Conserved motifs in the Msn2-activating domain are important for Msn2-mediated yeast stress response

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Summary
The Msn2 and Msn4 transcription factors play crucial roles in the yeast general stress response. Previous studies identified several large functional domains of Msn2, mainly through crude truncations. Here, using bioinformatics and experimental approaches to examine Msn2 structure–function relationships, we have identified new functional motifs in the Msn2 transcriptional-activating domain (TAD). Msn2 is predicted to adopt an intrinsically disordered structure with two short structural motifs in its TAD. Mutations in these motifs dramatically decreased Msn2 transcriptional activity, yeast stress survival and Msn2 nuclear localization levels. Using the split-ubiquitin assay, we found that these motifs are important for the interaction of Msn2 with Gal11, a subunit of the mediator complex. Finally, we show that one of these motifs is functionally conserved in several yeast species, highlighting a common mechanism of Msn2 transcriptional activation throughout yeast evolution.

Key words: Msn2, Saccharomyces cerevisiae, Stress response, Transcriptional activation domain, Unstructured proteins

Introduction
Cells respond to changes in environmental conditions by significant alterations of gene expression programs through the activation and repression of hundreds of different genes (Causton et al., 2001; Gasch et al., 2000). In yeast, a central stress response program is promoted by the transcription factor, Msn2, and its partially redundant parologue, Msn4 (Boy-Marcotte et al., 1998; Görner et al., 1998). Msn2 and Msn4 (Msn2/4) serve as classical master regulators, enabling the transcription of general stress response genes following yeast exposure to diverse environmental conditions, including starvation, heat, oxidative or osmotic stresses (Hasan et al., 2002; Kandror et al., 2004). These changes in gene expression programs allow yeast to rapidly adapt to the stress conditions, leading to a significant increase in yeast survival. Interestingly, Msn2 transcriptional activity in the absence of stress leads to a decreased yeast growth rate, highlighting the need for tight regulation of Msn2 activity (Durchschlag et al., 2004; Smith et al., 1998). Thus, regulation of Msn2 activity not only ensures its activation in the face of stress but also acts to repress Msn2 activity in the absence of stress.

Previous studies have shown that the regulation of Msn2/4 cellular localization is a highly efficient mechanism for controlling its transcriptional activity (Görner et al., 1998; Görner et al., 2002). In the absence of stress, Msn2/4 reside in the cytoplasm and are inactivated by protein kinase A (PKA)-mediated phosphorylation to prevent nuclear localization of these factors (Beck and Hall, 1999; Görner et al., 1998; Medvedik et al., 2007; Smith et al., 1998). However, following yeast exposure to different stress conditions, these proteins rapidly translocate to the nucleus where they promote the transcription of stress-induced genes. Several other mechanisms for the regulation of Msn2 activity have been previously identified in the nucleus, including proteasome-mediated degradation and Msn2 interaction with the mediator complex that is required for successful transcription (Durchschlag et al., 2004; Lallet et al., 2006). This interaction was shown to induce Msn2 hyperphosphorylation following yeast exposure to different stress conditions (Garreau et al., 2000; Lallet et al., 2006). Finally, following stress relief, Msn2 returns to the cytoplasm through a regulated export system involving the Msn5 nuclear export protein (Durchschlag et al., 2004). It was previously shown that under certain conditions, Msn2 undergoes rapid shuttling between the cytoplasm and the nucleus (Jacquet et al., 2003).

Previous studies focusing on Msn2 sequence-function relationships identified several functional regions of the protein, including the C-terminal zinc finger DNA-binding domain (DBD), the region containing the nuclear localization signal (NLS), the region containing the nuclear export signal (NES) and the essential Msn2 transcriptional-activating domain (TAD), located in the N-terminal region. It was previously shown that the TAD domain promotes Msn2 nuclear localization and physically interacts with Gal11, a subunit of the mediator complex (Lallet et al., 2006). Msn2 functional domains were mainly identified by truncation of large regions of Msn2 and by fusing Msn2 fragments to non-natural DNA-binding domains (Boy-Marcotte et al., 2006). However, despite such sequence-function insight, very little is known regarding the overall structural properties of Msn2. In particular, it is not clear what are the functional motifs and specific residues of the Msn2 TAD that control Msn2 nuclear localization and transcriptional activity. It is also not known whether these functional motifs are conserved in sequence and function throughout yeast evolution.

Here, we employed bioinformatics and experimental approaches to analyze structure–function relationships of Msn2. We report that
Msns2 is predicted to be an intrinsically unstructured protein containing two short structural motifs in its TAD that are important for Msns2 activity. Circular dichroism (CD) analysis of a synthetic peptide corresponding to one of these motifs shows that it can adopt an amphipatic $\alpha$-helical structure in solution. We found that mutations in this motif that disrupt its amphipatic nature lead to significant reductions in Msns2 transcriptional activity, yeast survival following exposure to extreme oxidative environment and Msns2 nuclear localization levels, demonstrating the importance of this motif for Msns2 regulation and activity. We also found that each of the structured motifs is important for Msns2 interaction with Gal11 and show that Gal11 plays a role in promoting Msns2 nuclear localization and retention. Finally, we show that these motifs are functionally conserved in several yeast species, providing common mechanisms for Msns2 transcriptional activation during yeast evolution.

Results

Msns2 is an unstructured protein containing two structural motifs in the N-terminal transcriptional-activating domain (TAD)

It has been previously shown that about one third of yeast proteins are predicted to be intrinsically unstructured and in many cases these proteins were experimentally characterized (Gspomer et al., 2008). These unstructured proteins can belong to protein families of diverse function, including transcription factors, signaling molecules, chaperones and cytoskeleton proteins (Dosztányi et al., 2005). To determine whether Msns2 is predicted to be a structured or intrinsically unstructured protein, we analyzed its sequence using IUPred software (Dosztányi et al., 2005). IUPred bioinformatics analysis allows the user to estimate the capacity of polypeptides to form stabilizing contacts and thus defines the probability of a protein sequence to assume a structured or unstructured conformation (Dosztányi et al., 2005). Such examination of the Msns2 sequence predicts that it mainly adopts intrinsically unstructured conformations, with few small structured regions (Fig. 1; supplementary material Fig. S1). In particular, we identified two short structured regions in the Msns2 N-terminal TAD located within the first 300 residues of the protein (Boy-Marcotte et al., 2006). The two predicted structured regions are found between residues 1–50 and residues 253–267 and are defined as motif A and motif B, respectively (Fig. 1). Bioinformatics analysis of the secondary structure of motif B predicts that it adopts an amphipatic $\alpha$-helical structure (supplementary material Fig. S2). We have also analyzed Msns4 using IUpred and found that, similar to Msns2, most of the protein is predicted to be intrinsically unstructured with several short structural regions (supplementary material Fig. S3). We found that the ordered region in ScMsns4 located between residues 235–246 is similar to motif B in Msns2 and is highly conserved in Msns4 from several yeast species (supplementary material Fig. S3). The high conservation of motif B between Msns2 and Msns4 highlights the possible importance of this motif in the functions of these proteins (see below for experimental analysis of motif B in Msns2).

To examine the importance of these regions for Msns2 activity, we first generated several truncated Msns2 variants in which the N-terminal Msns2 TAD is partially deleted. The truncated msn2 genes were integrated into the yeast genome and expressed under the control of the endogenous Msn2 promoter (see Materials and Methods). We then monitored the transcriptional activity of the N-terminal-truncated Msns2 proteins in vivo using flow cytometry analysis of the HSP12 reporter gene fused to green fluorescent protein (GFP) (Sadeh et al., 2011). HSP12 is a general stress response gene encoding a plasma membrane protein and its expression is highly dependent upon Msns2/4 transcriptional activity (supplementary material Fig. S3) (Pacheco et al., 2009; Welker et al., 2010). The HSP12 promoter contains several stress response elements (STRE) and was shown to be strongly induced following yeast exposure to diverse stress conditions (Martínez-Pastor et al., 1996; Pacheco et al., 2009). We have recently found that Hsp12 expression levels are tightly correlated to the expression of other Msns2 reporter genes, including PNC1 and TPS2, and to yeast stress survival, showing that HSP12 reporter gene can reflect overall Msns2 transcriptional activity (Sadeh et al., 2011). To examine the activity of Msns2 without the influence of Msns4, we measured levels of Hsp12-GFP fluorescence of the truncated Msns2 variants on the background of a yeast strain deleted of the Msn4 gene (supplementary material Fig. S4).

We found that deletion of the first 50 residues (Msns2-B) reduced Msns2 activity by 15–30%, relative to the activity of the full length Msns2 protein (Fig. 2; supplementary material Fig. S5). Further deletion of the first 250 residues (Msns2-C) did not lead to additional reduction in Msns2 activity, indicating that this region is not essential for Msns2 activity under the stress conditions examined. In contrast, we found that deletion of the first 260 residues (Msns2-D) leads to a dramatic 40–60% reduction in Msns2 activity, relative to that of the full-length protein (Fig. 2; supplementary material Fig. S5). Further deletion of the first 300 residues of Msns2 containing the entire TAD led to additional decrease in Msns2 activity (Msns2-E) (Boy-Marcotte et al., 2006). To further characterize the effect of the different truncations and mutations on Msns2 activity, we also examined Tps2-GFP expression levels in the different Msns2 mutant strains.
We found good correlation between the effects of Msn2 mutation on Hsp12-GFP and Tps2-GFP expression (Fig. 2; supplementary material Fig. S6A), indicating that the different truncations affect the general transcriptional activity of Msn2. Overall, our results show that the transcriptional activity of truncated Msn2 is correlated to deletions in the predicted structured regions in the Msn2 TAD (Figs 1, 2). To compare the expression of the truncated Msn2 proteins, relative to WT Msn2, we performed western blot and flow cytometry analysis of these mutants fused to GFP. Using these approaches, we found that most of the truncated versions of Msn2 are expressed at a level similar to that of the full length protein (supplementary material Fig. S7). However, in the case when 260 residues were deleted, we found ~25% less Msn2 protein, relative to the level of the WT protein.

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Site-directed mutagenesis of residues in motif B can reduce stress-induced Msn2 activity

We observed that deletion of a region in Msn2 TAD containing part of motif B leads to a significant reduction in Msn2 activity following yeast exposure to diverse stress conditions (Fig. 2). To directly examine the role of motif B in controlling Msn2 activity, we mutated residues within this motif and followed Hsp12-GFP reporter expression levels. We found that mutating three aspartate residues to alanines in motif B (Msn2-3DA: D264A, D265A and D270A) reduced Msn2 activity by 40–60% relative to wild-type Msn2 (Fig. 3A,B). In contrast, mutating these residues to glutamates (Msn2-3DE) had no effect on Msn2 activity, indicating that the negative charge of these residues is sufficient for Msn2 activity (Fig. 3B). To further examine whether the two adjacent serine residues in the motif are also

Fig. 2. Generation and examination of N-terminal-truncated Msn2 variants. (A) Schematic representation of Msn2 highlighting the different domains, including the transcriptional-activating domain (TAD), the domain containing the nuclear export signal (NES), the domain containing the nuclear localization signal (NLS) and the zinc finger (ZF)-binding domain. The two structured motifs, motif A and motif B, are highlighted below in grey boxes. (B) Schematic representation of N-terminal-truncated Msn2 examined in this study. (C) Activity of Msn2-truncated variants was analyzed by Hsp12-GFP reporter gene expression. All truncated Msn2 constructs were generated on the background of the msn4-deleted strain. Hsp12-GFP expression levels in the different strains were measured following yeast exposure to osmotic stress (0.5 M NaCl). Expression level of Hsp12-GFP in the absence of stress is depicted in grey and the increase in expression following exposure to osmotic stress is depicted in white. Values of Hsp12-GFP expression were normalized relative to the Msn2 expression (supplementary material Fig. S7). Asterisks indicate significant differences of Hsp12-GFP expression relative to the WT or the higher molecular weight Msn2 construct, as determined by Student’s t-test (P<0.05). t-test for all data was calculated using the Excel function for comparison between two samples of one tailed t-test with unequal variance. Values are means ± s.e.m.

Fig. 3. Mutational analysis of motif B located in the Msn2 transcriptional activation domain. (A) Msn2 motif B mutants examined in this study. (B) Msn2 mutant activity analyzed by Hsp12-GFP reporter gene expression. All mutated Msn2 proteins were generated on the background of the msn4-deleted strain (WT*). Hsp12-GFP expression levels in the different strains were measured following yeast exposure to osmotic stress (0.5 M NaCl). Expression level of Hsp12-GFP in the absence of stress is depicted in blue and the increase in expression following exposure to osmotic stress in red. (C) Survival and Hsp12-GFP expression levels of the different Msn2 mutant strains following exposure to extreme (3 mM of H2O2) and mild (0.6 mM of H2O2) oxidative stress, respectively. Survival and Hsp12-GFP expression levels were normalized to the levels of the msn4-deleted strain. Values are means ± s.e.m.
important for Msn2 activity, we mutated S268 and S269 to alanines (Msn2-2SA) (Fig. 3A). We found that these mutations had no effect on Msn2 activity, suggesting that any possible phosphorylation of these serine residues is not essential for Msn2 activity. To further support these results, we also analyzed the mRNA levels of Hsp12 and Pnc1 in the WT, Msn2-3DA mutant and the msn2-deleted strains by RNA extraction followed by real-time PCR (supplementary material Fig. S6B). We found that the transcript levels of HSP12 reporter gene correlates with the effect of the mutations on the respective protein levels. To verify that the decrease in the transcriptional activity of the Msn2-3DA mutant is not due to a significant reduction in the protein level, we compared the expression of the mutant relative to that of the WT. Using western blot analysis, flow cytometry and ELISA, we found that the level of expression of the mutant is very similar to that of WT Msn2 (supplementary material Fig. S7).

To examine the combined effects of inactivation of motifs A and B, we generated a double mutant of Msn2 deleted of the first 50 residues that include motif A and containing the three aspartate to alanine mutations in motif B (Msn2-B-3DA). This double mutant presented much lower Msn2 transcriptional activity, relative to the activity obtained upon inactivation of either motif alone, indicating that the two motifs contribute independently to Msn2 transcriptional activity (supplementary material Fig. S8). To further examine the ability of the two motifs to serve as activating domains, we established a yeast one-hybrid assay by fusing motif A (residues 1–50), motif B (residues 258–270) or 3DA-mutated motif B to the Gal4 DNA-binding domain and testing the ability of the respective strains to grow on selective agar plates lacking histidine (supplementary material Fig. S9). This allowed us to examine whether the two motifs can independently promote the transcription of the HIS3 gene. We found that both motif A and motif B are able to serve as transcriptional activators, whereas the 3DA mutation in motif B prevented growth on selective plates lacking histidine (supplementary material Fig. S9). These results highlight the function of both motifs as transcriptional activators and suggest that both motifs can contribute additively to the overall transcriptional activity of Msn2.

Next, we examined the effects of the different mutations on yeast stress survival following exposure of the msn2-mutated strains to extreme oxidative or heat stress. We found that the msn2-3DA mutant strain exhibits significantly reduced survival relative to the wild-type Msn2 strain. In contrast, the extent of survival of the msn2-3DE mutant strain following exposure to an extremely oxidative environment was similar to that of the wild-type Msn2 strain (Fig. 3C). The excellent correlation between stress survival and changes in Hsp12-GFP expression levels following yeast exposure to heat or oxidative stress indicates that the three aspartate to alanine mutations in motif B significantly reduced overall Msn2 activity (Fig. 3B,C; data not shown).

Msn2 motif B folds into an amphipatic α-helix
To experimentally determine whether wild-type and mutant motif B can adopt a secondary structure, we analyzed the secondary structure of two synthetic peptides derived from residues 255–271 of the Msn2 wild-type and Msn2-3DA proteins using circular dichroism (CD). Previously, CD studies of peptides derived from proteins with known secondary structures confirmed that peptide structures correlate well with the secondary structures of the corresponding regions of the protein (Dyson et al., 1992).

Analysis of the CD spectrum of the wild-type Msn2 peptide in buffer shows it to adopt a random coil structure reflected as a large negative band around 200 nm (Fig. 4A). This random coil structure is expected for a peptide composed of over 50% hydrophobic residues in an aqueous environment (Orlando and Ornellas, 1999). To stabilize the α-helical structure of the Msn2 peptide, we added increasing concentrations of 2,2,2-trifluorothanol (TFE). TFE is an organic polar solvent that was found to stabilize the α-helical structure of peptides by displacing water molecules from the peptide surface, leading to a more favorable formation of intrapeptide hydrogen bonds (Nelson and Kallenbach, 1986; Roccatano et al., 2002). We found that in a 25% TFE/water mixture (vol/vol), the CD spectrum of the wild-type motif B peptide is shifted towards that of an α-helical structure (Fig. 4A). At 50% and 75% TFE/water mixtures, the peptide adopts a much more pronounced α-helical conformation, indicated by the appearance of two negative peaks at 208 and 222 nm (Fig. 4A). In contrast, we found that the peptide derived from the Msn2-3DA mutant displayed a higher content of α-helical structure in the 25% TFE/water mixture, relative to the wild-type peptide that remained unchanged at higher TFE concentrations (Fig. 4B). These results confirm that motif B

![Peptide structures](image_url)

**Fig. 4.** Peptides corresponding to Msn2 motif B region analyzed by CD spectroscopy in the presence of TFE. (A) Peptide derived from the wild-type Msn2 motif B, corresponding to sequence NSLNIDSLDDYVSSDL (residues 255–271). (B) Peptide derived from mutated motif B, corresponding to sequence NSLNIDSLAAYVSSAL. Both peptides were examined at a concentration of 25 μM in 50 mM phosphate buffer, pH 7.5, at 25 °C and exhibit spectra characteristics of an α-helical structure in the presence of TFE. The measured CD values were converted to mean residue molar ellipticities and plotted as a function of the incident wavelength.
can adopt an α-helical structure in solution and show that the aspartic acid to alanine mutations within the motif dramatically affect Msn2 activity without disrupting the secondary structure of this region (Fig. 3).

**Conservation of Msn2 motif B throughout yeast evolution**

It was previously shown that Msn2 is present in many yeast species, including *Candida glabrata*, *Kluyveromyces lactis* and *Ashbya gossypii* (Roetzer et al., 2008). Functional analysis of *C. glabrata* Msn2 (CgMsn2) revealed that the environmental stress response (ESR) genes are partially conserved between these species and that *C. glabrata* and *S. cerevisiae* share many common Msn2 target genes (Roetzer et al., 2008). To examine whether Msn2 predicted structural motifs are conserved throughout yeast evolution, we performed bioinformatics and experimental analysis of Msn2 from *S. paradoxus*, *S. mikatae*, *C. glabrata*, *K. lactis* and *A. gossypii*. Bioinformatics analysis of Msn2 from these species using IUPred indicates that, similar to *S. cerevisiae* Msn2 (ScMsn2), all examined Msn2 orthologues are predicted to be unstructured with a short structural region at the N-terminal TAD domain, except for *K. lactis* Msn2 (KlMsn2) and *A. gossypii* Msn2 (AgMsn2) (supplementary material Fig. S10). Sequence alignment of Msn2 motif B from the different species shows that it is highly conserved in all Msn2 proteins from the examined *Saccharomyces* species and in CgMsn2, highlighting the possible importance of motif B for Msn2 function in these species (Fig. 5A).

### Figures

**Fig. 5.** Msn2 orthologues that contain motif B can complement the deletion of *MSN2* in *S. cerevisiae*. (A) Motif B is conserved in several yeast Msn2 orthologues. Sequence identity levels of Msn2 protein are shown in brackets. (B) Msn2 orthologue activity analysed by flow cytometry measurements of Hsp12-GFP reporter gene expression. All strains containing *MSN2* orthologues were generated on the background of *msn2-100* and *msn4-1* double deletion strain. The Hsp12-GFP expression levels in the different strains were measured following yeast exposure to osmotic stress (0.5 M NaCl). Expression level of Hsp12-GFP in the absence of stress is depicted in blue and the increase in expression following exposure to osmotic stress is depicted in red. Values are means ± s.e.m.

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<td><em>S. paradoxus</em> Msn2 (90.8%)</td>
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<td><em>S. mikatae</em> Msn2 (83.7%)</td>
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<td><em>C. glabrata</em> Msn2 (23.8%)</td>
<td>MSLDTMLDDYLVTE</td>
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<td><em>K. lactis</em> Msn2 (24.2%)</td>
<td>Motif B not found</td>
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<td><em>A. gossypii</em> Msn2 (16.9%)</td>
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Next, we considered the level of complementation of *MSN2* deletion in *S. cerevisiae* upon introduction of the different Msn2 orthologues, using the Hsp12-GFP reporter assay. We found that *S. paradoxus* Msn2 (SpMsn2) and *S. mikatae* Msn2 (SmMsn2) can fully complement the deletion of the *MSN2* gene in *S. cerevisiae*, while CgMsn2 exhibits only a partial complementation of ~50% (Fig. 5B). In agreement with the partial complementation ability of CgMsn2, we found that this gene is shorter than the ScMsn2 and does not contain motif A, located in the first 50 residues of ScMsn2 (supplementary material Fig. S10). To examine the effect of adding the ScMsn2 motif A to CgMsn2, we generated the Cgmsn2 chimeric gene encoding for 1–100 residues of ScMsn2, followed by the CgMsn2 sequence. We found that this chimeric protein displays 15–20% higher Hsp12-GFP expression than does wild-type CgMsn2, further supporting the contribution of the two motifs to Msn2 activity (supplementary material Fig. S11). Examination of Msn2 orthologues from more distantly related species showed that the expression of KlMsn2 and AgMsn2, that do not contain motif B, could not complement the deletion of *MSN2* in *S. cerevisiae* (Fig. 5B).

Finally, we examined the effects of mutations in motif B of SmMsn2 and CgMsn2 on the ability of these orthologues to complement the deletion of *MSN2*. We found that aspartic acid to alanine mutations in SmMsn2 and CgMsn2 (SmMsn2-3DA and CgMsn2-3DA, respectively) (Fig. 6A) significantly reduced the activity of the Msn2 orthologues, as indicated by a dramatic reduction in Hsp12-GFP reporter gene expression relative to the corresponding wild-type proteins (Fig. 6B). As expected, we also found that these msn2 mutant strains exhibit dramatic reduction in survival following exposure to extreme oxidative and heat stress, relative to strains containing the corresponding wild-type genes (Fig. 6C; data not shown). Overall, these results highlight the conservation of sequence and function of motif B in several yeast species across evolution.

### The Msn2 motif B is essential for proper stress-induced nuclear localization

One of the most prominent mechanisms of Msn2 regulation is stress-induced nuclear localization. It was previously shown that in the absence of stress, Msn2 is mainly localized in the cytoplasm. Following exposure to stress, Msn2 is rapidly translocated to the nucleus, where it facilitates the expression of stress-induced genes (Görner et al., 1998). To examine the effects of mutations in motif B on Msn2 localization, we expressed ScMsn2-GFP, ScMsn2-3DA-GFP, CgMsn2-GFP and CgMsn2-3DA-GFP fusion proteins in *S. cerevisiae*. We then followed the cellular localization of these chimeras in the presence of ethanol or osmotic stress and in the absence of stress. It was previously shown that the exposure to high ethanol concentration (7%) leads to significant Msn2 nuclear localization (Görner et al., 1998). As expected, we found that in the absence of stress, all of the proteins are localized to the cytoplasm (Fig. 7; supplementary material Fig. S12). Upon stress exposure, however, ScMsn2 and CgMsn2 rapidly translocate to the nucleus, while ~30 min following stress induction, both ScMsn2 and CgMsn2 are gradually exported out of the nucleus (Fig. 7; supplementary material Figs S12, S13) (Roetzer et al., 2008). In contrast, the ScMsn2-3DA mutant exhibited a much weaker level of nuclear localization following stress exposure and was excluded from the nucleus much earlier than was wild-type
Msn2 (Fig. 7). In addition, we found that the CgMsn2-3DA mutant did not accumulate in the nucleus following stress exposure. These results are in excellent agreement with the lower transcriptional activity of ScMsn2-3DA and CgMsn2-3DA, relative to the corresponding wild-type proteins (Fig. 5), indicating that the lack of activity of these mutants can be explained by their impaired nuclear localization and retention. Finally, we examined the effect of deletion of motif A, located in the first 50 residues of Msn2, on Msn2 nuclear localization. Examination of the truncated Msn2 fused to GFP indicated no significant effect on Msn2 nuclear localization following yeast exposure to stress (supplementary material Fig. S13).

Msn2 motif A and motif B are essential for Msn2 and Gal11 interaction

It was previously shown that Gal11 plays an important role in stress-induced hyper-phosphorylation of Msn2 and physically interacts with the Msn2 TAD (Boy-Marcotte et al., 2006). To examine whether motif A or motif B is essential for the physical interaction of Msn2 with Gal11, we have utilized the Split Ubiquitin yeast two-hybrid (Y2H) assay. The split ubiquitin is a Y2H assay that is based on conditional proteolysis of ubiquitinated Ura3-conjugated proteins (Snider et al., 2010). This assay was extensively used to monitor interactions between membrane proteins and between transcriptional-activating domains, such as Gal4 and Gcn4, with other cellular proteins (Lim et al., 2007; Snider et al., 2010). We utilized the Split Ubiquitin assay to detect interactions between Gal11 and ScMSN2, ScMsn2-3DA, CgMSN2 or CgMsn2-3DA. In this assay, the interaction between Gal11 and Msn2 leads to the assembly of two halves of ubiquitin, each fused to the interacting proteins, that in turn leads to the degradation of the fused Ura3 protein (Lim et al., 2007). We found that both ScMsn2 and CgMsn2 can bind to Gal11, leading to a lower growth level of the respective strains, relative to the control strains on uracil-lacking plates (Fig. 8). We also found that the deletion of the first 50 residues containing motif A or the presence of mutations in motif B (Fig. 3A) abolished the interaction of ScMsn2 with Gal11 (Fig. 8). Combining the deletion of motif A and the mutations in motif B had no further effect on the interaction of ScMsn2 with

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In the case of CgMsn2, the same pattern of interactions was observed, although the effect of the mutations in CgMsn2 motif B on the interaction with Gal11 was less pronounced. These results are in agreement with previous analysis of Msn2-Gal11 interactions that detected the interaction of Msn2 with Gal11 through immunoprecipitation (Lallet et al., 2006). Overall, our results indicate that motif A and motif B are essential for the interaction of ScMsn2 with Gal11 and that this mode of interaction is conserved between ScMsn2 and CgMsn2.

Finally, we examined whether Gal11 is involved in the nuclear localization of Msn2 following yeast exposure to oxidative or ethanol stresses. We found that deletion of GAL11 significantly reduced the level of the nuclear-localized Msn2-GFP, relative to the parental strain (supplementary material Fig. S14). Moreover, we found that localization of the Msn2-3DA-GFP mutant in the gal11-deleted strain was significantly lower than was the nuclear localization of the Msn2-3DA-GFP mutants on the background of the parental strain (supplementary material Fig. S14). These results strongly indicate that Msn2-Gal11 interaction is important for the translocation and/or retention of Msn2 in the nucleus following stress induction.

Discussion

In this work, we enlisted bioinformatics and experimental approaches to analyze Msn2 TAD structure–function relationships. We found that Msn2 is predicted to be an intrinsically unstructured protein but that its TAD contains two short structured motifs. Previously, bioinformatics analysis of all proteins from *S. cerevisiae* showed that a large proportion of these proteins are predicted to be intrinsically unstructured (Gsponer et al., 2008). The lack of structure of these proteins is thought to provide several advantages in terms of protein function, including a significant increase in the interaction surface area, conformational flexibility and accessible post-translational modification sites, including sites for phosphorylation or ubiquitination (Dosztányi et al., 2005; Kriwacki et al., 1996; Oldfield et al., 2005; Wright and Dyson, 1999). These properties are highly important for proteins participating in signaling and/or regulatory functions, including kinases and transcription factors. Thus, the predicted unstructured nature of Msn2 may promote its physical interaction with partners that tightly regulate Msn2 activity in the presence or absence of stress (Boy-Marcotte et al., 2006; Görner et al., 1998; Kaida et al., 2002; Lee et al., 2008; Medvedik et al., 2007). In addition, the high accessibility of serine or threonine residues in the unstructured regions of Msn2 could lead to its hyperphosphorylation following yeast exposure to stress (Garreau et al., 2000). We found that several Msn2 orthologues from different fungal species are also predicted to be unstructured, indicating that this property was conserved during yeast evolution (supplementary material Fig. S10).

Analysis of the predicted structured and unstructured regions of Msn2 enabled the identification of two structured motifs in the...
Ms2 TAD. Mutational analysis of motif B, located between residues 253 and 267, clearly shows that this motif plays an important role in enabling Ms2 transcriptional activity and yeast stress survival. We specifically found that mutations that eliminate most of the negative charge of motif B (Ms2-3DA) (Fig. 3) led to a significant decrease in Ms2 activity (Fig. 3). Interestingly, structural analysis of mutated motif B showed that these mutations did not affect the stability of its α-helical structure (Fig. 4). This result highlights the importance of the negative charge of motif B in promoting Ms2 activity. Our finding that mutation of these positions disrupts Ms2 interaction with Gal11 suggests that the aspartic acid residues in motif B can form salt bridges with positively charged residues in Gal11 to stabilize the Ms2-Gal11 interaction. Previous analysis of Ms2-Gal11 interactions had localized the interaction site to the entire TAD (i.e. residues 1–303) of Ms2 and to the N-terminal domain of Gal11 (residues 1–351) (Lallet et al., 2006). Our analysis provides much higher resolution in terms of Ms2 sites of interaction with Gal11 and shows that the two structured motifs, Ms2 motif A and motif B (Fig. 8), are important for this interaction.

In addition, we found that the Ms2-3DA mutant shows a much lower nuclear localization level and diffuses faster from the nucleus, relative to wild-type Ms2. The low level of nuclear localization and retention of the Ms2-3DA mutant was reduced on the background of the GAL11-deleted strain (supplementary material Fig. S14). These results highlight the possible importance of Ms2-Gal11 interaction for Ms2 nuclear localization and retention. It was previously shown that Ms2-Gal11 interaction is essential for Ms2 hyper-phosphorylation, a trait that is important for Ms2 activity (Lallet et al., 2006). Thus, one of the possible roles of Ms2 hyper-phosphorylation could be to prevent the rapid export of Ms2 from the nucleus, so as to enable the expression of stress-induced genes. Another possible role of the Ms2-Gal11 interaction could be to promote Ms2 nuclear localization by participating in active recruitment of Ms2 to the nucleus (Menon et al., 2005; Schmid et al., 2006). This ‘reverse recruitment’ model suggests that large protein complexes are anchored to the nuclear periphery to facilitate the actual transcription of DNA. According to this model, the mediator complex can take an active role in importing Ms2 into the nucleus, thus facilitating Ms2 nuclear localization following yeast exposure to stress conditions.

We have also shown that motif B is conserved in Ms2 from several yeast species, including SpMs2, SmMs2 and CgMs2 (Fig. 5). In contrast, we found that motif B is not present in more distantly related species, including AgMs2 and KlMs2. We found that the overall sequence identity between CgMs2 and ScMs2 is only around 24% and that despite this low identity, CgMs2 can partially complement the deletion of MSN2 in S. cerevisiae. We also found that aspartic acid to alanine mutations in CgMs2 abolishes its activity in S. cerevisiae (Fig. 6). Thus, the sequence and functional conservation of motif B in different yeast species suggests a common mechanism of transcriptional activation of Ms2 across yeast evolution. Previous studies using microarray analysis showed that S. cerevisiae and C. glabrata share many conserved Ms2 target genes (Roetzer et al., 2008). This study also identified a conserved region in Ms2, termed homology domain 1 (HD1) that is located at the NES domain and is responsible for the regulation of the Ms2 export from the nucleus. This domain was found to be phosphorylated and it was reported that mutations of serine to alanine residues in this domain decreased Ms2 nuclear export (Roetzer et al., 2008). In contrast, we showed that motif B is involved in nuclear import and is probably not regulated by phosphorylation, since mutations of two serine residues in the motif had no effect on Ms2 activity.

In summary, our combined bioinformatics and experimental approaches allowed the identification of conserved structured regions in Ms2 TAD. We have shown the high importance of these motifs for Ms2 transcriptional activity, yeast stress survival and nuclear localization. Our strategy can be utilized for the analysis of functional regions in many other unstructured proteins, including other transcription factors and signaling molecules that comprise a large proportion of the eukaryotic proteome.

Materials and Methods

Yeast strains and plasmids

The Saccharomyces cerevisiae strains used in this study are listed in supplementary material Table S1. The HIS12-GFP fusion (RS81) was generated by genomic integration of a PCR fragment amplified from the BY4741 strain (supplementary material Fig. S14). The HIS12-GFP fusion was transfected into the chromosomal MSN2 fused to GFP (Invitrogen) into the W303-1A strain. To construct the MSN2A (RS82), MSN4A (RS83) and the double deletion strain, MSN2::MSN4A (RS84), we replaced the complete MSN2 or MSN4 sequences or both of these genes with antibiotic resistance cassettes in strain W303-1A. The resistance cassettes for G418 and hygromycin B were amplified from plasmids pFA6a-kanMX6 and pAG32, respectively, with primers containing the flanking regions of MSN2 and MSN4 coding sequence, as required. The deletion cassettes were integrated by homologous recombination to generate the msn2::KanMX6 and msn4::hphMX4 single deletion strains or the msn2::KanMX6::hphMX4 double deletion strains. To generate a template plasmid for preparation of genomic integration of msn2-truncated strains (MTS1–MTS5), the Sbb221 plasmid (a gift from Shay Ben-Aroya) (Ben-Aroya et al., 2008) containing URA3 marker flanked by fragments of the KanMX6 gene was digested with BgIII and BamHI to remove the KanMX6 fragment located upstream to the URA3 gene. A PCR fragment containing the MSN2 ORF, together with 500 bp of the Ms2 promoter and 100 bp of the Ms2 terminator, was ligated into the digested plasmid using the In Fusion dry-down cloning kit, according to the manufacturer’s instructions (Clontech), yielding plasmid SBB221-Msn2. Generation of msn2-truncated strains was achieved using plasmid SBB221-Msn2 as a template for PCR and using the appropriate primers to amplify a fragment containing the Ms2-truncated gene, the URA3 marker and the fragment of the KanMX6 gene downstream of the URA3 gene. These PCR fragments were transformed into RGS4 strain (supplementary material Table S1) and integrated into the msn2::KanMX6 locus by homologous recombination. Yeast transformants were grown on selective media and proper genomic integration was verified using PCR. Generation of MSN2 and MSN4 orthologous strains (MSO1–MSO5) was performed by assembling a PCR product of the orthologue gene amplified from genomic DNA of the appropriate fungal strain and a PCR product containing the Ms2 terminator, the URA3 marker and a KanMX6 gene fragment amplified from the Sbb221-Msn2 plasmid. This PCR fragment was inserted into the msn2::KanMX6 locus by homologous recombination. Transformants were grown on selective media and proper genomic integration was verified using PCR. Strains SDS1–SDS7 were created by assembling together two fragments amplified from the Sbb221-Msn2 plasmids appending the relevant mutations.

To follow the cellular localization of Ms2, a plasmid containing the MSN2-GFP fusion under the control of a constitutive ADH1 promoter was used (pMSN2-GFP, a gift from Christoph Schuller, University of Vienna, Vienna, Austria) (Görner et al., 1998). The pMSN2-GFP plasmid was digested with Sall and NotI to release the MSN2-GFP gene, followed by amplification and homologous recombination of CgMSN2 and all mutated MSN2 genes into the linearized vector. To examine the interaction of Ms2 with Gal11, split-ubiquitin Y2H plasmids were obtained from Norbert Lehming (National University of Singapore, Singapore, Singapore) (Lim et al., 2007). To generate the Ms2 fusions to the N-terminal of ubiquitin, the PADNX-Nα-GAL80 plasmid was digested with NotI and Xhol to release the GAL80 gene (Laser et al., 2000). PCR products corresponding to ScMSN2 or CgMSN2 were amplified from the appropriate genomic DNA and inserted into the digested plasmid by homologous recombination in-frame with the N-terminal ubiquitin fragment. All msn2 mutants were generated by assembly PCR of mutated gene fragments and integrated into the PADNX-Nα digested plasmid by homologous recombination. All plasmids were verified using PCR and sequenced.

Identification of Ms2 orthologues

Ms2 orthologues were identified using the fungal orthogroups repository site (http://www.broadinstitute.org/cgi-bin/regev/orthogroups/show_orthogroup.cgi?orf=...
YMR037C). The Msn2 orthologous genes used in this study are: spar179-g2.1 (S. paradoxus), smk57-g8.1 (S. cerevisiae), GASL0509995g (C. glabrata), KLLA0F26961g (K. lactis) and ABR0899C (A. gossypii).

Hsp12-GFP or Tps2-GFP expression analysis

Strains were grown overnight in 5 ml synthetic complete (SC) medium, diluted 1:50 to OD600 = 0.1 in the same medium and grown for an additional 6 hours. Each strain culture was then divided and different conditions were applied to each fraction. The conditions applied were: 0.6 mM H2O2 (oxidative stress), 0.5 M NaCl (osmotic stress), temperature shift from 30˚C to 37˚C (heat stress). Reference samples where no stress was applied were used as controls. Following 40 minutes of stress application, the samples were analyzed by flow cytometry (FACS Calibur), and the Hsp12-GFP fluorescence intensity of 10,000 yeast cells was measured. The median fluorescence intensity of each strain population was calculated from the flow cytometry histograms. The fluorescence intensity of each strain following exposure to the different stress conditions was normalized to the median of the msn4-deleted strain measured under the same condition.

Western blot

Strains expressing either WT Msn2-GFP or its mutants were grown overnight in SC-Leu at 30˚C, diluted to OD600 of 0.1 in SC-Leu and grown to OD600 of 0.6 at 30˚C. Cells were then harvested by centrifugation and the cell pellet was resuspended in 500 μl cell lytic and glass beads (Sigma). Protein concentrations were measured using a standard BCA kit (Thermo Scientific) and equal concentrations of each lysate sample was loaded onto a 10% SDS-PAGE gel and transferred onto a PVDF membrane. Msn2-GFP fusion proteins or α-tubulin were detected using anti-GFP (Roche) or anti-α-tubulin (Sigma) antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies (Jackson). Immunoreactive bands were identified by chemiluminescence (Thermo Super Signal West Fento substrate) and analyzed using a Fujifilm LAS-3000 science imaging system.

Yeast one hybrid (Y1H) analysis

Plasmids expressing Msn2 motif B (residues 255-271), Msn2-3DA motif B or Msn2 Bades 1-50 fused to GAL4-BD were generated by homologous recombination to the pBD-GAL4 Cam plasmid (Stratagene). The resulting plasmids were transformed into the YRG-2 strain (Matα ura3-52 his3-200 ade2-101) by transformation. Total RNA was isolated using a MasterPure Yeast RNA Purification Kit (Epicentre) and reverse transcription was performed with RevertAid reverse transcriptase (Fermentas) and oligo dT primers, using 1.5 μg of each RNA. Quantitative PCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems), using specific primers directed against HSP12, PCNI and ACT1 as an internal control. Samples were run on the 7300 Real Time PCR System (Applied Biosystems) under standard PCR conditions. Data was analyzed with the 7300 System SDS Software.

Viability assay

The different strains were grown overnight in 5 ml of SC medium and then diluted 1:50 to OD600=0.1 and grown for an additional 6 hours at 30˚C to OD600=0.4. Oxidative stress was induced by adding H2O2 (3 mM, final concentration) to the cultures. For heat stress, the temperature was shifted from 30˚C to 49˚C. An aliquot of 70 μl of each strain was taken 0, 20, 40 and 60 minutes after stress application, diluted 1:1000, 1:1000 and 1:500 and then 70 μl of each dilution was plated onto YPD plates. The plates were incubated for 24 hours at both 30˚C and 30˚C and colonies were counted and averaged. The viability of each strain was calculated as the ratio of the number of colonies at the different time points to the number of colonies on the reference plates (i.e. time 0). The survival of each strain was normalized to that of the msn4-deleted strain at the same time point for assessment of the effect of deletion on yeast stress survival.

ELISA for the analysis of Msn2 expression

ELISA plates (Greiner Microlon 96W) were coated with 0.2 μg/ml streptavidin (Pierce) and 0.1 μg/ml of a 700 bp HSP12 promoter fragment containing Msn2-binding sites amplified with 5′ biotinylated primer. The plates were then washed with PBS supplemented with 0.05% Tween-80 (PBST) and blocked by incubation with 100 μl of PBS supplemented with 3% skimmed milk for 1 hour. Yeast cell extracts were generated from 0.5 l of logarithmic cultures using conventional methods. Briefly, cell pellets were lysed with Cell Lytic (Sigma) supplemented with protease inhibitors (Sigma) and glass beads. Following centrifugation, cell extracts were collected and protein concentration was determined by the BCA method. Following DNA coating, the plates were incubated with the cleared lysate at a concentration of 2 mg/ml of total protein and shaken at room temperature for 1 hour. Plates were then washed with PBS and each well was incubated with mouse anti-GFP-tag antibodies (Roche) diluted by a factor of 1:2000 and then with secondary HRP-conjugated goat anti-mouse antibodies (Jackson, 1:2000). The HRP chromogenic TMB substrate solution (Dako) was added and the reaction was stopped by the addition of 1 ml of 1 M sulfuric acid and recorded at 450 nm using a Tecan Infinite M200 plate reader. Values represent averages of at least three independent repeats.

Split-ubiquitin assays

Ten-fold serial dilution of W303 cells expressing the Nα fusions to the different Msn2 variants and Gal11-Cα-RUra3p fusions were spotted onto control plates lacking leucine and tryptophan. These strains were also spotted onto plates lacking leucine, tryptophan and uracil, to detect Msn2-Gal11 interactions, which were indicated by lack of growth, as the interaction leads to the degradation of the RUra3p reporter.

MSN2 localization analysis

Strains expressing a GFP-tagged protein were grown overnight in SC medium with 2% glucose. The strains were diluted to OD600=0.1 and grown for 4 hours at 30˚C. The strains were then diluted to an O.D. of 0.2 and 100 μl of cell suspension were added to each well of a 96-well glass-bottomed plate and allowed to settle for 10 minutes. Fluorescent images were collected in four separate fields using a Olympus IX81 Scan system fitted with a CAM-ORCA-R2 camera and a 40× objective, NA 0.90, short working distance. Following collection of images of the samples, 100 μl of 1.0 M NaCl (final concentration of 0.5 M) or 100 μl of 14% ethanol (final concentration of 7%) were added in synthetic medium with 2% glucose to the cells and fluorescent images were collected.

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References


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Fig. S1. Computational prediction of unstructured regions in Msn2 (residues 1-300) using the IUPred (red) web server, metaPrDOS (blue) and POODLE-S (gold). The predictor score is plotted against the residue number. The threshold is 0.5 and residues with higher and lower scores are considered as being found in disordered and ordered regions, respectively. Two regions that are predicted to be structured are highlighted in turquoise and termed motif A (residue 1-50) and motif B (residue 253-267).
**Fig. S2.** Helical wheel representation of the motif B amino acid sequence derived from *S. cerevisiae* Msn2 (ScMsn2, A) and *C. glabrata* Msn2 (CgMsn2, B). The helical wheel representation shows the amphipathic nature of the helices which consist of hydrophobic, non-polar residues on one side of the helical cylinder and hydrophilic and polar residues on the other side. The coloring key for the amino acids is: Blue - polar uncharged, purple - polar charged, green- histidine and gold - hydrophobic. The helical wheel representation was generated using [http://www-nmr.cabm.rutgers.edu/bioinformatics/Proteomic_tools/Helical_wheel/](http://www-nmr.cabm.rutgers.edu/bioinformatics/Proteomic_tools/Helical_wheel/).
**Fig. S3.** Computational prediction of unstructured regions in Msn4 using the IUPred web server. Motif B, predicted to be structured, is highlighted by a circle (A). Sequence alignment of motif B (Fig. 3) in Msn2 and Msn4 from several yeast species, including *S. cerevisiae* (Sc), *S. mikatae* (Sm), *S. paradoxus* (Sp), *S. bayanus* (Sb) and *C. glabrata* (Cg), shows the high conservation of this region between Msn2 and Msn4 (B).
Fig. S4. The effect of deleting *MSN2, MSN4* or both genes on *HSP12* expression. Hsp12-GFP expression levels in the different single or double deletion strains following exposure to multiple stress conditions. Reporter gene expression levels were normalized to that of the wild type strain.
Fig. S5. Analysis of the activity of Msn2-truncated variants by flow cytometry measurement of Hsp12-GFP reporter gene expression. All truncated Msn2 proteins were generated on the background of the msn4-deleted strain. Hsp12-GFP expression levels in the different strains were measured following yeast exposure to oxidative stress (0.6 mM of H$_2$O$_2$, A) or heat stress (temperature shift from 30°C to 37°C, B). Expression of Hsp12-GFP in the absence of stress is depicted in blue and the increase in expression following exposure to stress is depicted in red. Values of Hsp12-GFP expression were normalized, relative to Msn2 expression (Fig. S6). Asterisks indicate significant differences of Hsp12-GFP expression, relative to the WT or the higher molecular weight Msn2 construct, as determined by Student’s $t$-test (P<0.05). Student’s $t$-tests for all data were performed using the appropriate Excel function for comparing two samples by one-tailed $t$-test with unequal variance.
Fig. S6. Examination of N-terminally-truncated Msn2 variants and the Msn2 3DA mutant (Figs 2, 3). (A) The activity of Msn2-truncated variants was analyzed by flow cytometry measurement of Tps2-GFP reporter gene expression. All truncated Msn2 proteins were generated on the background of the \textit{msn4}-deleted strain. Tps2-GFP expression levels in the different strains were measured following yeast exposure to three different stress conditions, including oxidative stress (0.6 mM H$_2$O$_2$), osmotic stress (0.5 M NaCl) and heat stress (temperature shift from 30°C to 37°C) and normalized to the levels seen in the \textit{msn4}-deleted strain. (B) Analysis of mRNA expression levels in the WT, Msn2 3DA and \textit{MSN2}-deleted strains using real-time PCR. All experiments were performed in triplicate and results are normalized to WT expression levels.
Fig. S7. Analysis of the expression of the different Msn2 truncations and the Msn2-3DA mutant. (A) Western blot analysis of GFP-tagged Msn2, indicating the expression of Msn2 and the differences in molecular weight of the Msn2-truncated proteins (abbreviations: 3DA-Msn2-3DA, B-Msn2-B lacking the first 50 residues, C-Msn2-C lacking the first 250 residues, D-Msn2-D lacking the first 260 residues and E-Msn2-E lacking the first 300 residues). (B) Flow cytometry measurements of the levels of Msn2-GFP fluorescence in strains containing the different Msn2 mutants. Experiments were performed in triplicate and the level of background fluorescence of cells that do not express GFP was subtracted in each case. The background signal was 34 ± 1 AFU (C) Schematic illustration of the ELISA used to detect the relative expression of the Msn2-containing strains. (D) The ELISA signal for the WT Msn2 and the 3DA mutant. Signals at 450 nm are presented following subtraction of the background signal from cells that do not express tagged Msn2 (see Materials and Methods for a detailed description). The background signal was 0.3 OD. The values shown indicate similar expression levels and represent averages of at least 3 independent repeats.
Fig. S8. Deletion of motif A (residues 1-50, *msn2-B*) and mutation of three aspartic acid residues to alanines in motif B (*msn2-3DA*) has an additive effect on Msn2 transcriptional activity. The activity of Msn2 variants was analyzed by flow cytometry measurement of Hsp12-GFP reporter gene expression. All Msn2 variants were generated on the background of the *msn4*-deleted strain (WT*). Hsp12-GFP expression levels in the different strains were measured following yeast exposure to three different stress conditions, including oxidative stress (0.6 mM H₂O₂), osmotic stress (0.5 M NaCl) and heat stress (temperature shift from 30°C to 37°C) and normalized to the level seen with the *msn4*-deleted strain (WT*) strain.
Fig. S9. Yeast single hybrid (Y1H) analysis showing that motif A (composed of Msn2 residues 1-50) and motif B (composed of Msn2 residues 258-270) are independently able to promote the transcription of the \textit{HIS3} gene. Motif A, motif B and the motif B-3DA mutant were fused to the Gal4 DNA-binding domain (BD). Ten-fold serial dilutions of cells expressing the different motifs fused to BD were spotted onto plates lacking tryptophan (left) or lacking tryptophan and histidine (right) and incubated for three days. Activation of transcription by the motifs leads to expression of the \textit{HIS3} gene, allowing for growth on selective agar plates lacking tryptophan and histidine.
Fig. S10. Computational prediction of unstructured regions in Msn2 from different yeast species using the IUPred web server (see text for details). The computational prediction was performed for Msn2 from *S. cerevisiae* (A), *S. paradoxus* (B), *S. mikatae* (C), *C. glabrata* (D), *K. lactis* (E) and *A. gossypii* (F). The predictor score is plotted against the residue number. The threshold is 0.5 and residues with higher and lower scores are considered as being found in disordered and ordered regions, respectively. Two regions that are predicted to be structured are circled and termed motif A (residue 1-50) and motif B (residue 253-267); *S. cerevisiae* Msn2 numbering.
Fig. S11. The addition of 1-100 residues of ScMsn2 to CgMsn2 leads to increased CgMsn2 transcriptional activity in *S. cerevisiae*. Msn2 variants activity was analyzed by flow cytometry measurement of Hsp12-GFP reporter gene expression. All Msn2 variants were generated on the background of the *msn4*-deleted strain (WT*). The Hsp12-GFP expression levels in the different strains were measured following yeast exposure to three different stress conditions, including oxidative stress (0.6 mM H$_2$O$_2$), osmotic stress (0.5 M NaCl) and heat stress (temperature shift from 30°C to 37°C) and normalized to the level obtained by the *msn4*-deleted strain (WT*) strain.
Fig. S12. Aspartic acid to alanine mutations in Msn2 motif B (Msn2-3DA) significantly reduce Msn2-GFP nuclear localization levels and retention in *S. cerevisiae*. Distribution of the wild type and mutated Msn2-GFP fusion proteins from *S. cerevisiae* and *C. glabrata* was examined in the absence and following 1, 15 and 29 minutes of stress. To induce stress, cells were exposed to media containing 0.5 M NaCl.
Aspartic acid-to-alanine mutations in Msn2 motif B (Msn2-3DA) significantly reduce Msn2-GFP nuclear localization levels and retention in *S. cerevisiae*. In contrast, truncation of Motif A, located at amino acids 1-50 (Msn2-B, Fig. 2) does not significantly reduce Msn2-GFP nuclear localization. Distribution of the wild type and mutated Msn2-GFP fusion proteins was examined in the absence and following 1, 15 and 29 minutes of applied stress. To induce stress conditions, the cells were exposed to 7.5% ethanol in the medium. To mark yeast nuclei, a plasmid encoding mCherry fused to a nuclear localization signal (NLS) was expressed under the control of a constitutive *S. cerevisiae* promoter.
Fig. S14. Msn2-GFP nuclear localization and retention is reduced in a gal11-deleted strain, relative to the parental strain (A,B). The nuclear localization levels of the Msn2-3DA-GFP mutant on the background of a gal11-deleted strain are further reduced, relative to the parental strain following stress (B–D). Distribution of the wild type and mutated Msn2-GFP fusion proteins was examined in the different background strains in the absence and following 1, 15 and 29 minutes of stress. To induce stress, cells were exposed to media containing 7.5% ethanol.
Table S1. List of strains used in this study.

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