBD with a winged-helix-turn-helix motif (32), and two activation domains located at the N- and C- termini of the protein (27, 112). Recent studies show there are three types of HSEs, and Hsf1p recognizes all three types: 1) the perfect type, nTTCnnGAAnnTTCn (7, 118, 162, 163), 2) the gap type, nTTCnnGAAn(5 bp)nGAAn (132, 149), and 3) the step type, nTTCn(5 bp)nTTCn(5 bp)nTTCn (164), where n represents any nucleotide. Since Hsf1p contains a flexible linker region located between the DNA-binding and trimerization domains, this accounts

for its ability to recognize three different classes of HSEs (47, 164). It is proposed that the regulation of Hsf1p for some heat shock proteins is dependent on protein kinase A which represses *HSF1* expression and maintains Hsf1p activity at constitutively low levels in the absence of heat shock stress (44).

1.4.2 Msn2p and Msn4p, the General Stress Response Factors

The heat shock stress response is not limited to Hsf1p activation alone. Studies show that other transcription factors, in addition to Hsf1p, respond to heat shock stress. One particular study identified a novel *cis*-regulatory element, CCCCT, in the promoter region of the DNA damage responsive gene *DDR2* that responded not only to heat shock stress, but also to DNA damage stress (76). Further studies showed that this same regulatory element in the promoter region of the cytosolic catalase T gene *CTT1* responded to nitrogen and carbon starvation, osmotic stress, oxidative stress, and weak acid in addition to heat shock (99, 102). The major binding factors for this Hsf1p/HSE-independent element, named STRE for stress response element, are the C_2H_2 zinc finger proteins Msn2p and its structural homologue, Msn4p (42, 77, 102, 134). Studies show that components of the Ras/cAMP pathway negatively regulate Msn2p and Msn4p (52, 99, 141). As Msn2p and Msn4p are general stress response regulators, it is not surprising that they regulate a small set of heat shock genes also regulated by Hsf1p, namely *HSP12*, *HSP26*, and *HSP104* (22, 54).

1.4.3 Yap1p, the Oxidative Stress Response Factor

Oxidative stress results from exposure to reactive oxygen species that cause damage to DNA, protein, and membrane lipid content (127). Aside from Msn2p/4p, the bZIP protein Yap1p also plays a major role in the oxidative stress response. The bZIP protein family is characterized by a DBD containing a leucine zipper that mediates dimerization (20, 108). Loss of Yap1p results in decreased resistance to hydrogen peroxide (82, 135). Yap1p directly activates transcription of several genes involved in the oxidation status of the cell, including *GSH1*, *TRX2*, *YCF1*, and *GLR1*, by binding to a consensus site

(TTAG/CTAA) within the promoters of these target genes (82, 155, 161). It is interesting to note that although Yap1p is the only member of its family to activate oxidative stress genes, several members of Yap family of bZIP proteins act in other stresses, including metal and osmotic stress (127).

1.4.4 War1p, the Weak Acid Response Factor

Weak acids are naturally occurring compounds that prevent microbial growth and cause an extended lag phase and cell stasis in yeast cells (144). The weak acid stress response is mediated through Pdr12p, an ABC transporter that effluxes weak acids (122). The zinc cluster protein War1p recognizes the weak acid response element (WARE; CGG-N_{23}-CCG) in the *PDR12* promoter and activates transcription of this gene (81). A recent genome-wide analysis showed the existence of other weak acid stress response genes in addition to *PDR12*, and that many of these genes were regulated independently of War1p and Msn2p/4p (136). Therefore, it is very likely that other unknown factors contribute to various stress responses and that perhaps other zinc cluster proteins like War1p may play a role.

Section 2:

Rationale and Objectives

The phenomenon of drug resistance is well-known in the world of medicine. For example, cancer cells that acquire multi-drug resistance can render some chemotherapy treatments to become useless (30, 53). However, the mechanisms that cause drug resistance are poorly understood. In yeast, studies show that zinc cluster proteins are involved in mediating PDR (14, 38, 147). Furthermore, zinc cluster proteins are uniquely fungal, making them excellent targets for anti-fungal drugs where fungal infections put the lives of immunocompromised patients at risk. Therefore, the knowledge that we gain from simple eukaryotic systems, like yeast, can be used to address medical concerns in higher eukaryotic systems.

As discussed in Section 3, the first objective of this study is to better understand the roles of zinc cluster proteins in PDR. A previous analysis of single deletion mutants yielded several new players with PDR phenotypes (4). To expand upon this study, we have created double deletion mutants to examine the interactions among zinc cluster proteins within the PDR network.

As presented in Section 4, the second objective of this study is to characterize putative zinc cluster proteins. There are over 50 putative zinc cluster proteins in *Saccharomyces cerevisiae*, yet many of them have unknown functions. Since many of the known proteins function as transcriptional regulators in a variety of cellular processes, it is likely that many of the uncharacterized members have roles in regulation as well.

Section 3:

A phenotypic analysis of double deletion mutants reveals functional relationships among zinc cluster proteins that mediate pleiotropic drug resistance in *Saccharomyces cerevisiae*

3.1 Abstract

Zinc cluster proteins belong to a sub-family of the zinc finger transcriptional regulators. Although some members of this family function in well-known processes (e.g. Gal4p regulation of galactose metabolism), many putative zinc cluster proteins have unknown roles. A previous analysis assigned phenotypes to some genes encoding zinc cluster proteins, yet many genes remained uncharacterized, possibly due in part to functional redundancy in the yeast genome. Redundant functions can often be uncovered by synthetic genetic interactions, in which a specific mutant is examined for secondary mutations that alter the original mutant phenotype. In this study, we attempted to characterize eight zinc cluster proteins (Ecm22p, Hal9p, Pdr1p, Pdr3p, Rds2p, Stb5p, Upc2p, and Yrr1p) with known or potential involvement in pleiotropic drug resistance. We conducted a phenotypic comparison between double deletion mutants and their respective single mutants by monitoring cell growth in the presence of caffeine, cycloheximide, ketoconazole, and 4-nitroquinoline *N*-oxide. Among other relationships, our results show that Upc2p and Yrr1p may have functional redundancy in mediating cycloheximide resistance, and that Hal9p and Upc2p may functionally compensate for each other in the presence of caffeine. In conclusion, our analysis provided insight into functional relationships among zinc cluster proteins involved in pleiotropic drug resistance.

3.2 Introduction

Of the 6200 known *Saccharomyces cerevisiae* genes, only 1100 have essential functions (40). Singular deletion of the remaining 5100 genes has no major effect on cell viability, providing evidence of molecular mechanisms that have evolved to buffer the phenotypic consequences of genetic variation (15). Redundant functions can often be uncovered by synthetic genetic interactions, in which a specific mutant is examined for secondary mutations that either suppress or enhance the original mutant phenotype (37). In other words, if a combination of two mutations causes cell death where neither of the singular mutations is lethal, this could imply that the mutated genes act in a single biochemical pathway, or that the genes interact in independent pathways which can functionally compensate for each other (15). Recently, Tong *et al.* developed a method for systematic construction of double deletion strains (37). Termed synthetic genetic array (SGA) analysis, this method crosses one mutation to an array of approximately 4700 deletion mutants to create double-mutant meiotic progeny. Inviable progeny would indicate a functional relationship between both genes of interest.

Previously, we attempted to characterize proteins belonging to the zinc cluster or binuclear cluster protein family of transcriptional regulators (3, 4). Fungal proteins belonging to this family contain a well-conserved DNA-binding motif (Cys-X_2-Cys-X_6-Cys-X_{5-16}-Cys-X_2-Cys-X_{6-8}-Cys) (32, 38) and regulate processes that include galactose metabolism (16, 20, 27), cellular respiration (31), and mediation of pleiotropic drug resistance (PDR) (6, 11, 35). However, the function of many putative zinc cluster proteins is unknown. To better understand their roles, we performed a phenotypic analysis on 33 strains carrying zinc cluster gene deletions. Our studies showed that, amongst other phenotypes, nine of these deletion strains were either resistant or sensitive to at least one drug (3, 4). Although many conditions were tested in our analysis, we could not assign phenotypes to many zinc cluster genes. We believe that this may be due in part to functional redundancy among members of the zinc cluster protein family.

It is known that nearly 16% of the yeast proteome consists of paralogous protein pairs (41). Paralogous transcription factors, aside from having similar protein structure, often interact with similar promoters. For example, Pdr1p and Pdr3p, two members of the zinc cluster protein family known to mediate PDR, have highly similar zinc finger domains and activate similar sets of target genes by recognizing pleiotropic drug resistance elements (PDREs) (12, 13, 18, 25). Yrr1p and its homologue, Yrm1p, are two more examples of zinc cluster proteins that regulate similar target genes (22). However, in this case, Yrm1p interacts with promoters of target genes only in the absence of Yrr1p.

By employing a modified method of SGA analysis, this study attempts to characterize putative zinc cluster proteins through a phenotypic comparison between double deletion mutants and their respective single mutants on various compounds including caffeine, cycloheximide, ketoconazole, and 4-nitroquinoline *N*-oxide (4-NQO).

3.3 Materials and Methods

Media – Media were prepared according to Adams *et al.* (1). YPD medium contained 1% yeast extract, 2% peptone, and 2% glucose. SD medium 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%. Pre-sporulation medium contained 0.8% yeast extract, 0.3% peptone, and 10% glucose. Minimal sporulation medium contained 1% potassium acetate and was supplemented with methionine, lysine, histidine, uracil, and leucine at a final concentration of 0.001%.

Strains – Derivatives of BY4741 (*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) were used to construct strains designed specifically for this study (Table I) (8).

(i) ***Construction of* MFA1pr-HIS3 *cassette.*** A cassette containing a *MFA1* promoter and *HIS3* selection marker was created for integration at the *kanMX4* module (containing a geneticin (G418) selection marker) of deletion strains derived from BY4741 and obtained from Research Genetics (Huntsville, AL) (40). To create this cassette, the *MFA1* promoter was amplified by PCR from a Y3656 genomic DNA template (isolated according to Talibi and Raymond (36)) using the primers ATCAGGAGCTCCAGGATAGTGTGCAACGTGG and CGGGATCCTTCTATTCGATGGCTTTGTA. This PCR product was digested with *Sac*I and *Bam*HI and subcloned into the plasmid pBlueScriptII KS+ (Stratagene) cut with the same enzymes to create pBlue-*MFA1pr*. The *HIS3* selection marker was amplified by PCR from a *Pichia pastoris* genomic template using the primers CGGGATCCATGACAGGAGAACATAAACG and AGATTACTCGAGTGTGAGTACTAAGGTCATCT. The *Pichia pastoris HIS3* gene was used to prevent recombination at the *HIS3* locus in yeast. This PCR product was digested with *Bam*HI and *Xho*I and subcloned into the plasmid pBlue-*MFA1pr* cut with the same enzymes to create pBlue-*MFA1pr-HIS3*. This plasmid was then used as a template to amplify a *MFA1pr-HIS3* cassette containing flanking regions homologous to the *kanMX4* module. The primers used in this PCR amplification were

TTGCCTCGTCCCCGCCGGGTCACCCGGCCAGCGACATGGAGGCCCCAGGATA GTGTGCAACGTGG and TTAGTATCGAATCGACAGCAGTATAGCGACCAGCATTCACATACGTGACCAT GATTACGCCAAGC.

(ii) ***Integration of* MFA1pr-HIS3 *cassette***. Eight BY4741-derived deletion strains (*Δecm22-a*, *Δhal9-a*, *Δpdr1-a*, *Δpdr3-a*, *Δrds2-a*, *Δstb5-a*, *Δupc2-a*, and *Δyrr1-a*) were transformed with the *MFA1pr-HIS3* PCR product and transformants were selected on SD medium lacking histidine. Homologous recombinants (*HIS3* strains) were verified by sensitivity to YPD medium containing 400 μg/ml G418 (Table I).

(iii) ***Mating and Random Spore Analysis***. *HIS3* strains were crossed with deletion strains of the opposite mating type (mating type α, BY4742 background) on YPD medium to obtain 28 diploid strains. These diploid strains contained all possible double deletion combinations of the 8 previously mentioned genes. The diploid colonies were then grown on pre-sporulation medium for 2 days at 30°C prior to growth on minimal sporulation medium for 1 week at room temperature. Individual spores were isolated by random spore analysis (5). Briefly, cells from sporulation plates were incubated in a zymolyase-100T solution (1 mg/ml) at 30°C overnight to lyse unsporulated diploid cells. Nonidet P-40 was then added and cells were sonicated to release haploid spores from their asci. Cells were plated on YPD and grown for 2 days at 30°C. Haploid double deletion strains were obtained by first selecting colonies that grew on SD medium lacking histidine and then, from those colonies, selecting ones showing resistance to G418 at 400 μg/ml. These *HIS3*-G418^R colonies were then screened for a BY4741 background.

Growth assays – Sensitivity to drugs was assayed on YPD plates supplemented with 0.15% caffeine, 0.2 μg/ml cycloheximide (prepared in 100% ethanol), 4 μg/ml ketoconazole, or 0.35 μg/ml 4-NQO (prepared in dimethyl sulfoxide). All drugs were obtained from Sigma. Wild-type, single deletion, and double deletion strains were grown overnight in liquid YPD, spun, and resuspended in water. Cells were then serially diluted (approximately 5×10^3, 1×10^3, 2×10^2, and 4×10^1), spotted on appropriate plates, and grown for 2 to 4 days at 30°C.

3.4 Results

The roles of many putative zinc cluster proteins are unknown. Previous studies attempted to examine the phenotypes of 33 strains carrying deletions of genes encoding zinc cluster proteins on various compounds including caffeine, cycloheximide, ketoconazole, chloramphenicol, 4-NQO, rhodamine 6-G, and oligomycin (3, 4). From these analyses, nine deletion strains were found to be either resistant or sensitive to at least one drug. Of these nine deletion strains, we chose six of them (*Δecm22, Δhal9, Δrds2, Δstb5, Δupc2,* and *Δyrr1*) and, along with *Δpdr1* and *Δpdr3*, constructed all possible combinations of double deletion mutants to determine whether functional relationships exist between these eight genes by phenotype analysis.

Double deletion strain construction. We took advantage of the single deletion strain library (8) and the SGA method developed by Tong *et. al* (37) to construct double deletion strains. Firstly, a *MFA1pr-HIS3* cassette was created for integration at the *kanMX4* module in single deletion strains of a BY4741 (mating type a) background (Fig. 1*A*, Table I). Next, the single deletion strains containing the *MFA1pr-HIS3* cassette were crossed with deletion strains of the opposite mating type (BY4742 background) to obtain all possible double deletion combinations of the previously mentioned genes, resulting in 28 diploid strains (Fig. 1*B*). Diploid colonies were grown on sporulation media and individual spores were isolated by random spore analysis. Haploid double deletion strains were obtained by selecting for growth on media lacking histidine and for G418 resistance.

Sensitivity to cycloheximide. Cycloheximide is a potent inhibitor of translation which acts by binding to the 60S ribosomal subunit to inhibit initiation and elongation (14). As expected from our previous study, the single deletion strains *Δhal9, Δpdr1, Δpdr3, Δrds2,* and *Δstb5* showed sensitivity to cycloheximide, while *Δecm22, Δupc2* and *Δyrr1* showed normal growth comparable to the wild-type strain (Table II). However, a *Δupc2Δyrr1* double deletion strain showed sensitivity to cycloheximide while the deletion strains *Δecm22Δupc2* and *Δecm22Δyrr1* were resistant.

Sensitivity to 4-NQO. With the exception of Δ*yrr1*, all single deletion strains showed slightly inhibited or normal growth comparable to the wild-type strain when spotted on YPD plates containing 4-NQO, a DNA-damaging agent (Table III) (33). The double deletion strains Δ*ecm22*Δ*pdr1*, Δ*ecm22*Δ*stb5*, Δ*pdr1*Δ*pdr3*, Δ*pdr1*Δ*rds2*, Δ*pdr1*Δ*stb5*, Δ*pdr1*Δ*upc2*, Δ*pdr3*Δ*stb5*, and Δ*stb5*Δ*upc2* showed sensitivity to 4-NQO.

Sensitivity to ketoconazole. Ketoconazole belongs to the azole anti-fungal drug family and inhibits the *ERG11* gene product involved in ergosterol synthesis. Ergosterol is a major component in yeast cell membranes. Although the concentration of ketoconazole used in this spotting assay (4 μg/ml) was most likely too high (as shown by wild-type strain hypersensitivity to this compound), it is interesting to note that the double deletion strain Δ*rds2*Δ*yrr1* showed hyperresistance whereas their respective single deletion strains were sensitive (Table IV, Fig. 2).

Growth on caffeine. Growth sensitivity to caffeine is associated with defects in components of MAP kinase pathways (14). Deletion strains were tested for sensitivity to caffeine. As expected, all single deletion strains showed no sensitivity to caffeine except for a Δ*stb5* strain, which is consistent with results obtained previously (Table V) (4). Furthermore, a Δ*stb5* deletion in combination with Δ*ecm22*, Δ*pdr1*, Δ*rds2* and Δ*yrr1* showed similar levels of sensitivity to caffeine as the single Δ*stb5* deletion, with Δ*pdr3*Δ*stb5* showing moderate growth on caffeine. However, Δ*stb5* with either Δ*hal9* or Δ*upc2* suppressed its growth defect on caffeine, but a Δ*hal9*Δ*upc2* double deletion strain was sensitive to caffeine (Fig. 3). Interestingly, Δ*pdr1*Δ*rds2* and Δ*pdr3*Δ*upc2* showed moderate growth on caffeine.

3.5 Discussion

PDR relates to the ability of an organism to become resistant to a wide range of structurally and functionally unrelated cytotoxic compounds (7). Cells that have acquired PDR consistently show higher levels of expression of drug efflux pumps belonging to either the Major Facilitator Superfamily (MFS) or the ATP-binding cassette (ABC) family of transporters (19, 42).

Cycloheximide resistance is mediated primarily by overexpression of the ABC transporter Pdr5p (21). *PDR5* expression is regulated by the zinc cluster proteins Pdr1p, Pdr3p, and Stb5p (2, 3, 17, 29). The results presented in this study are consistent with these findings as Δ*pdr1*, Δ*pdr3*, and Δ*stb5* showed hypersensitivity to cycloheximide (Table II). In a previous study, we showed that deletion of the zinc cluster genes *ECM22*, *HAL9*, and *YRR1* resulted in cycloheximide sensitivity (3). Although our results are consistent for *HAL9,* we did not see cycloheximide sensitivity for Δ*ecm22* nor Δ*yrr1* (Table II). This discrepancy may result from differences in cycloheximide concentrations used (0.2 μg/ml in this study versus 1 μg/ml in our previous study). In analyzing double deletion phenotypes, we observed cycloheximide hypersensitivity of a Δ*upc2*Δ*yrr1* strain whereas strains carrying a single deletion of either gene showed normal growth. Furthermore, Δ*ecm22*Δ*upc2* and Δ*ecm22*Δ*yrr1* strains showed slightly inhibited and normal growth on cycloheximide, respectively. Taken together, these results suggest that Upc2p and Yrr1p may have functional redundancy. *UPC2* encodes an activator involved in sterol biosynthesis, while *YRR1* modulates expression of the ABC transporters Snq2p and Yor1p, which confer resistance to 4-NQO and oligomycin, respectively (9, 39, 43). Sterols are major components of the fungal plasma membrane and help maintain membrane integrity and fluidity (23, 30). As a result, a Δ*upc2*Δ*yrr1* strain with an altered plasma membrane and reduced drug efflux pumps would show hypersensitivity to cycloheximide. It is interesting to note that *UPC2* and *ECM22* encode homologous proteins with functions in sterol biosynthesis, but some findings suggest that they have different and specific targets, as is the case in this study (3, 24, 34, 39).

Resistance to 4-NQO is normally mediated by overexpression of *SNQ2* (10). *SNQ2* expression is regulated by Pdr1p, Pdr3p, Stb5p, and Yrr1p (3, 9, 25). Our results are consistent with previous findings that a complex interplay exists among regulators of drug resistance (2). In this study, we observed 4-NQO sensitivity of a Δ*yrr1* strain, but little or no sensitivity of Δ*pdr1*, Δ*pdr3*, nor Δ*stb5* strains. Yrr1p may therefore act as a major regulator for 4-NQO resistance as all double deletion strains in combination with Δ*yrr1* also showed little to no growth (Table III). Since Pdr1p forms heterodimers with Pdr3p and Stb5p, it is expected that the toxicity of 4-NQO is increased in strains carrying double deletions of these genes (i.e. Δ*pdr1*Δ*pdr3*, Δ*pdr1*Δ*stb5*, and Δ*pdr3*Δ*stb5*), which is consistent with our results (2, 26). *ECM22*, *RDS2*, and *UPC2* may show some functional overlap with known regulators of 4-NQO resistance as the double deletion strains Δ*ecm22*Δ*pdr1*, Δ*ecm22*Δ*stb5*, Δ*pdr1*Δ*rds2*, Δ*pdr1*Δ*upc2*, and Δ*stb5*Δ*upc2* showed sensitivity to 4-NQO.

As stated above, ketoconazole inhibits the P450 cytochrome-dependent lanosterol demethylase encoded by *ERG11*. Upc2p and Ecm22p are paralogous proteins that target the *ERG2* and *ERG3* genes by acting through sterol response elements (SREs) in their promoters (39). We have shown previously that the deletion strains Δ*upc2* and Δ*rds2* are sensitive to ketoconazole (3). Due to high concentrations of ketoconazole used in this study, we were unable to confirm these phenotypes because all single deletion strains (with the exception of Δ*pdr1*) and the wild-type strain showed sensitivity to ketoconazole (Table IV). However, the double deletion strain Δ*rds2*Δ*yrr1* showed hyperresistance to ketoconazole (Fig. 2). *RDS2* encodes a zinc cluster protein which, upon deletion, shows hypersensitivity to ketoconazole, which is contrary to our results (3). This unique phenotype could implicate both *RDS2* and *YRR1* as regulators in the ergosterol biosynthetic pathway and deletion of Rds2p and Yrr1p may uncover other proteins that regulate this pathway.

Caffeine is a purine analog and displays toxicity on cells through inhibition of the MAP kinase pathway and phosphodiesterase of the cAMP pathway (14). Consistent with other findings, a Δ*stb5* deletion strain was shown to exhibit hypersensitivity to caffeine

(4). However, a *Δstb5* deletion in combination with either *Δhal9* or *Δupc2* showed resistance, whereas a *Δhal9Δupc2* double deletion strain showed sensitivity to caffeine (Fig. 3). Hal9p activates transcription of the *ENA1* gene which encodes a Na^+/Li^+ extrusion pump (28). These results show that Hal9p and Upc2p may functionally compensate for each other, and that deletion of these genes, in combination with *Δstb5*, may uncover other genes involved in a stress response to caffeine. Furthermore, the zinc cluster pairs Rds2p/Pdr1p and Pdr3p/Upc2p may also have redundant functions as double deletions show sensitivity, but single deletions do not.

As zinc cluster proteins are known to interact with other members of this family, it is worth exploring the relationships of the eight genes analyzed in this study with other zinc cluster genes not necessarily involved in PDR or even uncharacterized putative zinc cluster genes. For example, Yrr1p is known to mediate PDR, but only recently has Yrm1p, another zinc cluster protein with 41% homology to Yrr1p, been characterized as a potential regulator of drug resistance (22). In this case, Yrm1p overexpression led to a decreased level of expression of Yrr1p target genes. Furthermore, in the absence of Yrr1p, Yrm1p transcriptional activity upregulated 23 genes, 14 of which are also Yrr1p targets that include *SNQ2* and *YOR1* (22). This example of cross-protection could explain why many of the single and double deletion strains analyzed in this study showed no differences in growth compared to the wild-type strain.

In the case with Yrr1p and Yrm1p, the function of Yrm1p is masked in the presence of Yrr1p. We believe that the unusual phenotypes of *Δrds2Δyrr1* showing hyperresistance to ketoconazole and *Δhal9Δstb5* and *Δstb5Δupc2* showing resistance to caffeine may be due in part to unmasked proteins. Therefore, one experiment that could be used to reveal masked zinc cluster proteins is a genome-wide expression analysis coupled with chromatin immunoprecipitation. For this experiment, we could determine the binding of a particular zinc cluster protein to its target genes in the absence of another zinc cluster protein (i.e. in a single deletion strain background). We could then compare the binding targets of this particular protein in a wild-type background with those in a single deletion strain background and potentially identify new target genes for further investigation.

In this study, we identified several possible relationships among zinc cluster proteins that have implications in PDR. To further analyze these relationships, microarray analyses for single deletion versus double deletion strains could be performed. These analyses could then be used to identify common and different target genes between strains. Microarray analyses could also be coupled to chromatin immunoprecipitation experiments under wild-type and single deletion strain backgrounds to unmask functions of proteins that may otherwise be hidden in the presence of other proteins. Other future experiments could include the generation of more double deletion strains, particularly for genes that have neither assigned function nor phenotype, and the generation of triple deletion strains as redundancy of the yeast genome may go beyond paralogous pairs.

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3.7 Tables and Figures

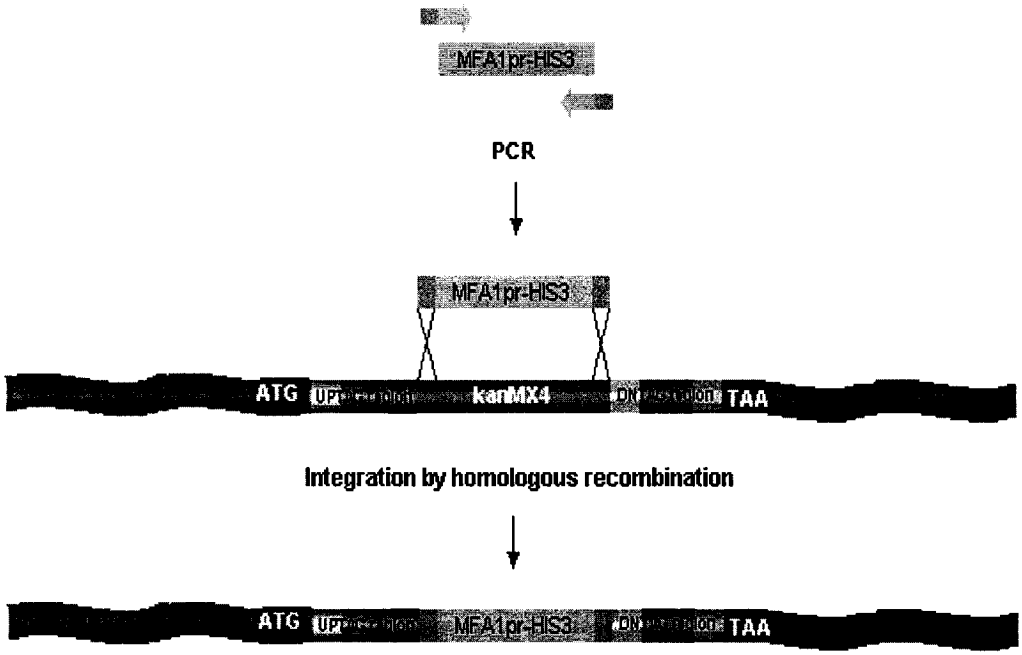
TABLE I. **Strains used in this study**

Strain Name	Genotype	Reference
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	8
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	8
Δecm22-a	BY4741 *ecm22Δ::kanMX4*	8
Δhal9-a	BY4741 *hal9Δ::kanMX4*	8
Δpdr1-a	BY4741 *pdr1Δ::kanMX4*	8
Δpdr3-a	BY4741 *pdr3Δ::kanMX4*	8
Δrds2-a	BY4741 *rds2Δ::kanMX4*	8
Δstb5-a	BY4741 *stb5Δ::kanMX4*	8
Δupc2-a	BY4741 *upc2Δ::kanMX4*	8
Δyrr1-a	BY4741 *yrr1Δ::kanMX4*	8
Δecm22	BY4741 *ecm22Δ::MFA1pr-HIS3*	This study
Δhal9	BY4741 *hal9Δ::MFA1pr-HIS3*	This study
Δpdr1	BY4741 *pdr1Δ::MFA1pr-HIS3*	This study
Δpdr3	BY4741 *pdr3Δ::MFA1pr-HIS3*	This study
Δrds2	BY4741 *rds2Δ::MFA1pr-HIS3*	This study
Δstb5	BY4741 *stb5Δ::MFA1pr-HIS3*	This study
Δupc2	BY4741 *upc2Δ::MFA1pr-HIS3*	This study
Δyrr1	BY4741 *yrr1Δ::MFA1pr-HIS3*	This study
Δecm22-α	BY4742 *ecm22Δ::kanMX4*	8
Δhal9-α	BY4742 *hal9Δ::kanMX4*	8
Δpdr1-α	BY4742 *pdr1Δ::kanMX4*	8
Δpdr3-α	BY4742 *pdr3Δ::kanMX4*	8
Δrds2-α	BY4742 *rds2Δ::kanMX4*	8
Δstb5-α	BY4742 *stb5Δ::kanMX4*	8
Δupc2-α	BY4742 *upc2Δ::kanMX4*	8
Δyrr1-α	BY4742 *yrr1Δ::kanMX4*	8
Δecm22Δhal9	BY4741 *hal9Δ::MFA1pr-HIS3 ecm22Δ::kanMX4*	This study
Δecm22Δpdr1	BY4741 *ecm22Δ::MFA1pr-HIS3 pdr1Δ::kanMX4*	This study
Δecm22Δpdr3	BY4741 *pdr3Δ::MFA1pr-HIS3 ecm22Δ::kanMX4*	This study
Δecm22Δrds2	BY4741 *ecm22Δ::MFA1pr-HIS3 rds2Δ::kanMX4*	This study
Δecm22Δstb5	BY4741 *stb5Δ::MFA1pr-HIS3 ecm22Δ::kanMX4*	This study
Δecm22Δupc2	BY4741 *ecm22Δ::MFA1pr-HIS3 upc2Δ::kanMX4*	This study
Δecm22Δyrr1	BY4741 *ecm22Δ::MFA1pr-HIS3 yrr1Δ::kanMX4*	This study
Δhal9Δpdr1	BY4741 *hal9Δ::MFA1pr-HIS3 pdr1Δ::kanMX4*	This study

Δhal9Δpdr3	BY4741 *hal9Δ::MFA1pr-HIS3 pdr3Δ::kanMX4*	This study
Δhal9Δrds2	BY4741 *rds2Δ::MFA1pr-HIS3 hal9Δ::kanMX4*	This study
Δhal9Δstb5	BY4741 *hal9Δ::MFA1pr-HIS3 stb5Δ::kanMX4*	This study
Δhal9Δupc2	BY4741 *upc2Δ::MFA1pr-HIS3 hal9Δ::kanMX4*	This study
Δhal9Δyrr1	BY4741 *hal9Δ::MFA1pr-HIS3 yrr1Δ::kanMX4*	This study
Δpdr1Δpdr3	BY4741 *pdr3Δ::MFA1pr-HIS3 pdr1Δ::kanMX4*	This study
Δpdr1Δrds2	BY4741 *rds2Δ::MFA1pr-HIS3 pdr1Δ::kanMX4*	This study
Δpdr1Δstb5	BY4741 *stb5Δ::MFA1pr-HIS3 pdr1Δ::kanMX4*	This study
Δpdr1Δupc2	BY4741 *pdr1Δ::MFA1pr-HIS3 upc2Δ::kanMX4*	This study
Δpdr1Δyrr1	BY4741 *yrr1Δ::MFA1pr-HIS3 pdr1Δ::kanMX4*	This study
Δpdr3Δrds2	BY4741 *rds2Δ::MFA1pr-HIS3 pdr3Δ::kanMX4*	This study
Δpdr3Δstb5	BY4741 *pdr3Δ::MFA1pr-HIS3 stb5Δ::kanMX4*	This study
Δpdr3Δupc2	BY4741 *upc2Δ::MFA1pr-HIS3 pdr3Δ::kanMX4*	This study
Δpdr3Δyrr1	BY4741 *yrr1Δ::MFA1pr-HIS3 pdr3Δ::kanMX4*	This study
Δrds2Δstb5	BY4741 *stb5Δ::MFA1pr-HIS3 rds2Δ::kanMX4*	This study
Δrds2Δupc2	BY4741 *rds2Δ::MFA1pr-HIS3 upc2Δ::kanMX4*	This study
Δrds2Δyrr1	BY4741 *yrr1Δ::MFA1pr-HIS3 rds2Δ::kanMX4*	This study
Δstb5Δupc2	BY4741 *upc2Δ::MFA1pr-HIS3 stb5Δ::kanMX4*	This study
Δstb5Δyrr1	BY4741 *yrr1Δ::MFA1pr-HIS3 stb5Δ::kanMX4*	This study
Δupc2Δyrr1	BY4741 *yrr1Δ::MFA1pr-HIS3 upc2Δ::kanMX4*	This study

FIGURE 1. **Construction of double deletion mutants.** *A*, A *MFA1pr-HIS3* cassette was created by PCR with primers containing flanking regions homologous to the *kanMX4* module and the pBlue-*MFA1pr-HIS3* plasmid as a template (see "Materials and Methods"). The *MFA1pr-HIS3* cassette was integrated into the BY4741-derived single deletion strains *Δecm22-a*, *Δhal9-a*, *Δpdr1-a*, *Δpdr3-a*, *Δrds2-a*, *Δstb5-a*, *Δupc2-a*, and *Δyrr1-a* by homologous recombination with *kanMX4*. This figure is modified from http://www-sequence.stanford.edu/group/yeast_deletion_project/PCR_strategy.html. *B*, Single deletion strains containing the *MFA1pr-HIS3* cassette with gene deletion "xxx" were crossed to deletion strains carrying gene deletion "yyy". The resulting diploids were then sporulated and haploid double deletion strains were selected as described in "Materials and Methods". This figure is modified from Tong *et al.* (37).

A


MFA1pr-HIS3
PCR
MFA1pr-HIS3
ATG
kanMX4
TAA
Integration by homologous recombination
ATG
MFA1pr-HIS3
TAA

B

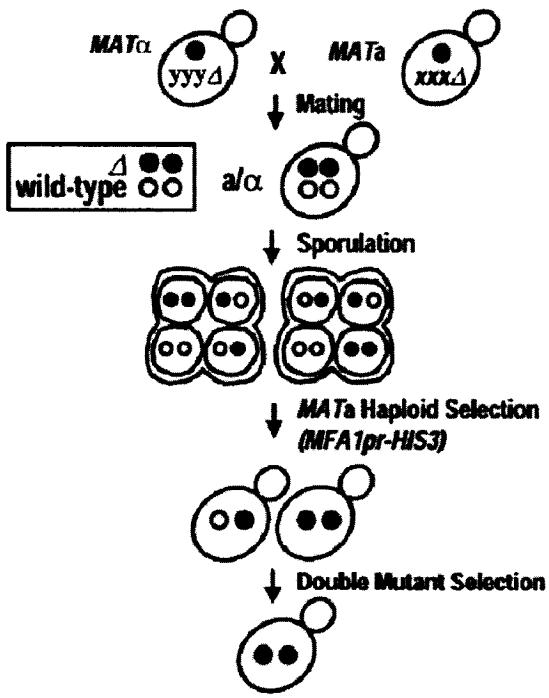

MATα
yyyΔ
X
MATa
xxxΔ
Mating
wild-type
a/α
Sporulation
MATa Haploid Selection
(MFA1pr-HIS3)
Double Mutant Selection

TABLE II. **Phenotypes of deletion strains in the presence of cycloheximide.** Single and double deletion strains were serially diluted, spotted on YPD plates containing 0.2 μg/ml cycloheximide, and grown for 4 days at 30°C. Growth is as indicated: +++ for normal growth; ++ for slightly inhibited growth; + for moderate growth; and – for no growth (or severely impaired growth).

	Single KO	Double KO							
		Δyrr1	*Δupc2*	*Δstb5*	*Δrds2*	*Δpdr3*	*Δpdr1*	*Δhal9*	*Δecm22*
Δecm22	+++	+++	++	-	+++	-	+	-	
Δhal9	+	-	+	+	+	-	+		-
Δpdr1	+	++	+++	-	-	-		+	+
Δpdr3	-	-	-	-	-		-	-	-
Δrds2	-	-	+	-		-	-	+	+++
Δstb5	-	-	+		-	-	-	+	-
Δupc2	+++	+		+	+	-	+++	+	++
Δyrr1	++		+	-	-	-	++	-	+++
WT	+++								

TABLE III. **Phenotypes of deletion strains in the presence of 4-NQO.** Single and double deletion strains were serially diluted, spotted on YPD plates containing 0.35 μg/ml 4-NQO, and grown for 2 days at 30°C. Growth is as indicated: +++ for normal growth; ++ for slightly inhibited growth; + for moderate growth; and – for no growth (or severely impaired growth).

	Single KO	Double KO							
		Δyrr1	*Δupc2*	*Δstb5*	*Δrds2*	*Δpdr3*	*Δpdr1*	*Δhal9*	*Δecm22*
Δecm22	+++	+	++	+	++	+++	+	++	
Δhal9	+++	-	+++	++	+++	++	++		++
Δpdr1	++	-	+	-	+	+		++	+
Δpdr3	+++	-	++	+	+++		+	++	+++
Δrds2	++	-	++	++		+++	+	+++	++
Δstb5	++	+	+		++	+	-	++	+
Δupc2	+++	+		+	++	++	+	+++	++
Δyrr1	+		+	+	-	-	-	-	+
WT	+++								

TABLE IV. **Phenotypes of deletion strains in the presence of ketoconazole.** Single and double deletion strains were serially diluted, spotted on YPD plates containing 4 μg/ml ketoconazole, and grown for 4 days at 30°C. Growth is as indicated: +++ for normal growth; ++ for slightly inhibited growth; + for moderate growth; and – for no growth (or severely impaired growth).

	Single KO	Double KO							
		Δyrr1	*Δupc2*	*Δstb5*	*Δrds2*	*Δpdr3*	*Δpdr1*	*Δhal9*	*Δecm22*
Δecm22	-	+	-	-	-	++	-	-	
Δhal9	+	-	-	-	-	-	-		-
Δpdr1	++	++	++	++	+	++		-	-
Δpdr3	-	+	-	-	-		++	-	++
Δrds2	-	+++	-	-		-	+	-	-
Δstb5	-	-	-		-	-	++	-	-
Δupc2	-	-		-	-	-	++	-	-
Δyrr1	-		-	-	+++	+	++	-	+
WT	-								

FIGURE 2. **Growth of selected strains on YPD containing 4 μg/ml ketoconazole.** Wild-type and deletion strains were grown overnight in YPD, washed, serially diluted, and spotted on YPD plates with or without ketoconazole as indicated.

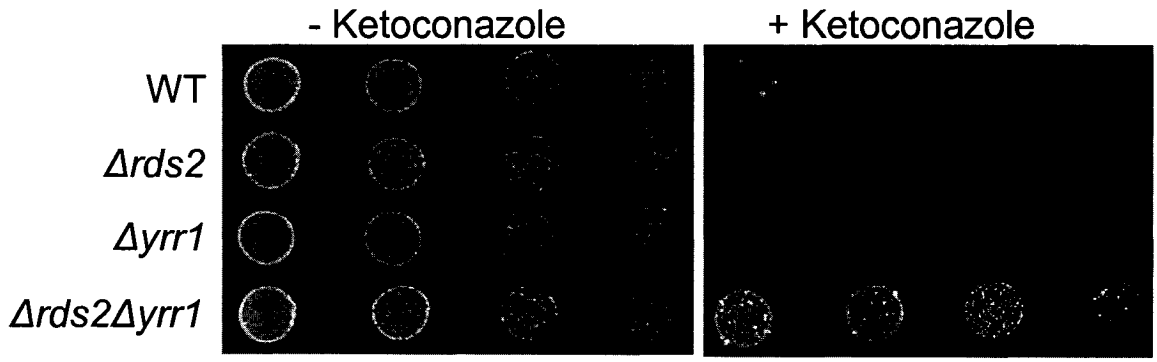
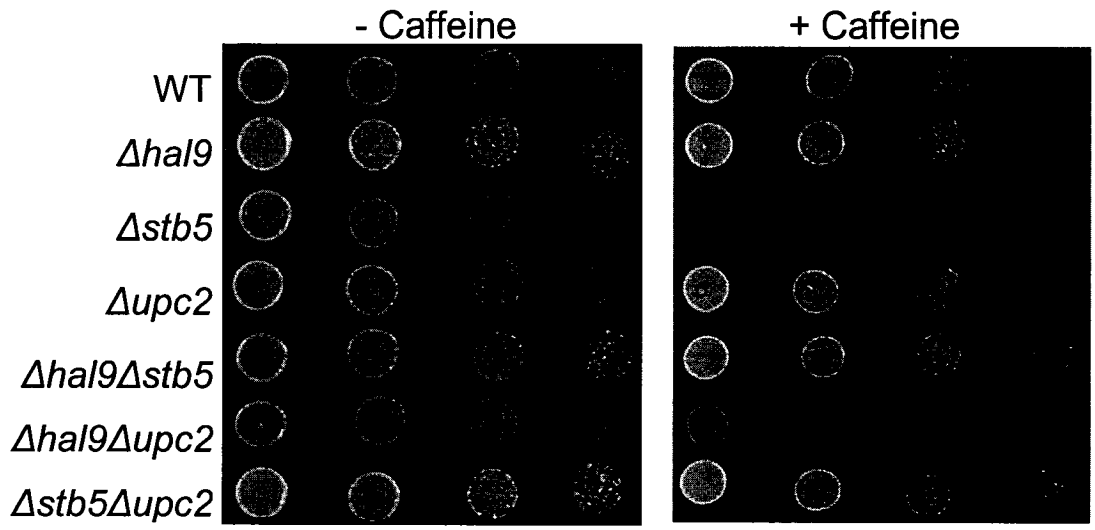


TABLE V. **Phenotypes of deletion strains in the presence of caffeine.** Single and double deletion strains were serially diluted, spotted on YPD plates containing 0.15% caffeine, and grown for 2 days at 30°C. Growth is as indicated: +++ for normal growth; ++ for slightly inhibited growth; + for moderate growth; and – for no growth (or severely impaired growth).

	Single KO	Double KO							
		Δyrr1	*Δupc2*	*Δstb5*	*Δrds2*	*Δpdr3*	*Δpdr1*	*Δhal9*	*Δecm22*
Δecm22	+++	+++	+++	-	+++	++	+++	++	
Δhal9	+++	++	+	+++	++	+++	++		++
Δpdr1	+++	+++	+++	-	-	+++		++	+++
Δpdr3	+++	++	+	+	+++		+++	+++	++
Δrds2	+++	++	+++	-		+++	-	++	+++
Δstb5	-	-	+++		-	+	-	+++	-
Δupc2	+++	+++		+++	+++	+	+++	+	+++
Δyrr1	+++		+++	-	++	++	+++	++	+++
WT	+++								

FIGURE 3. **Growth of selected strains on YPD containing 0.15% caffeine.** Wild-type and deletion strains were grown overnight in YPD, washed, serially diluted, and spotted on YPD plates with or without caffeine as indicated.



Connecting Text

It is well known that zinc cluster proteins play a role in mediating PDR. In the previous section, we identified functional relationships among well-characterized and newly identified members of PDR. However, zinc cluster proteins have roles in other cellular processes. In the next section, we identify a previously uncharacterized zinc cluster protein, Asg1p, as a potential regulator involved in a novel stress response pathway.

Section 4:

The zinc cluster protein Asg1p is a putative regulator of stress response genes in *Saccharomyces cerevisiae*

4.1 Abstract

All living organisms have evolved mechanisms to detect and respond to adverse environmental conditions. In *Saccharomyces cerevisiae*, the general stress response regulators, Msn2p and Msn4p, as well as the heat-shock response regulator, Hsf1p, respond to such stresses as heat, ethanol, and weak acid exposure. In this study, we have characterized a member of the zinc cluster protein family of transcriptional regulators, Asg1p (Activator of Stress Genes, systematic name: *YIL130W*), which activates a set of stress genes independent of Msn2p, Msn4p, and Hsf1p regulation. Microarray and Northern blot analyses revealed and confirmed six target genes (*HSP30, STP4, TPO2, YER130C, YLR297W, YRO2*) with significantly lower mRNA levels in an Δ*asg1* deletion strain compared to the wild-type strain. Three of the six identified genes are involved in known stress responses. Using a *YER130C-lacZ* reporter, we found decreased β-galactosidase activity in the Δ*asg1* deletion strain compared to the wild-type strain. Furthermore, deletion analysis of upstream noncoding sequences revealed a *cis*-acting response element in the *YER130C* promoter. Using electrophoretic mobility shift assays, we found that the purified DNA-binding domain (DBD) of Asg1p likely binds *in vitro* to the *cis*-acting response element CGG-N_8-CGG in the *YER130C* promoter. We further demonstrated that Asg1p-DBD directly binds to this element in the promoter of *STP4*, another target gene identified in this study. Our results show that Asg1p directly regulates *YER130C* and *STP4*. We postulate that *STP4* and *YER130C* gene products may regulate the other stress genes identified in the microarray analysis. Taken together, these data suggest that we have identified a novel stress response pathway in yeast that may be independent of Msn2p/4p and Hsf1p regulation.

4.2 Introduction

All organisms ranging from bacteria to humans have developed several types of responses to survive under threatening conditions. These conditions include high temperature, oxidative stress, osmotic stress, and nutrient depletion, to name a few. The cellular response to these and other stresses consists of two lines of defense. The first line of defense acts rapidly and involves the modulation of pre-existing proteins to produce changes (47). This early response typically recruits low molecular weight components and small proteins that function as chaperones and in repair systems necessary for immediate survival (38). As part of the immediate response, stress signals may activate signal transduction pathways, which then trigger the second line of defense. The delayed secondary response induces transcription of genes encoding factors with protective functions (38, 44). The combination of early and late responses enables cells to adapt and resume growth under both mild and severe conditions.

In *Saccharomyces cerevisiae*, general responses to stresses, including mild heat shock, starvation, osmotic stress, oxidative stress, alcohol, and weak acids, depend on the functionally redundant C_2H_2 zinc finger transcription factors Msn2p and Msn4p (14, 35, 38). Upon exposure to stresses, Msn2p and Msn4p accumulate in the nucleus (18) and activate stress-responsive genes via stress response elements (STRE; CCCCT) in their promoters (14, 28, 38, 46). Studies show that protein kinase A of the Ras/cAMP pathway negatively regulates this general stress response through phosphorylation and cytoplasmic retention of Msn2p and Msn4p (18, 35, 50).

In addition to the general stress response regulators Msn2p and Msn4p, there exist regulators for specific stresses. The essential heat shock transcription factor, Hsf1p, responds to rapid increases in temperature by activating transcription of genes encoding heat shock proteins (Hsps) (32, 51, 59). Hsf1p recognizes three different classes of heat shock elements (HSEs) in the promoters of heat shock genes (4, 45, 65). War1p and Pdr1p/Pdr3p, transcriptional regulators belonging to the zinc cluster protein family, activate genes in response to weak acids and drugs, respectively (6, 30). The weak acid

response is mediated through War1p-binding to the weak acid response element (WARE; CGG-N_{23}-CGG) in the *PDR12* promoter, which encodes an ATP-binding cassette (ABC) transporter that effluxes weak acids (30, 42). Pdr1p, and its homologue Pdr3p, positively control expression of the ABC transporter genes *PDR5*, *SNQ2*, and *YOR1*, whose gene products confer drug resistance to cycloheximide, 4-nitroquinoline *N*-oxide, and oligomycin, respectively (7, 11, 13, 25, 29, 33, 62). Pdr1p and Pdr3p activate transcription through *cis*-acting elements called pleiotropic drug resistance elements (PDRE; CCGCGG) present in target gene promoters (12, 13, 22, 26, 33, 61). However, there exist several stress genes (e.g. *HSP30*) that have unknown mechanisms of regulation.

In addition to War1p and Pdr1p/Pdr3p, zinc cluster proteins regulate a wide variety of cellular processes. For example, Gal4p activates genes involved in galactose metabolism (21). The *S. cerevisiae* sequencing project identified over fifty proteins belonging to this family (55), yet the function of many of these putative proteins remains unknown. In our attempt to characterize these unknown proteins, we performed whole-genome analysis of gene expression with DNA microarrays on several zinc cluster gene deletion strains. Here we suggest that the gene product of *YIL130W*, a previously uncharacterized zinc cluster protein, is an activator of several stress response genes.

4.3 Materials and Methods

Strains and Media – The wild-type strain used was BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) (9). The deletion strain BY4742 *Δyil130w::kanMX4* was obtained from Research Genetics (Huntsville, AL) (60). Media were prepared according to Adams *et al.* (1). YPD medium contained 1% yeast extract, 2% peptone, and 2% glucose. SD medium 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%. 2×YT medium contained 1.6% peptone, 1% yeast extract, and 0.5% NaCl.

Microarray Analysis – Yeast cells were grown in YPD medium to an A_{600} of 0.8 to 1.0 and total RNA was isolated by the hot phenol procedure (23). Briefly, cells were incubated with TES (10 mM Tris-Cl, pH 7.5, 10 mM EDTA, 0.5% SDS) solution and acid phenol at 65°C for 1 h. After centrifugation, the aqueous phase was extracted with acid phenol-chloroform (5:1) and chloroform-isoamyl alcohol (25:1). RNA was precipitated with 3 M sodium acetate and ethanol and resuspended in diethylpyrocarbonate (DEPC)-treated water. RNA was further purified with Qiagen columns according to the manufacturer's protocol except that RNA was eluted twice for 15 min per elution. Microarray analysis was performed by the Microarray Facility at the Genome Quebec Innovation Centre (Montreal, Canada) and gene expression changes were assessed using Affymetrix GeneChip S98 oligonucleotide microarrays (Affymetrix, Santa Clara, CA). The results presented in Table I are an average of two independent experiments performed with independent RNA preparations.

Northern Blot Analysis – The same RNA isolated for the microarray analysis was used for Northern blot analysis (5). Approximately 30 μg of total RNA were loaded per lane on a 1% agarose denaturing gel containing formaldehyde. The gel was washed with water for 1 h and with 20× SSC (3 M NaCl, 0.3 M Na_3citrate•$2H_2O$, pH 7) for 45 min before the RNA was transferred to a nitrocellulose membrane (Hybond-N) by upward capillary action overnight at room temperature. The membrane was baked at 80°C for 2 h and incubated in pre-hybridization solution (50% formamide, 5.6× SSC, 5× Denhardt's

solution, 0.5% SDS) at 42°C prior to hybridization. Hybridization was performed at 42°C in 50% formamide, 1 M NaCl, 2.8× Denhardt's solution, 0.5% SDS, and 10% dextran sulphate. After overnight incubation, the membrane was washed with wash buffer 1 (2× SSC, 0.1% SDS) at 37°C for 15 min and then wash buffer 2 (0.1× SSC, 0.5% SDS) at 65°C for 45 min. Results were obtained using the Phosphorimager system (Molecular Dynamics).

Probes for Northern Blot Analysis – The following oligonucleotides were used to obtain probes by PCR with a BY4742 genomic DNA template:
HSP30 – CATGGCCTGGATATGCACAT and AAAGATAGCCTCACCGTCTG;
STP4 – GAGACGATGTCTTTACGGAG and GACGATGAATACCTGCTTGT;
TPO2 – TTGAGGGCCAACAACCTCAA and CGCAAGGGATATTGAAGATG;
YER130C – GACGATGAGCTTGAAGACTC and AGATCTGACATGCCTCTTCA;
YLR297W – GGTCGAAGGTGATTTTGTCG and CATGCTTTTTCAGCAAGTGC; and
YRO2 – AACCGGTGCTGATTTCCACA and CTGGCAAGATAGATAGGATC. PCR products were labeled using a Hexa-labeling kit according to the manufacturer's instructions (MBI Fermentas). The 2.8 kb *ACT1* fragment was excised from plasmid pBR322-ACTIN (provided by Beatrice Magee, University of Minnesota) by digestion with *Eco*RI and *Hind*III.

LacZ Reporters – To construct reporters, the following oligonucleotides were used to amplify 700 bp of the promoter region upstream of the ATG by PCR:
HSP30-LacZ – HSP30-A (ATCGACTCGAGTGCATAAGTAAGAATAACTA) and HSP30-B (GAAGATCTGGTCATTTGAAATTTGTTGTTTT);
STP4-LacZ – STP4-A (ATCGACTCGAGCGGGCCGACACTTACACGAG) and STP4-B (CGGGATCCGGTCATAGGGGAAGCCAAAGAAA);
TPO2-LacZ – TPO2-A (ATCGATCGTCGACCCTTAAAAATGCGAGAGCGC) and TPO2-B (CGGGATCCGGTCATTTTGATGCTTTTTTTAA);
YER130C-LacZ – YER130C-A (ATCGACTCGAGCGATGAAACAGATAACTGCC) and YER130C-B (CGGGATCCGGTCATGCTTGTCTGTCTATGTT);

YLR297W-LacZ – YLR297W-A (ATCGATCGTCGACTGTCGCTTTCTTTTACTCAA) and YLR297W-B (CGGGATCCGGTCATTTTCTGTTTTTATATTT); and *YRO2-LacZ* – YRO2-A (ATCGACTCGAGTCTCCGGTCCAATGCATGAA) and YRO2-B (CGGGATCCGGTCATTTTGATGCTTTTTTTAA). A BY4742 genomic template was used. For the deletion analysis with truncated *YER130C-LacZ* reporters, the following forward oligonucleotides were used to amplify promoter regions by PCR with the original *YER130C-LacZ* reporter as a template (with YER130C-B as a reverse oligonucleotide for all reporters; regions of promoter amplified relative to the ATG start site are shown in brackets):
YER130CΔ1 (-548 to +1 bp) – ATCGACTCGAGTTTTTGGCGCCTGTCATACC;
YER130CΔ2 (-382 to +1 bp) – ATCGACTCGAGGCTCCATGCGGATATACTTG;
YER130CΔ3 (-328 to +1 bp) – ATCGACTCGAGATGGCCCAATTGGGTGCTGT;
YER130CΔ4 (-279 to +1 bp) – ATCGACTCGAGCGCTTCGGAAATGGAGCAAA;
YER130CΔ5 (-210 to +1 bp) – ATCGACTCGAGTTTTGCCCAAGTTGTTGCCC; and
YER130CΔ6 (-178 to +1 bp) – ATCGACTCGAGTAGCGTGGCCATCCAAGATA. All PCR products were cut with *Xho*I and *Bam*HI and subcloned into the plasmid pSLFΔ178K, a high copy plasmid with a *URA3* selection marker (16), cut with the same enzymes to remove the minimal *CYC1* promoter. All reporters were sequenced to guard against PCR errors.

β-Galactosidase Assays – BY4742 and *Δasg1* strains were transformed with reporters and grown on selective medium lacking uracil. Transformed colonies were grown overnight in YPD and diluted in SD medium supplemented with the appropriate amino acids and adenine. β-galactosidase assays were performed with permeablized cells (19) and values obtained were the average of at least 2 independent experiments performed in duplicate.

Electrophoretic Mobility Shift Assay (EMSA) – (i) ***Expression vector***. A DNA fragment encoding the N-terminal 140 amino acids of Asg1p (corresponding to the putative zinc finger of the protein) was synthesized by PCR with the oligonucleotides CGGGATCCATGCCAGAACAAGCGCAACA and

GGAATTCCGACCTCATCAAATATTTGCT, using BY4742 genomic DNA as a template. The PCR product was digested with *Bam*HI and *Eco*RI and subcloned into plasmid pGEX-F (22) cut with the same enzymes to give pGST-ASG1. pGST-ASG1 was sequenced and found to be error-free.

(ii) ***Protein purification***. pGST-ASG1 was transformed into *Escherichia coli* BL21. A 20 ml portion of overnight culture was diluted in 200 ml of 2×YT containing 100 μg/ml ampicillin and grown for 1 h at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) and $ZnSO_4$ were then added at final concentrations of 0.5 mM and 100 μM, respectively. The cells were grown for 2 h at room temperature and used for protein purification. GST fusion proteins were purified essentially as described previously (5). The purified GST fusion proteins bound to beads were treated with thrombin (150 μg/ml) for 1 h at room temperature and then overnight at 4°C. The beads were then centrifuged and the supernatant was mixed with 1 volume of 2× storage buffer (20% glycerol, 4 mM dithiothreitol (DTT), 10 μM $ZnSO_4$, 2 μg/ml bovine serum albumin (BSA)) and stored at -80°C.

(iii) ***Probes***. The DNA sequences of the *YER130C* probes used in EMSA promoter dissection are the following (positions of probes relative to the ATG start site are shown in brackets; reverse complements are not shown):

YER130C-1 (-355 to -315 bp) –
TCGATGCATAGCCGCCCGGCGTTAATCACACATGGCCCAATTGG;

YER130C-2 (-330 to -290 bp) –
TCGAACATGGCCCAATTGGGTGCTGTTTTTTTACATTTACGGTT;

YER130C-3 (-305 to -265 bp) –
TCGATTTACATTTACGGTTACGAGTGCGACGCTTCGGAAATGGA;

YER130C-4 (-280 to -240 bp) –
TCGACGCTTCGGAAATGGAGCAAAAAATTTCAGCGGATAACCCA;

YER130C-5 (-255 to -215 bp) –
TCGATCAGCGGATAACCCACGGAAAGGTGGCTTTTACTGTTCCG;

YER130C-6 (-230 to -190 bp) –
TCGAGCTTTTACTGTTCCGAGGGCTTTTGCCCAAGTTGTTGCCC;

YER130C-7 (-205 to -165 bp) –
TCGACCCAAGTTGTTGCCCGGCGCGGTTCCTAGCGTGGCCATCC; and
YER130C-8 (-180 to -140 bp) –
TCGACTAGCGTGGCCATCCAAGATATATATAGATAGCTGAATGG. The DNA sequences of the *STP4* probes used are the following (reverse complements are not shown, CGG triplets are in bold, and mutations are in bold and underlined):
STP4-wt – TCGATTTTTTTC**CGG**GAACGCCG**CGG**CTTTGCAA;
STP4-mut1 – TCGATTTTTTTC**CAG**GAACGCCG**CGG**CTTTGCAA;
STP4-mut2 – TCGATTTTTTTC**CGG**GAACGCCG**CAG**CTTTGCAA; and
STP4-mut1+2 – TCGATTTTTTTC**CAG**GAACGCCG**CAG**CTTTGCAA.
(iv) ***EMSA***. Double-stranded oligonucleotides were end-labeled with T4 polynucleotide kinase (New England Biolabs). Binding buffer contained 4% glycerol, 4 mM Tris-HCl pH 8.0, 40 mM NaCl, 4 mM $MgCl_2$, 10 μM $ZnSO_4$, 0.5 μg of sheared salmon sperm DNA, approximately 60 ng of each radiolabeled probe, and 0.2% bromophenol blue. Each reaction contained 2-20 μl protein extract and binding was performed for 20 min at room temperature. The samples were then loaded on a 4% polyacrylamide gel in 0.5× Tris-borate-EDTA (TBE) that was prerun at 120V for 2 h at room temperature. The gel was run for approximately 2 h, dried by vacuum for 45 min, and exposed to film in an autoradiography cassette (Fisher Scientific).

4.4. Results

Whole-genome analysis of YIL130W. Prior to this study, the only data available for the *YIL130W* gene were that it contains a putative C6 zinc cluster motif (55) and that a Δ*yil130w* deletion strain shows impaired growth on non-fermentable carbon sources and sensitivity to calcofluor white (2). Since many known zinc cluster proteins are transcriptional regulators, we attempted to characterize the gene product of *YIL130W.* To identify genes regulated by the gene product of *YIL130W*, RNA was isolated from the wild-type strain BY4742 and a strain carrying a deletion of *YIL130W.* We performed a whole-genome analysis of gene expression using DNA microarrays. Data were obtained on approximately 6000 genes and are an average from two independent experiments. These data revealed six genes with more than 2.5-fold decreased expression in the deletion strain compared to the wild-type (Table I). Of these six genes, three of them (*HSP30*, *YRO2*, and *TPO2*) encode proteins involved in stress response. Hsp30p is a plasma membrane protein induced by heat shock stress (40), Yro2p is a putative protein homologue of Hsp30p, and Tpo2p is a polyamine transport protein (56). Two of the six genes (*YER130C* and *STP4*) encode putative zinc finger proteins similar to the general stress response regulators Msn2p and Msn4p. The remaining gene (*YLR297W*) is a hypothetical ORF. Our results suggest that the *YIL130W* gene product is a transcriptional activator for genes involved in a stress response. We have therefore renamed the *YIL130W* gene as *ASG1* for Activator of Stress Genes.

Confirmation of Microarray results by Northern blot analyses. To confirm the microarray results, we performed Northern blot analyses with the same RNA isolated from wild-type and Δ*asg1* strains as in the microarray analysis (Fig. 1). *HSP30*, *YRO2*, and *TPO2* mRNA levels decreased in the deletion strain compared to wild-type with values equivalent to those observed in the microarray. Similarly, *YER130C* and *STP4* mRNA levels decreased in the Δ*asg1* strain and these results are in close agreement to those obtained in the microarray analysis. For *YLR297W*, we were unable to obtain results by Northern blot analysis. Equal loading and transfer of RNA isolated for wild-type and deletion strains were shown by similar signals obtained with an actin probe (Fig. 1).

Activity of lacZ reporters. To determine if changes in mRNA levels were due to altered promoter activity, we constructed *lacZ* reporters. These reporters contained 700 bp of the promoters belonging to the six genes identified in the microarray analysis. The reporters were transformed into wild-type and Δ*asg1* strains and assayed for β-galactosidase activity (Fig. 2). Of the six reporters tested, only one reporter (*YER130C-lacZ)* gave consistently lower activity (approximately 2.5-times lower) in the deletion strain compared to the wild-type strain. None of the other reporters showed significant differences in activity between the wild-type and deletion strains, likely owing to these reporters lacking sufficient promoter regions (as discussed later).

5' Deletion mapping of the YER130C promoter. We wanted to ascertain how the transcription of the *YER130C* gene is regulated. Firstly, to map regulatory elements in the *YER130C* promoter, we performed a deletion analysis. A set of reporters containing progressively longer 5' truncations of the *YER130C* promoter were constructed and transformed into the wild-type and Δ*asg1* strains (Fig. 3). Removal of *YER130C* DNA between -700 bp and -328 bp did not have significant effects on activity in the wild-type or deletion strains. However, removal of DNA between -328 bp and -279 bp resulted in a 2-fold reduction of *YER130C* activity in both the wild-type and Δ*asg1* strains. Further removal of DNA between -279 bp and -210 bp resulted in a 7-fold and 5-fold decrease in activity in the wild-type and Δ*asg1* strains, respectively. Removal of DNA between -210 bp and -178 bp did not result in any further decrease in activity. Therefore, the observed reduction in activity upon removal of the regions -328 bp to -279 bp and -279 bp to -210 bp suggests that the segment of promoter between -328 bp and -210 bp contains at least one regulatory element.

Asg1p binds to a regulatory element in the YER130C promoter. To pinpoint a more specific regulatory element in the region between -328 bp and -210 bp (as identified in the 5' deletion analysis) and to determine if Asg1p binds to this element, we performed electrophoretic mobility shift assays (EMSAs). The DNA-binding domain (DBD) of Asg1p (amino acids 1 to 140) was expressed in bacteria, purified, and assayed in the presence of eight different probes that were each 40 bp in length and collectively spanned

the *YER130C* promoter region between -355 bp and -140 bp (Fig. 4*A*). Each consecutive probe overlapped the preceding probe by 15 bp and two different amounts of purified protein (2 μl and 10 μl) were tested. Asg1p bound strongly to the probe spanning the region between -255 bp and -215 bp and weakly to the probe spanning the region between -280 bp and -240 bp. Examining the sequences of these 2 probes revealed a region common to both probes that contained one CGG triplet (Fig. 4*B*). Recognition of CGG triplets is common to many zinc cluster proteins (55) and many zinc cluster proteins recognize CGG triplets as homodimers (36, 37). Therefore, it is not surprising to observe weak binding to a probe carrying one CGG triplet and strong binding to a probe carrying two CGG triplets (spanning from -255 to -215 bp), in which Asg1p recognizes two CGG triplets as a homodimer. Also, the relative orientation and spacing between CGG triplets has been shown to be important (34, 48). In this case, Asg1p appears to recognize direct CGG triplets spaced by 8 bp (CGG-N_8-CGG).

Asg1p binds to a regulatory element in the STP4 promoter. We examined the sequences of the other five genes identified in the microarray and found that the promoter region of *STP4* contained the element CGG-N_8-CGG (WT, Fig. 5*A*). To determine if Asg1p recognizes this element in the *STP4* promoter, we designed a probe containing this element (WT). We also designed probes with each or both CGG triplet(s) mutated to determine if the CGG triplets are important for binding (mut1, mut2, and mut1+2; Fig. 5*A*). EMSAs were performed using the same Asg1p purified protein (amino acids 1 to 140) as the previous EMSA (Fig. 5*B*). Asg1p bound strongly to the *STP4*-WT probe (containing the CGG-N_8-CGG element) and *STP4*-mut2 probe (containing the element CGG-N_8-CAG with the 2nd CGG mutated), weakly to the *STP4*-mut1 probe (containing the element CAG-N_8-CGG with the 1st CGG mutated), and not at all to the *STP4*-mut1+2 probe (containing both mutated CGGs). This indicates that Asg1p recognizes the element CGG-N_8-CGG and that the first CGG is more important for Asg1p binding than the second CGG triplet. As well, since almost no binding was seen for the *STP4*-mut1+2 probe but weak binding was observed for *STP4*-mut1, this indicates that both CGG triplets are important for binding. This provides evidence that CGG-N_8-CGG is a regulatory element in the *YER130C* and *STP4* promoters.

4.5 Discussion

In this study, we identified 6 target genes presumably under transcriptional regulation by the zinc cluster protein Asg1p. *HSP30* and its putative homologue, *YRO2,* were identified in the microarray analysis and confirmed by Northern blot analysis as having decreased mRNA levels in the *Δasg1* strain compared to the wild-type. *HSP30* encodes a heat shock-induced regulator of the plasma membrane H^+ATPase in which Hsp30p induction leads to downregulation of this pump (43). When induced by heat shock, yeast Hsp30p is the only protein that substantially copurifies with the plasma membrane (40). Furthermore, its regulation is independent of Msn2p/4p activation as the transcription of *HSP30* is unaffected by loss of Msn2p/4p or with mutations in STRE-like consensus sequences within its promoter (49). Recently, a study by Hahn *et. al* using genome-wide expression analyses in combination with chromatin immunoprecipitation experiments showed *HSP30*, amongst many other genes, to be bound by Hsf1p under heat shock induction (20). As mentioned earlier, Hsf1p, the essential heat shock transcription factor, recognizes three types of HSEs (the perfect, gap, and step types (4, 41, 45, 54, 63-65)) due to the flexible linker region located between the DBD and trimerization domain (15, 65). In the study by Hahn *et. al*, Hsf1p recognizes the step type HSE in *HSP30* (20). Although Hsf1p may bind to *HSP30*, other studies show that it does not activate *HSP30* transcription under heat shock induction (49). Therefore, Asg1p may be the transcriptional regulator of *HSP30*, but more studies need to be done to confirm this.

TPO2, another gene identified in our microarray analysis, is a member of the Major Facilitator Superfamily (MFS) of transporters (17). It encodes a polyamine transport protein that localizes to the cytosolic membrane along with its homologues Tpo1p, Tpo3p, and Tpo4p (3, 56). Polyamines are important for cell proliferation and differentiation, but are toxic at higher concentrations (53). Our results show that Asg1p may activate transcription of *TPO2*. No other studies have been conducted on the regulation of *TPO2*.

Another gene identified in our microarray analyses was *YLR297W*. Although it is classified as a hypothetical ORF, a recent study on mechanisms of toxicity using microarrays revealed an increase in *YLR297W* mRNA levels upon exposure to the genotoxic stress inducers methyl methanesulfonate (MMS), bleomycin, and cisplatin (10). MMS is an alkylating agent and genotoxicity to this compound is mainly attributed to 7-methylguanine adduct formation (8); bleomycin causes oxidative damage to nucleotides, resulting in single- and double-stranded DNA breaks; and cisplatin is a cross-linking agent (10). The gene product of *YLR297W* was also found to interact with Tpo3p in a two-hybrid assay, in which *TPO3* is a homologue of *TPO2* (24). Therefore, these studies implicate *YLR297W* as a player in stress response, particularly to DNA damage. As well, the *YLR297W* gene product may interact with polyamine transport proteins (encoded by *TPO1-TPO4*) to elicit a more general response to stress with Asg1p as a putative master regulator in this particular pathway.

Of the six genes identified, *STP4* and *YER130C* contain C_2H_2 zinc fingers and show sequence similarity to the general stress response regulators Msn2p and Msn4p. *STP4* is homologous to *STP1* and *STP2*, and studies show that Stp1p and Stp2p, which were originally identified as nuclear factors required for pre-tRNA maturation (57, 58), activate transcription of amino acid permease genes (39). By promoter deletion analyses, our study revealed a *cis*-regulatory element that appears in both the *STP4* and *YER130C* promoters. By EMSAs, we showed that the DBD of Asg1p directly recognizes two direct CGG triplets spaced by 8 bp (CGG-N_8-CGG) in the *STP4* promoter, and that Asg1p-DBD likely recognizes the same element in the *YER130C* promoter. These findings are consistent with other studies showing zinc cluster protein recognition of CGG triplets (55). However, this brings up the question of whether Asg1p directly or indirectly regulates the six identified target genes. Since the gene products of *STP4* and *YER130C* contain DNA-binding zinc finger domains similar to those of the general stress response regulators Msn2p and Msn4p, it is possible that they are regulating the other 4 genes, rather than Asg1p regulating all 6 genes directly. Also, we were unable to identify the CGG-N_8-CGG element in the promoters of *HSP30, YRO2,* and *YLR297W. TPO2* contained this CGG-N_8-CGG element, but when assayed by EMSAs, Asg1p-DBD did not

bind this regulatory element in *TPO2* (data not shown). However, we cannot conclude that Asg1p does not regulate *HSP30*, *YRO2*, *TPO2*, and *YLR297W* directly. Asg1p may recognize an element that is different from CGG-N_8-CGG, possibly using a mechanism similar to that of Hsf1p which recognizes 3 different kinds of regulatory elements (4, 15, 41, 45, 54, 63-65). Therefore, we propose that Asg1p exerts its action through either a direct or indirect pathway (Fig. 6). One possible way to deduce the mechanism of action is by comparing mRNA levels of *HSP30, TPO2, YRO2,* and *YLR297W* between wild-type, Δ*stp4*, and Δ*yer130c* strains. If the regulation is direct, there will be no difference in mRNA levels; if the regulation is indirect, mRNA levels will be lower in the Δ*stp4* and Δ*yer130c* strains compared to the wild-type strain. Another experiment is to compare mRNA levels of *HSP30, TPO2, YRO2,* and *YLR297W* between overexpression strains of *YER130C* and *STP4* with the wild-type strain. In this case, if the regulation is indirect, mRNA levels will be higher in the overexpression strains compared to the wild-type strain, whereas no differences will be seen in direct regulation.

Another question that must be addressed is whether or not we have found a novel stress response pathway. From our results, we can only conclude that expression of our target genes is Asg1p-dependent at a basal level (i.e. in the absence of stress). However, the target genes we identified have been known to act in stress situations. Therefore, we want to know whether stress-induced expression of these genes is Asg1p-dependent and, if so, the kinds of stress that trigger Asg1p to induce expression of these genes. We attempted to identify possible stress inducers using heat shock, ethanol, sodium chloride, acetaldehyde, and sorbic acid in Northern blot analyses by comparing *HSP30* mRNA levels of wild-type and Δ*asg1* strains in the presence and absence of stress. We also examined these same stress inducers in β-galactosidase assays with wild-type and Δ*asg1* strains carrying various reporters. However, we were unable to obtain any differences in *HSP30* mRNA levels or *lacZ* activity between the wild-type and Δ*asg1* strains (data not shown). These problems may be due to strain specificity as previous studies in stress induction typically use strains with a W303-1A background whereas our studies use a BY4742 background.

Also, β-galactosidase assays did not reveal any regulatory elements in the promoters of *HSP30, YRO2, TPO2,* or *YLR297W.* Since only 700 bp of promoter were tested for *lacZ* activity, it is likely that our reporters did not contain enough promoter regions. Furthermore, the *STP4-lacZ* reporter containing 700 bp also did not show *lacZ* activity in the β-galactosidase assays, which is likely due to the location of the regulatory element being more upstream (at approximately -960 bp) of the start site. It is known that most regulatory elements in yeast are located 50 to 500 bp upstream of the ATG (52), but some elements, such as the *cis*-acting site for *HO* expression, can be located up to 1.5 kb upstream of the start site (31). Therefore, new reporters containing more promoter region could be constructed and tested for differences in *lacZ* activity between wild-type and Δ*asg1* strains.

However, it is also likely that other regulators may be involved in this stress response pathway. A previous study identified *TPO2, YRO2, YLR297W,* and *YER130C* as having decreased mRNA levels in a Δ*haa1* strain compared to a wild-type strain when induced by acetaldehyde (27). Haa1p was identified as having homology to the copper-regulatory domain of Ace1p, but was unaffected by the copper status of cells (27). Therefore, it is possible that Haa1p may have functional redundancy with the gene products of *ASG1, YER130C,* or *STP4* (depending on the mechanism of action). Also, we cannot rule out that Msn2p/Msn4p may regulate some of these genes as both *YER130C* and *STP4* contain STREs in their promoters.

In summary, we have most probably identified a novel Msn2p/Msn4p/Hsf1p-independent stress response pathway in yeast. Our results show that the DBD of Asg1p directly regulates *STP4* through the *cis*-regulatory element CGG-N_8-CGG, and that Asg1p-DBD likely recognizes this same element in the *YER130C* promoter. We postulate that the *YER130C* and *STP4* gene products may regulate other stress genes. However, we do not rule out the possibility that cross-regulation by other gene products may be involved.

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4.7 Tables and Figures

TABLE I. **Microarray analysis: genes whose expression is altered in a *Δyil130w* strain compared to wild-type.** Genes whose mRNA levels are decreased more than 2.5-fold by deletion of the *YIL130W* gene are listed. "Expression" refers to the ratio of a given mRNA level in the *Δyil130w* strain as compared to the wild-type strain BY4742.

Systematic Name	Gene	Gene Product	Expression (*Δyil130w*/WT)
YCR021C	***HSP30 (YRO1)***	Hydrophobic plasma membrane protein induced by heat shock, negatively regulates H+-ATPase Pma1p	- 3.1
YDL048C	***STP4***	Putative protein homologue of Stp1p and Stp2p which regulate amino acid permease genes, contains C_2H_2 zinc finger motif	- 3.6
YGR138C	***TPO2***	Polyamine transport protein	- 4.6
YBR054W	***YRO2***	Putative plasma membrane protein, homologue of *HSP30*	- 3.7
YER130C	-	Msn2-/Msn4-like protein, contains C_2H_2 zinc finger motif	- 3.1
YLR297W	-	Hypothetical ORF	- 4.6

FIGURE 1. **Northern blot analysis of selected genes**. Wild-type (BY4742) and *Δasg1* (*Δyil130w*) strains were grown in rich medium, RNA was isolated by the hot phenol method, and approximately 30 μg of total RNA were loaded per lane for Northern blot analysis (see "Materials and Methods"). Strains are indicated *above* the autoradiograms, while probes are indicated *below* the autoradiograms. Actin (*ACT1*) was used as a loading control.

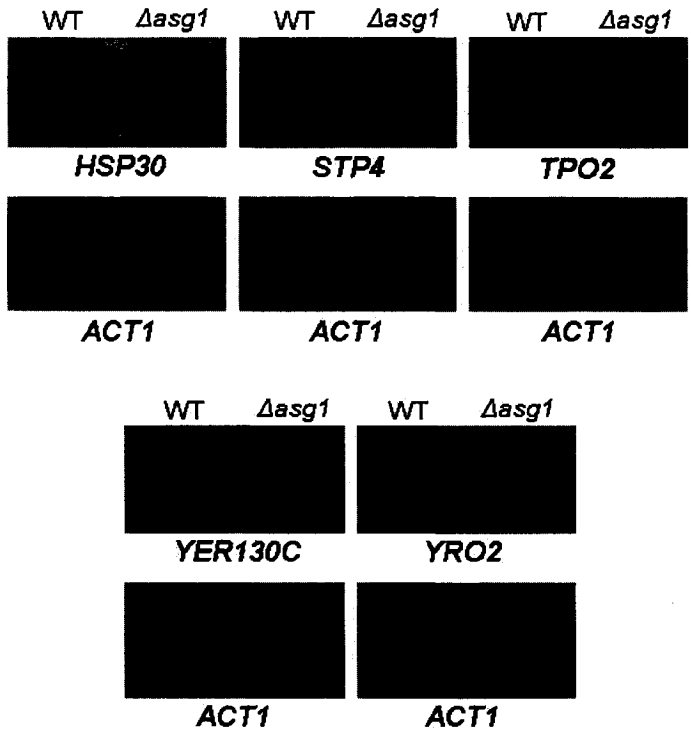


FIGURE 2. **Activity of a *YER130C-lacZ* reporter is decreased in an *Δasg1* strain.** *β*-galactosidase activity was measured in wild-type (WT; BY4742, *black bars*) and *Δasg1* strains (*hatched bars*) containing reporters as shown on the left-hand side of the figure. The activity values reported are averages of at least 2 independent experiments performed in duplicate.

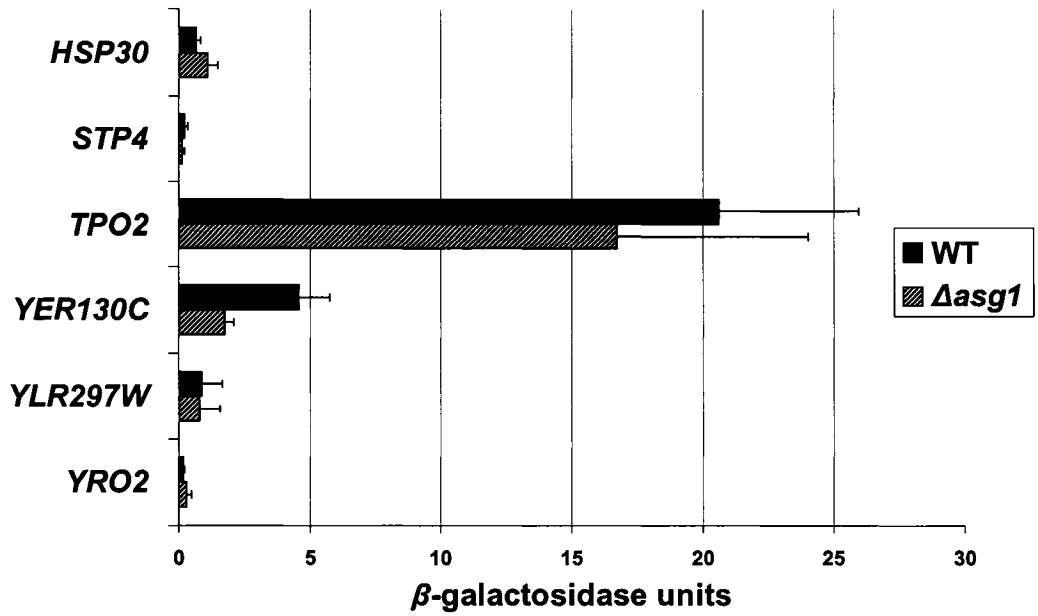


FIGURE 3. **Deletion mapping of the *YER130C* promoter region.** Schematic representations of the *YER130C* promoter deletion constructs are shown. β-galactosidase activity was measured in wild-type (WT; BY4742, *black bars*) and Δ*asg1* strains (*hatched bars*) containing reporters as shown on the left-hand side of the figure. The extent of *YER130C* 5' non-coding DNA remaining in each deletion mutation is indicated by the number at the deletion endpoint. The *YER130C*-dependent β-galactosidase activity was determined for each construct and the values reported are averages of at least 2 independent experiments performed in duplicate.

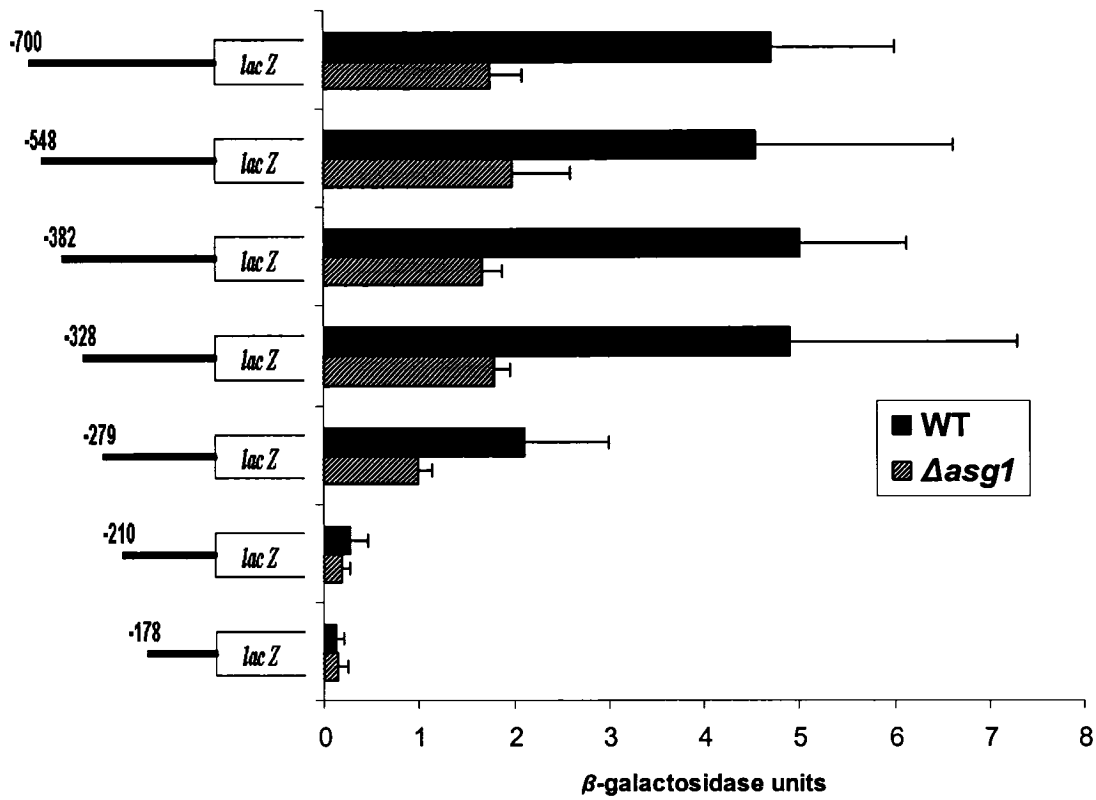
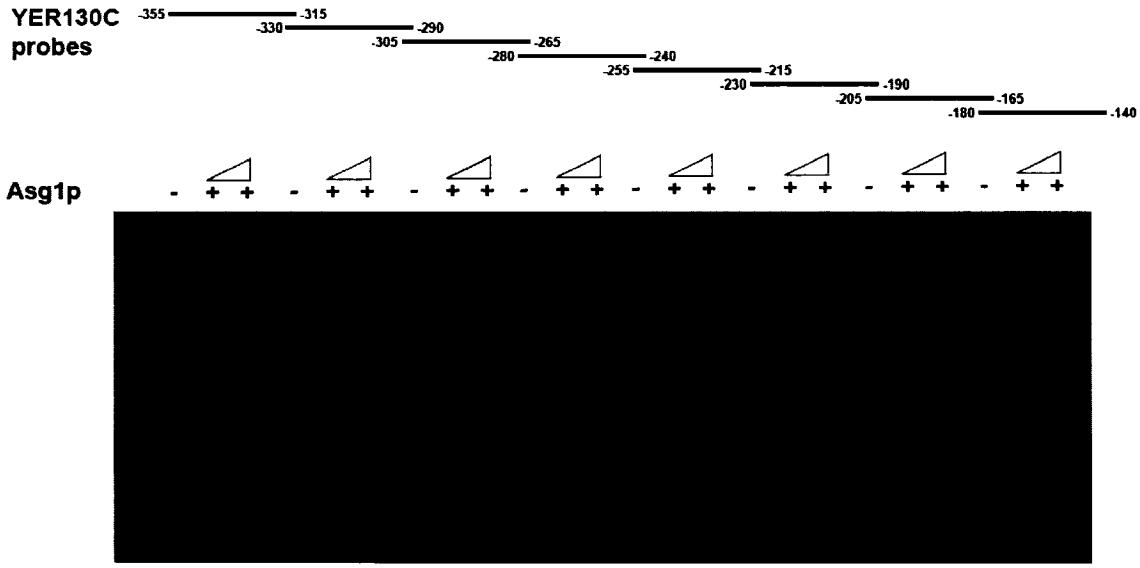


FIGURE 4. **Binding of Asg1p to probes containing 40 bp segments of the *YER130C* promoter.** *A*, EMSA was performed with the DBD of Asg1p (amino acids 1-140) and 40 bp probes spanning a region of the *YER130C* promoter. Each consecutive probe overlapped the preceding probe by 15 bp. *Triangles* represent increasing protein amounts (2 μl and 10 μl). *B*, Sequences of probes *YER130C-4* (from -280 to -240 bp) and *YER130C-5* (from -255 to -215 bp) showing the 15 bp overlapping region. CGG trinucleotides are shown in bold.

A



B

-280 -240

CGCTTCGGAAATGGAGCAAAAAATTTCAG**CGG**ATAACCCA

TCAG**CGG**ATAACCCA**CGG**AAAGGTGGCTTTTACTGTTCCG

-255 -215

FIGURE 5. **Binding of Asg1p to a CGG-N_8-CGG element in the *STP4* promoter.** *A*, Sequences of *STP4* probes used in EMSAs. CGG trinucleotides are shown in bold and their respective mutations in mutant probes are shown in bold and underlined. *B*, EMSA was performed with the DBD of Asg1p and probes mapping to the STP4 promoter. 20 μl of protein extract was used per binding reaction.

A

STP4 probes:

WT:
TTTTTTTC**CGG**GAACGCCG**CGG**CTTTGCAA

mut1:
TTTTTTTC**CAG**GAACGCCG**CGG**CTTTGCAA

mut2:
TTTTTTTC**CGG**GAACGCCG**CAG**CTTTGCAA

mut1+2:
TTTTTTTC**CAG**GAACGCCG**CAG**CTTTGCAA

B

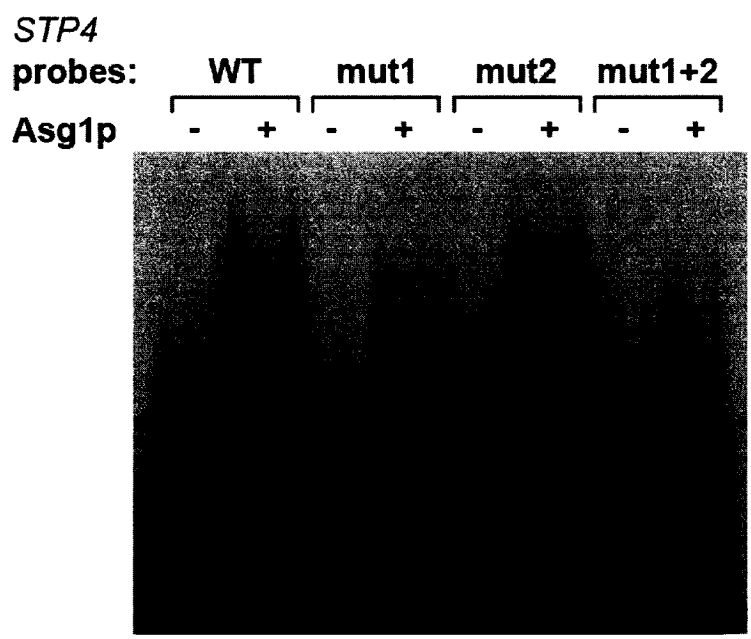
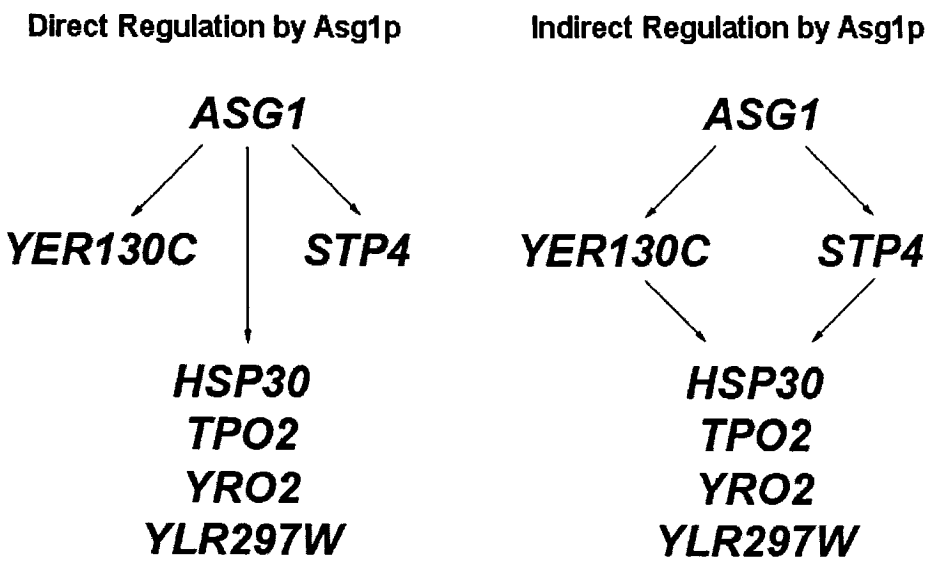


FIGURE 6. **Two possible mechanisms of regulation by Asg1p.**



Section 5:

Conclusions

The focus of this project was to better understand the roles of zinc cluster proteins in transcriptional regulation of genes. In two independent studies, we provided insight into functional relationships between zinc cluster proteins involved in PDR by a phenotypic analysis of double deletion mutants, and we also assigned a potential role in stress response to the previously uncharacterized zinc cluster protein, Asg1p. However, in both these studies, we discovered that the roles of zinc cluster proteins are more complex than originally anticipated. A great deal of interaction exists among themselves and other transcriptional regulators not belonging to this family.

One such complexity involves both similar and dissimilar functions among paralogous pairs. As mentioned previously, many members of the zinc cluster protein family bind and activate similar target genes: Pdr1p and Pdr3p regulate the ABC drug transporters encoded by *PDR5* and *SNQ2* (73, 96); Upc2p and Ecm22p activate *ERG2* and *ERG3* of the ergosterol biosynthetic pathway (153); and Yrr1p and Yrm1p also regulate 14 similar genes (91). However, paralogous proteins also have distinct characteristics in terms of their gene targets and how they regulate genes. For example, a recent paper showed that upon treatment with lovastatin (a competitive inhibitor which decreases sterol levels and induces expression of *ERG* genes), more Upc2p was present than Ecm22p and a greater fraction of *ERG3* promoters were occupied by Upc2p than by Ecm22p (33). In the case with Yrr1p and Yrm1p, Yrm1p activation occurs only in the absence of Yrr1p (91). Furthermore, overproduction of Yrm1p in the presence of a wild-type or a gain-of-function allele of *YRR1* leads to decreased expression of all Yrr1p target genes, indicating that Yrm1p can act as a specific inhibitor of Yrr1p (91). In our phenotypic analysis of double deletion mutants, we discovered some zinc cluster protein pairs with possible functional overlap. For example, *UPC2* and *YRR1* may have redundant roles in mediating cycloheximide resistance as a *Δupc2Δyrr1* strain showed hypersensitivity to cycloheximide, whereas strains carrying a single deletion of either gene, or in combination with *Δecm22* (i.e. *Δecm22Δupc2*, *Δecm22Δyrr1*) showed normal growth. *HAL9* and *UPC2* may also have functional redundancy in caffeine tolerance as a *Δhal9Δupc2* strain showed hypersensitivity to caffeine, whereas strains carrying a single deletion of either gene, or in combination with *Δstb5* (i.e. *Δhal9Δstb5*, *Δstb5Δupc2*)

showed normal growth. To better understand the interactions among zinc cluster proteins, we could perform microarray experiments comparing target genes of single deletion strains with double deletion strains. For the most part however, most double deletion strains did not give any new information on redundant functions, perhaps owing to the regulation of genes being extremely tight and complex. To this end, we could construct triple deletion strains and examine their phenotypes under various growth conditions.

In contrast to complexity from paralogous pairs is that from autoregulation. Many genes such as *PDR3* and *YRR1* contain *cis*-regulatory elements that are recognized and bound by their own gene products (36, 166). It would be interesting to determine whether Asg1p regulates itself. Although the promoter region of *ASG1* does not contain the regulatory element CGG-N_8-CGG (which is the element found in the promoters of *YER130C* and *STP4* and bound by the DBD of Asg1p), Asg1p may recognize other elements within its own promoter. To determine this, we could construct a *lacZ* reporter containing the promoter region of *ASG1* and perform β-galactosidase assays in wild-type and Δ*asg1* strains. Then we could use EMSAs to determine a specific regulatory element that Asg1p directly binds to *in vitro*.

In addition to autoregulation is cross-regulation. Cross-regulation describes the ability of a transcriptional regulator to activate or repress other transcriptional regulators. For example, *YRR1* is not only auto-regulated, but also cross-regulated by Pdr1p and Pdr3p (166). It is possible that cross-regulation may be occurring in our novel stress pathway regulated by Asg1p. Asg1p may not only regulate the six identified target genes directly, but also indirectly through the regulators encoded by *YER130C* and *STP4*. It would be interesting to see the mechanism of Asg1p regulation in Δ*yer130c* and Δ*stp4* single deletion strains, as well as in a Δ*yer130c*Δ*stp4* double deletion strain. As well, the mechanism of Asg1p regulation could be deduced by using strains overexpressing *YER130C* and *STP4*.

As a result of this complex interplay among transcriptional regulators, one question we must address is how does Asg1p regulate its target genes? Aside from elucidating the mode of regulation as direct or indirect (through Stp4p and/or the gene product of

YER130C), we also must consider the involvement of other proteins in the regulation of our identified target genes. For example, upon exposure to acetaldehyde, *TPO2* and *YRO2* were induced in a Haa1p-dependent manner, while *HSP30* induction was Haa1p-independent (10). It is not unusual to observe two non-homologous proteins that regulate the same gene. Msn2p/4p and Hsf1p share some target genes involved in heat shock such as *HSP12, HSP26,* and *HSP104* (22, 54). However, many studies strengthen our hypothesis that Asg1p regulates a novel stress pathway. For instance, three of Asg1p's target genes (*HSP30, TPO2,* and *YRO2*) were found to be regulated independent of Msn2p, Msn4p, and War1p under weak acid induction (136), and *HSP30* induction under heat shock is independent of Msn2p/4p and Hsf1p (99, 102, 140). Therefore, it seems that Asg1p may be activated upon certain kinds of stress, but that under specific circumstances (e.g. in the presence of acetaldehyde), other regulators may be recruited to offer cross-protection.

In addition to the mechanism of Asg1p regulation, we also want to know if stress-induced expression is Asg1p dependent and if so, what kinds of stress induce Asg1p. From previous findings, we know that the target genes of Asg1p are involved in various stress responses. For example, all six of the target genes can be induced by acetaldehyde (10). Acetaldehyde is produced during *Saccharomyces cerevisiae* metabolism under natural conditions, but can inhibit cell growth at high concentrations. Also, three of the target genes (*HSP30, TPO2,* and *YRO2*) can be induced by weak acid (136). Furthermore, *HSP30* was originally described as being induced by heat shock, ethanol, sodium chloride, and glucose limitation in addition to acetaldehyde and weak acid (123, 140). However, a recently published study showed contradictory results in which *HSP30* is downregulated in response to sodium chloride and ethanol (24). Therefore, we hypothesize that these stresses potentially induce Asg1p activation and that Asg1p regulates a novel pathway for a general response to stress. To test this hypothesis, we used Northern blots to determine differences in *HSP30* mRNA levels between wild-type and *Δasg1* strains when induced by heat shock, weak acid, ethanol, sodium chloride, and acetaldehyde. However, no difference was seen for *HSP30* mRNA levels between the two strains (data not shown). We speculate that this problem may be related to strain

specificity as our strains have a BY4742 background whereas most published data use strains with a W303-1A background. Therefore, we will repeat the Northern blot analysis using mRNA isolated from W303-derived strains.

As drugs are also considered a stress to cells, it is plausible that our novel stress pathway may overlap with the PDR network. For example, *TPO1* and *TPO4*, two homologues of *TPO2*, are regulated by Pdr1p (40). Tpo1p, the most well-characterized member of this group, shows typical multi-drug transporter behaviour with its ability to transport eight different compounds including polyamines, quinidine, cycloheximide, and nystatin (129). To determine whether the target genes of Asg1p may be involved in PDR, we could do a phenotypic analysis of strains containing single deletions of the 6 target genes. By spotting these deletion strains on media containing various drugs and looking for hypersensitivity, we can then visually determine whether these genes may play roles in mediating drug resistance.

In summary, this study has increased our knowledge on the functions of zinc cluster proteins in *Saccharomyces cerevisiae*. We have seen that the interaction among zinc cluster proteins is complex amongst known regulators (e.g. those involved in PDR) and newly identified regulators (e.g. Asg1p). In the future, we hope to further our understanding of the networks that exist between zinc cluster proteins and apply this knowledge to higher eukaryotic systems.

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When I first arrived in this lab, I was unaware of how difficult graduate school would be. I did not expect my experiments to fail most of the time; I did not anticipate the number of hours that would be spent repeating and modifying experiments until they gave positive results; I did not know that this was the norm for a graduate student. However, if it were not for the people who supported me at times I thought I would never get through, who offered me excellent advice on experiments, and who helped me find the balance between work and play, I would not have succeeded. Therefore, I would like to acknowledge those individuals who have helped me along the way. First, I would like to thank my supervisor, Dr. Bernard Turcotte, for giving me the opportunity to conduct challenging research and for always being available to help. Next I would like to acknowledge Dr. Mark Featherstone and Dr. Martine Raymond for acting on my Research Advisory Committee and for offering insightful suggestions. I would also like to acknowledge past and present members of the lab and the lab-next-door: Dr. Bassel Akache for convincing me that I am good enough to keep going, Delphine Fessart for her kindness, friendship, and excellent French cooking, Karen Hellauer for her support, Dr. Marc Larochelle for being an excellent role model, Karine Lebel for always listening to and laughing at my stories, Sarah MacPherson for being my partner-in-crime, Nitnipa Soontorngun for her positivity and encouragement, Marc-André Sylvain for introducing me to the world of independent music, and Hector Valderrama-Carvajal for introducing my hips to salsa. Lastly, I would like to thank my family for always believing in me, and my friends Germaine Lowe, Natalie Nguyen, and Nancy So for making me realize that even scientists need to have fun.

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