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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Differential Activation of Homologous Transcription Factors Diversifies Stress Mediated Gene Expression Programs in *S. cerevisiae*

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Joydeb Sinha

Committee in charge:

Professor Nan Hao, Chair
Professor James Kadonaga
Professor Roy Wollman

2016

The Thesis of Joydeb Sinha is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2016

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Material in this thesis, in part, is currently being prepared for submission for publication. AkhavanAghdam, Zohreh; Sinha, Joydeb; Tabbaa, Omar P.; Hao, Nan. Joydeb Sinha was a primary co-author of this work.

ABSTRACT OF THE THESIS

Differential Activation of Homologous Transcription Factors Diversifies Stress Mediated Gene Expression Programs in *S. cerevisiae*

by

Joydeb Sinha

Master of Science in Biology

University of California, San Diego, 2016

Professor Nan Hao, Chair

Homolog transcription factors have emerged from genome duplication events in evolutionary history, resulting in the co-existence of many similar proteomic pairs in eukaryotic cells. Although a large number of homologs have diversified over time to obtain unique roles from their partners, others have remained relatively conserved, limiting their downstream interactions to identical target genes. The functional advantages arising from the retention of such redundancy in the proteome have remained elusive in the context of biological design. Here, using quantitative single-cell imaging and microfluidics, we find

that the yeast general stress response transcription factor Msn2 and its seemingly redundant homolog Msn4 play distinct roles in mediating the gene expression in response to environmental stressors, which is contrary to previous belief. We observe at the single cell level, that although Msn4 exhibits identical translocation dynamics to Msn2, the level of its nuclear translocation during stress is significantly more heterogeneous than that of Msn2. As a result, while target genes with fast kinetics are expressed in most of the cells, slower target genes are induced only in the fraction of cells with high Msn4 activity. We further show that this Msn4-dependent diversification in the gene expression program in single cells is important for the adaptive response to environmental stresses.

INTRODUCTION

In order for a cell to thrive in a complex and dynamic environment it must be able to efficiently transmit enough information from its extracellular environment to the nucleus, where the information is processed, to elicit an appropriate response. For example, during environmental stress, cells will undergo a global change in gene expression programs to promote survival and adaptation in the specific suboptimal condition it is faced with (Berry and Gasch, 2008). Although much of the intricacies of cellular decision making still remain elusive, recent research seems to indicate cells, at least in part, rely on transcription factor dynamics as a means of encoding information about cues in their surroundings, and potentially alleviating signaling bottlenecks that may emerge from routing large amounts of information through a handful of conserved signaling nodes (Purvis and Lahav, 2013; Selimkhanov et al. 2014).

In many biological systems, the dynamics of transcription factor translocation seems to be a key driver of specific cellular responses. For example, in mammalian cells, a pulsatile nuclear localization of p53 results in cell cycle arrest and DNA damage response, whereas sustained nuclear localization of p53 results in the activation of downstream pathways leading to senescence and apoptosis (Purvis et al. 2012). More specifically, it has been found that cells can encode some environmental information, such as the identity and severity of a stress, by controlling the temporal dynamics of transcription factors (TFs) (Behar and Hoffmann, 2010; Hao and O'Shea, 2012; Hansen and O'Shea, 2013). Interestingly a recent proteome wide study in budding yeast has revealed that many transcription factors that exhibit pulsatile translocation dynamics also happen to exist as pairs or homologs**,** suggesting potential mechanisms by which the coordinated dynamics of two distinct yet similar TFs may serve functional roles in information processing as well as controlling many facets of cell fate (Dalal, et al. 2014; Lin et al. 2015).

In *Saccharomyces cerevisiae*, homolog TFs are thought to have arisen during a whole-genome duplication in history, and many have been maintained by natural selection (Kellis et al. 2004). Although many diverse classes of homolog TFs exist, the particular ones of interest in our study are those which contain similar DNA binding domains leading to the binding of identical target genes as well as shared

upstream regulation and are thus thought to be seemingly redundant. Previous studies of seemingly redundant homolog TFs have focused on comparing their relative expression levels in the steady state using genome sequencing or microarray techniques (Singh and Hannenhalli, 2008). It has been consistently noted that many of these redundant homolog TFs exhibit expression patterns where one homolog gene is expressed significantly higher than the other suggesting that cells may have evolved these homolog TFs as a way to fine-tune dosage (or concentration of cellular TFs), which can have implications in how well cells route and decode extracellular information transmitted through signaling nodes (e.g. PKA) (Lan and Pritchard, 2015).

Here we are primarily interested in investigating how changes in homolog TF dosage reshape the ability of cells to encode information from a signaling node to specific target genes. As a model system we use the budding yeast, *Saccharomyces cerevisiae* and its homolog stress response TFs, Msn2 and Msn4 (Msn2/4), which are both modulated by a common upstream PKA node. It is known that in the absence of stress, PKA activity is high, resulting in the maintenance of phosphorylated Msn2/4 and its consequent localization in the cytoplasm. However, when cells are exposed to a wide variety of stressors, or experience suboptimal environmental conditions, PKA activity is significantly inhibited, resulting in dephosphorylation of Msn2/4 and its subsequent translocation to the nucleus. In the nucleus, Msn2/4 binds to specific motifs known as stress response elements (STREs) in its target promoters and induces gene expression to adapt to suboptimal conditions (Martinez-Pastor et al. 1996; Gorner et al. 1998).

Previous studies have shown that Msn2 encodes stress identity and intensity of stressors with different dynamic schemes. For example, in response to osmotic stress, increasing stress intensity leads to an increase in the duration of time Msn2 resides in the nucleus without changing the amplitude of nuclear translocation, whereas in response to oxidative stress, increasing stress intensity leads to an increase in nuclear Msn2 concentration, but not duration of Msn2 nuclear localization. It has also been found that the Msn2 translocation dynamics shape the gene expression profiles of target genes differently based on the gene's respective promoter kinetics, which characterize a promoter based on its activation time

corresponding to various TF inputs (Hao and O'Shea, 2012; Hansen and O'Shea, 2013). While Msn2/4 target genes with fast kinetic promoters can effectively respond to transient inputs of TF, genes with slow kinetic promoters inherently filter out these inputs due to their slow activation times. These types of promoter activation kinetics are result of differing promoter architectures including unique arrangements of TF binding motifs and nucleosomes (Hansen and O'Shea, 2015a). As most previous studies have just focused on Msn2, the functional role of its homolog Msn4 is still poorly understood in the context of encoding and decoding stress signals. Thus, we aim to compare the Msn2/4 translocation dynamics in response to natural stresses and determine how these seemingly redundant transcription factors reshape the gene expression profiles of target promoters with distinct kinetic properties.

RESULTS

Msn4 is required for the activation of slow kinetic genes on physiologically relevant timescales

To study the combinatorial gene regulation by Msn2 and Msn4, we genetically engineered yeast strains to observe the dynamics of both TFs simultaneously in single cells. To this end, Msn2 and Msn4 were C-terminally tagged with fluorescent proteins mCherry (RFP) and mCitrine-V163A (YFP) respectively so nuclear localization in response to stressors could be quantified at the single cell level with live time-lapse microscopy in our microfluidics device **(Figure 1A).** Our strains also contained analog sensitive mutations on all three isoforms of Protein Kinase A (PKA), so a previously developed chemical genetics approach could be utilized to artificially perturb our system using a small cell permeable inhibitor molecule, 1-NM-PP1 to systematically control nuclear localization of Msn2/4 with micro-molar concentrations (Hao et al. 2013; Hao and O'Shea 2012). This drug can specifically and reversibly inhibit PKA and therefore be used to artificially induce nuclear localization of the TFs through temporal control of Msn2/4 phosphorylation **(Figure 1B).**

In order to investigate the role of each homolog, it was necessary to decouple Msn2 and Msn4 from one another before looking at downstream gene expression since their targets are shared. Thus we created knockout strains lacking either Msn2 or Msn4 by replacement of the open reading frame of the gene with a URA3 nutritional marker. We then transformed these knockout strains with a cyan fluorescent protein (CFP) reporter, so promoter activation from Msn2/4 specific genes could be monitored downstream at the single cell level as a result of transcription factor translocation **(Figure 2A).** As a proxy for gene activation we chose to look at expression of our reporter from both the DCS2 promoter (pDCS2) and the SIP18 promoter (pSIP18). We chose these promoters in particular, not only because these two candidates are specific targets of our transcription factor pair, but also because their distinct promoters kinetics have been well characterized in the context of the Msn2 system, making them canonical choices (Hansen and O'Shea, 2013). For a given TF input, the pDCS2 promoter can be activated 5 times faster when compared to the timescales of pSIP18 (Hansen and O'Shea 2013). Thus in this study we consider pSIP18 a slow kinetic promoter and pDCS2 a fast kinetic promoter respectively.

Using our chemical genetics approach, we controlled the translocation of Msn2/4 in wild type (WT) and knockout strains. Our experiment was set up to keep a fixed concentration of inhibitor (3M) and change the duration of time cells were exposed to the inhibitor, effectively varying the nuclear localization time Msn2/4. Using our microfluidics device, we flowed media for the first five minutes to obtain a fluorescence background baseline, before exposing cells to different durations of inhibitor. Single cells were imaged using time-lapse fluorescence microscopy and subsequent gene expression was monitored over the course of 3 hours. Surprisingly, based on single cell distributions of our reporter induction we found differing roles of Msn4 depending on the target gene promoter kinetics

We observed that given a 30min pulse of our inhibitor treatment, pDCS2 gene expression was not significantly hindered by deletion of either Msn2 or Msn4 when compared to that of WT cells **(Figure 2B).** In other words, the presence of either homolog TF was sufficient to fully activate the fast kinetic promoter within physiologically significant timescales (30mins of nuclear TF). In contrast, given a similar 30 min input of inhibitor, we found that the full induction of the slow kinetic pSIP18 required both Msn2 and Msn4; a single deletion of either homolog abolishes gene expression all together **(Figure 2C)**. This suggests that while Msn4 is redundant for gene expression from fast kinetic pDCS2 within the tested timescales, it plays an independent role from Msn2 in the activation of slow kinetic promoters and is specifically required to activate pDCS2. Surprisingly, we note that given a long enough duration (1-hour of nuclear localization), the Msn4 deletion strains appear to regain their ability to activate the slow kinetic gene to a similar degree to that of WT strains. Thus it appears that Msn4 plays a non-redundant role in gene expression during the physiologically relevant time window and appears to shift the timescales of gene activation of the slow kinetic pSIP18 to enable activation with a shorter duration of input.

To determine whether our results are a direct consequence of promoter kinetics, we utilized a strain with a mutated fast kinetic promoter (pSIP18-A4) through rearrangement and introduction of STRE sites upstream of the TATA box. This mutation converts the WT slow kinetic pSIP18 into a fast kinetic promoter (Anders and O'Shea, 2015a). In our pSIP18-A4 strain we saw strong gene expression was

restored at the 30min time point despite the deletion of Msn4 **(Figure 2D)**. Thus Msn4 appears to play an essential role in gene activation depending on promoter kinetics. As an additional control for our results we also tested another pair of previously characterized Msn2/4 specific promoters, the fast promoter DDR2 (pDDR2) and the slow kinetic promoter TKL2 (pTKL2) (Anders and O'Shea 2013). Consistent with our previous results, we found that for a 30min input duration of inhibitor, while either Msn2 or Msn4 was sufficient for full gene induction from pDDR2, both Msn2 and Msn4 were required to fully activate the slow kinetic pTKL2 **(Figure 2E)**. These findings confirm that the Msn4 dependence of slow kinetic promoter activation is not simply gene specific, but rather a general phenomenon governed by promoter kinetics.

To verify these results are relevant to cells during environmental stress response, we also exposed cells to different doses of KCL, which leads to osmotic stress as well as transient pulse of Msn2/4 localization (Hao et al., 2013; Hao and O'Shea 2012). Since osmotic stress is duration-modulated stress, the concentration for KCL was adjusted as to match the duration of our Msn2/4 input from our inhibitor experiment. A 30 min pulse of Msn2/4 induction similar to our inhibitor experiment was analogous to a continuous 0.5M KCL treatment. We found that consistent with our inhibitor experiments, while Msn4 was not required for the high level of fast kinetic pDCS2 activation, it was requirement for full induction of slow kinetic pSIP18 during KCL **(Figure 3)**. This was evident based on differences in gene expression profiles of Msn4 deletions and WT cells in 0.5M KCL. Thus our findings establish that Msn2 and Msn4 are not simply redundant homolog TFs, as their role in gene activation depends on the promoter kinetics of their target genes. In particular, slow kinetic promoters depend upon both homologs to fully induce gene expression in response to transient inputs corresponding to physiologically relevant timescales.

Msn4 exhibits a similar yet more heterogeneous nuclear translocation compared to Msn2

Having shown that Msn2 and Msn4 play non-redundant roles, we investigate possible upstream differences in induction of each factor in single cells that may account for our observations at the gene expression level. To this end, we monitor the nuclear translocation dynamics of each TF in individual cells

in response to a transient input of inhibitor in our microfluidics device. To directly compare stoichiometric amounts of Msn2 and Msn4 as a result of nuclear translocation, we performed control experiments to normalize the nuclear fluorescence intensities of YFP and RFP into "normalized a.u." **(Figure S1)**. We find that although Msn2 and Msn4 exhibit very similar nuclear translocation dynamics, at the single cell and population level, we consistently noted greater levels of heterogeneity in Msn4 induction compared to that of Msn2 during the exposure to both of natural stressors of KCL and ethanol. In other words, while Msn2 was usually induced at relatively similar degrees across all cells in the population within a given stress, nuclear levels of Msn4 were much more variable from cell to cell.

To quantify the heterogeneity in Msn4, we exposed cells to continuous inputs of either 0.25M KCL or 3% ETOH and monitored translocation of both TFs in single cells **(Figure 4A i, 4B i)**. Since Msn2 is expressed at different levels than Msn4 in our cells, we represent the standard deviation of the single cell traces scaled by the to the mean nuclear amount of each TF at the time point to visualize the variability in TF induction. We note that significantly noisy translocation of Msn4 persists in both natural stresses, suggesting cells will normally induce differential amounts of Msn4 in response to environmental stressors **(Figure 4A ii, 4B ii)**. Interestingly we also note that despite KCL being a duration modulated stress, the heterogeneity in Msn4 nuclear induction in the stress was more significant than its cell-cell differences in nuclear duration, indicating primary noise in Msn4 translocation is a result of differential amounts of nuclear Msn4 from cell to cell during stress.

We wanted to further study this heterogeneity in a systematic way, while avoiding possible crosstalk associated with natural stressors. Thus we chose to also utilize our chemical genetics approach to probe the dynamics of Msn2 and Msn4 by isolating its upstream regulation to just that from PKA (which is known to be its primary regulator). Using the inhibitor, we induced Msn2 and Msn4 translocation for 20 minutes and quantified differences in Msn2/4 nuclear translocation across cells. We found that the level of Msn4 translocation was again significantly more heterogeneous across cells than that of Msn2 when exposed to our inhibitor **(Figure 4C i, Figure 4C ii).** Taken as a whole, these time traces of Msn2 and

Msn4 induction illustrate that despite having temporally correlated translocation, levels of Msn4 nuclear induction intrinsically varies much more form cell to cell than that of its homolog during natural stress. With these results, we wanted to see whether this naturally occurring variation in Msn4 induction could generate heterogeneity at the gene expression level between individual cells.

MSN2 and MSN4 play distinct regulatory roles for activation of slow kinetic promoters

Given the differences in cell-cell variability of Msn4 translocation, we investigated whether differential activation of homologous TFs could reflect population level heterogeneity during stress mediated gene expression. Using our microfluidics setup, we exposed WT and Msn4 deleted cells to a 30-minute input of inhibitor and measured the level of single cell gene expression from our pSIP18 and pDCS2 reporters. Reporter induction and nuclear Msn2/4 levels from individual cells were monitored over the course 3 hours. To quantify single cell response in a quantitative manner, we classified all cells from our experiments into three groups based on their level of maximal level of gene expression, labeling them as "non-responders" (blue), "weak responders" (green), or "strong responders" (red) **(Figure 5).** To enable a full spectrum of TF translocation levels, various doses of inhibitors (0.25μM, 0.5μM, 0.75μM and 1μM) were used, and single cell data from all treatments were compiled together. With this, we produced a three dimensional scatter plot for each stress where single-cell levels of maximal Msn2/4 translocation were plotted on the x-y axis as a function of gene expression, which was represented based on our 3 color classification.

We find for the fast kinetic pDCS2, either an adequate amount of Msn2 or Msn4 activation can lead to gene expression in a majority of cells in a mostly dose dependent manner till saturation **(Figure 5A i)**. In contrast, for the slow kinetic pSIP18, fully induced gene expression is only limited to a fraction of cells with high levels of Msn4 **(Figure 5B i)**. Our results indicate that while Msn4 appears redundant for fast kinetic promoter activation, it appears to increase the probability of slow kinetic promoter activation given a fixed concentration of Msn2. While a sufficient amount of Msn2 induction must be present for pSIP18 activation, it appears that cells with very high levels of Msn2 do not exhibit gene expression

regardless of their level of Msn4, suggesting that there may be some level of competition involved between both Msn2 and Msn4 for promoter binding. Binning cells based on their individual Msn2 and Msn4 induction as a function of each gene shows distinctly different regulatory roles of each TF based on promoter kinetics of the target gene **(Figure 5A ii, Figure 5B ii)**. For pSIP18, Msn4 activation is strongly correlated with pSIP18 reporter expression in a graded fashion, increasing linearly within level of the TF. However, Msn2 appears to be play the role of a low threshold switch; it activates the slow promoters only after a certain amount of TF is present, but does not contribute to the level of gene expression once this threshold has been reached. In contrast, for the activation of pDCS2, both transcription factors appear to show a similar graded response with gene expression, indicating that increased nuclear induction of either TF can contribute to an overall increased level of gene expression.

To determine whether Msn4 also governs the expression of slow kinetic promoters during natural stress, in accordance with our inhibitor treatment we also generated a similar scatter plot from time-lapse experiments in which cells were exposed in our microfluidics to a continuous 3 hours of 4% ethanol or 0.5M KCL. We found that consistent with our inhibitor experiments, cells exhibiting higher levels of pSIP18 reporter expression were correlated exclusively with those with high levels of Msn4 in the same cells (strong responders). Furthermore, there was no correlation with Msn2 induction and pSIP18 expression, confirming that Msn4 solely plays the distinct role in tuning the level of gene expression in slow kinetic promoters **(Figure 6A i, Figure 6B i)**. We note a significantly higher fraction of non-responders in ethanol due to the harsh nature of the stress that may have led to translational arrest in a large subpopulation of our cells.

When cells were binned based on their Msn2 and Msn4 induction, we noticed that the level of pSIP18 expression was again, positively graded with Msn4 level while independent of Msn2 **(Figure 6A ii, Figure 6B ii)**. Thus the probability of pSIP18 induction is independent of Msn2 activity, but increases linearly with cells with moderate to high Msn4. For natural stressors, a switch like behavior for Msn2 persists, although a “basal off” state was not seen similar to what was observed during inhibitor treatment.

This was due to our natural stressors inducing Msn2 to greater levels than the inhibitor treatments used, and thus surpassing its low activation threshold. This indicates that during moderate to intense natural stress, nearly all cells induce Msn2 above the activation threshold and heterogeneity in slow kinetic gene expression is primarily governed by differences in Msn4 induction across a population.

Msn4 plays an important role during adaptive stress response

Since previous studies suggest that the primary function of Msn2 and Msn4 is to serve a preparative role so cells can adapt to environmental stressors, we investigate whether the presence of Msn4 could specifically play a physiological role in terms of enhancing stress resistance as a result of differential gene expression (Berry and Gasch, 2008; Kandror et al., 2004). To determine whether Msn4 could help cells in adapting, we chose to look specifically at the osmotic stress response, since it is a well-characterized system. In these experiments we preconditioned cells to an initial perturbation of 3μM of inhibitor for 30mins, inducing the nuclear localization of both Msn2 and Msn4. After "priming" the cells with inhibitor treatment they were exposed to a sustained osmotic stress using 0.75M KCL. We left a 1-hour break where cells were exposed to pure media (no stress) in between our inhibitor treatment and KCL treatment to facilitate possible protein synthesis as a result of Msn2/4 induction. To quantitatively measure cell adaptation during osmotic stress, we tagged a well-characterized osmolyte activated kinase, Hog1, with CFP in our stains. In response to osmotic stress, Hog1 will translocate into the nucleus, and activate stress response genes to generate glycerol in order to relieve turgor pressure in the cytosol (Muzzey et al., 2009). When the cell has balanced osmolality and has adapted to changes in salt concentration, Hog1 will either degrade or shuttle out of the nucleus, making it a commonly used in vivo proxy for osmotic stress.

We found that when WT cells were "primed" with our inhibitor treatment, they were significantly more resistant to the subsequent osmotic stress than cells that were not **(Figure 7A).** This was evident by Hog1 localization persisting for around 40% longer in cells that were not preconditioned as well as unprimed cells maintaining a higher steady-state level of Hog1 after adaptation. Thus preconditioning or priming clearly plays a physiological role in lessening the degree to which WT cells respond to a secondary

stress due to adaptation. More importantly, our results show that this effect is dependent on the presence of Msn4, since cells with a deletion of the TF exhibit a dramatically reduced priming phenotype **(Figure 7B).** In other words, when Msn4 deleted cells were preconditioned, they did not perform significantly better than their unprimed counterparts in regards to stress adaptation. In contrast, primed WT cells exhibited a much more dramatic acquired stress resistance as a result of much lower steady state levels of Hog1 and a shorter duration of its nuclear localization. Furthermore, the "basal" or unprimed values of both Hog1 duration and steady state levels do not differ significantly between our WT and Msn4 deletion strains allowing a direct comparison can be made between the two. These results underscore the physiological importance of Msn4 in the adaptive stress response.

DISCUSSION

Here we show that homolog transcription factors Msn2/4 play distinct roles in gene regulation and are not functionally redundant, as previous studies have suggested. In response to a transient pulse of stimulus, Msn2 and Msn4 respond in tandem to coordinate different gene expression programs depending on promoter kinetics of their target gene. For a fast kinetics promoters we see that both homologs contribute to the overall gene expression in a mostly dose dependent fashion and exhibit characteristics of a "rheostat", in that the amount of either factor can be tuned to express the gene. In contrast, for a slow kinetic gene, each homolog plays an independent role, where Msn2 functions as a low threshold switch to prevent any gene expression until a stress has been induced, while Msn4 acts as the rheostat to fine-tune the response and generate a diverse population of responders when a stress is present. This coupling of homologs to different gene expression programs is analogous to logic gate systems commonly found in digital circuits; slow kinetic genes behave as an AND gate, requiring the presence of both Msn2 and Msn4 for gene expression while the fast kinetic genes behave as an OR gate, becoming fully activated, with sufficient amount of either factor.

Despite both homologs exhibiting near identical dynamics to all stressors tested, we note Msn4 exhibits a significantly more noisy induction than does Msn2, although at this time we cannot conclude whether this noisy expression is a result of differential response of cells to stress of simply cell-cell variability in Msn4 protein abundance. Nonetheless, nuclear presence of both factors, play a key role in gene activation at slow kinetic promoters. A detailed biochemical analysis is required for the mechanistic understanding of how these homologs function in tandem, however we speculate one possible mechanism could involve the initial recruitment of specific chromatin remodelers by Msn2 which can essentially play the part of a low threshold switch based on nucleosome occupancy; initial Msn2 binding could lead to the opening up of the tightly packed nucleosome, characteristic of slow kinetic genes. This could subsequently be followed by a variable amount of Msn4, which facilitates the recruitment of additional chromatin modifiers that prime and stabilize the DNA region for transcription of the gene.

We believe the use of two separate transcription factors to activate shared target genes is an especially practical form of biological design, as it endows cells with the ability to diversify their response during times of stress without succumbing to the costs associated with the inherent leaky 'background' gene expression, often seen in purely noise driven systems. By having Msn2 as a low threshold switch, cells can efficiently keep slow kinetic genes under tight control until an actual environmental stress is present. We speculate many of these slow kinetic genes are resource intensive for the cell, and thus suboptimal to express unless truly called for by environmental conditions. However, simply controlling genes with a switch like response limits the degree to which heterogeneity can be generated in a population and prevents cells from bet hedging against unpredictable environmental conditions. In these conditions, it may be an advantage at the population level to induce high levels of gene expression in a subpopulation to safeguard against potentially harsh conditions, while continuing to maintain a large population of non-responders which will retain a fitness advantage if it simply a sub-lethal and transient stressor. In this regard, Msn4 introduces a second layer of regulation, by producing of a controlled form of heterogeneity at the level of cellular response allowing for the population of responders to diversify the degree to which they express slow kinetic genes. This allows for an efficient bet hedging mechanism during the first onset of stress and provides a means for population survival against rapidly changing environmental conditions.

We also show that combinatorial gene expression through Msn2/4 also plays a physiological role in adaptive stress response through shifting the timescales of slow kinetic gene activation allowing for an increased sensitivity (dynamic range) of stress response. This was evident with our KCL treatment as WT cells with both Msn2 and Msn4 could activate slow kinetic genes at lower doses of osmotic stress than our Msn4 deletion strain. In addition, more intense stressors such as ethanol or oxidative stress which lead to prolonged Msn2/4 nuclear localization also induce global translational arrest in cells, limiting the window of time in which TFs can induce gene expression programs (Ding et al., 2009; Hao and O'Shea, 2012; Shenton et al., 2006). Thus in these cases, it is critical to protect at least a subpopulation of cells by activating stress response genes during the very first onset of stress. These fractions of responding cells could be generated by cells with highly induced Msn4 to safeguard against lethal conditions and obtain

cross protection to different stresses they may encounter in the future as demonstrated by our priming experiments.

Interestingly, studies suggest Msn4 may also have unique regulators apart from the ones shared with Msn2, and may also be serve as an integration point for additional information about cellular state (Gasch et al., 2000; Reinke et al., 2006). Thus combined with our findings here, Msn4 level may very well be tuned at the single cell level and thus play a role in encoding a rudimentary form of memory through a cell's individual history to previous stressors. Given the abundance of homolog TF's in the proteome, our results may reveal a general biological design principle by which cells effectively regulate gene expression in a synergistic manner through activation of closely related TFs.

Material in this thesis, in part, is currently being prepared for submission for publication. AkhavanAghdam, Zohreh; Sinha, Joydeb; Tabbaa, Omar P.; Hao, Nan. Joydeb Sinha was a primary co-author of this work.

FIGURES

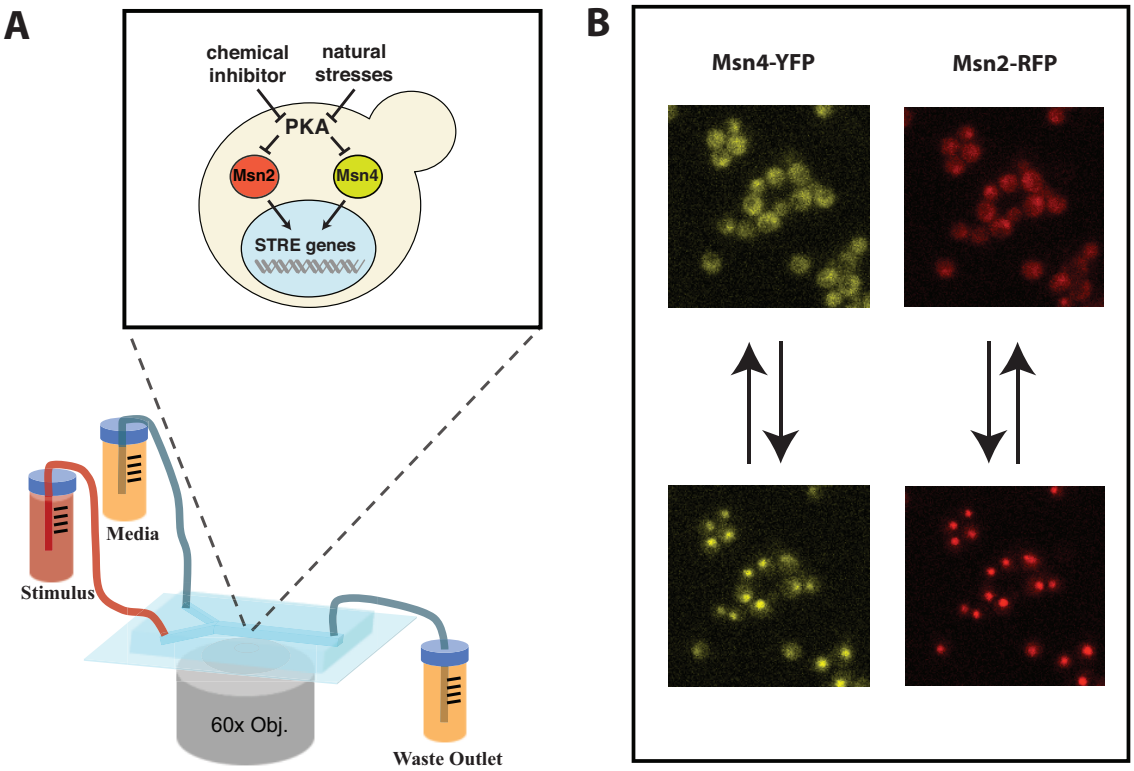


Figure 1: Experimental setup to study Msn2/4 dynamics in single cells

A schematic illustrating the experimental approach used in this project to study transcription factor dynamics in single cells. **(A)** Msn2 and Msn4 are tagged with fluorescent proteins RFP and YFP respectively and their nuclear localization is temporally controlled using a microfluidics device to administer natural stressors or 1-NM-PP1, a PKA inhibitor. **(B)** Under conditions of stress or PKA inhibition, Msn2 and Msn4 exhibit visible nuclear localization in our microfluidics device imaged using time-lapse microscopy. The localization is reversible with clearing of stress or cellular adaptation to environmental conditions.

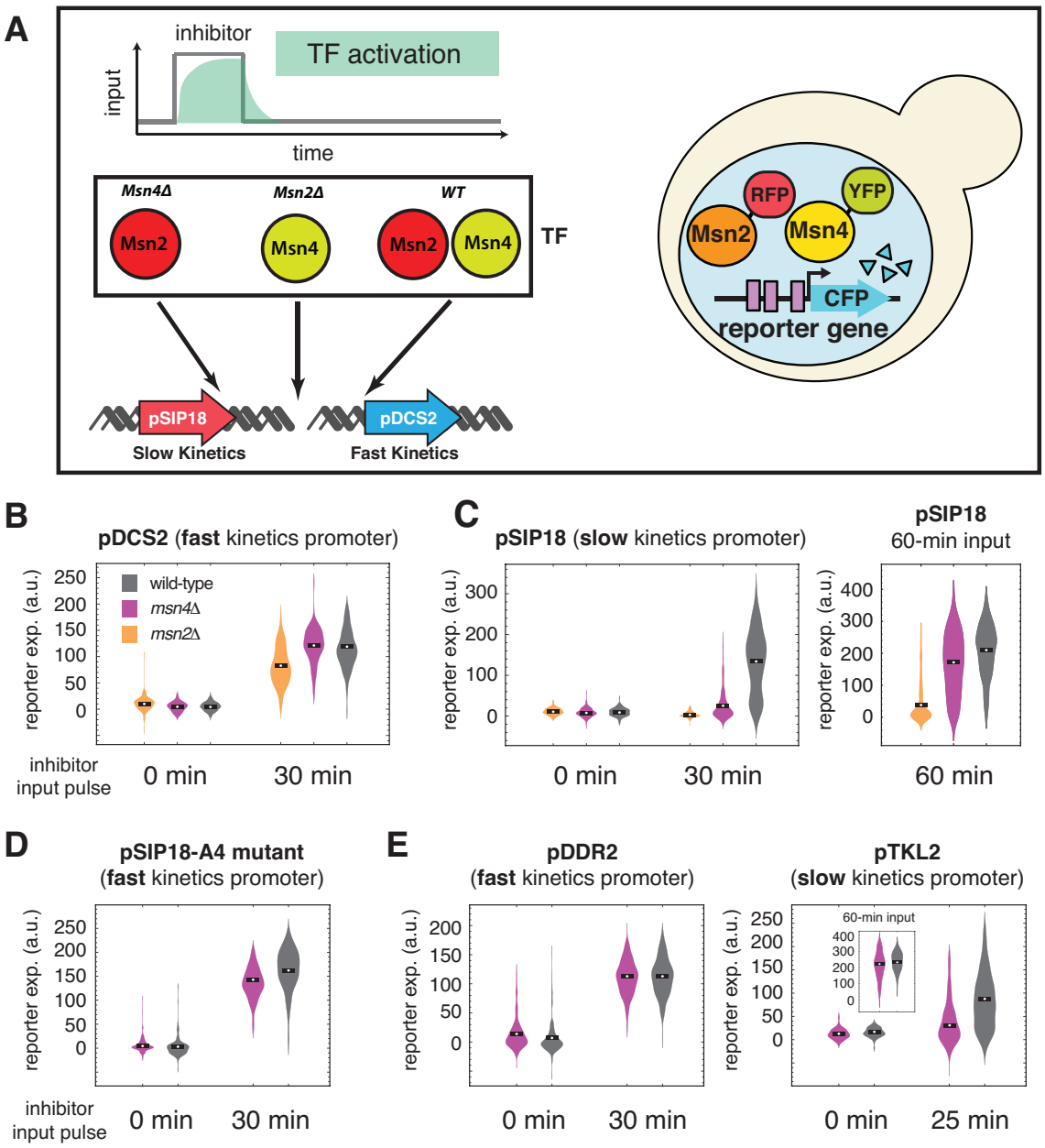


Figure 2: Msn4 shifts the timescales of slow kinetic promoter activation

(A) A diagram of the experimental approach used to systematically isolate downstream contributions of Msn2 and Msn4 to both slow and fast kinetic promoters pSIP18 and pDCS2. Each promoter drives the expression of a CFP reporter, which is used as a measure of promoter activation. **(B-E)** Violin plots showing distributions in reporter expression across cells from Msn2/4 specific target promoters in response to a transient pulse of 3μM inhibitor.

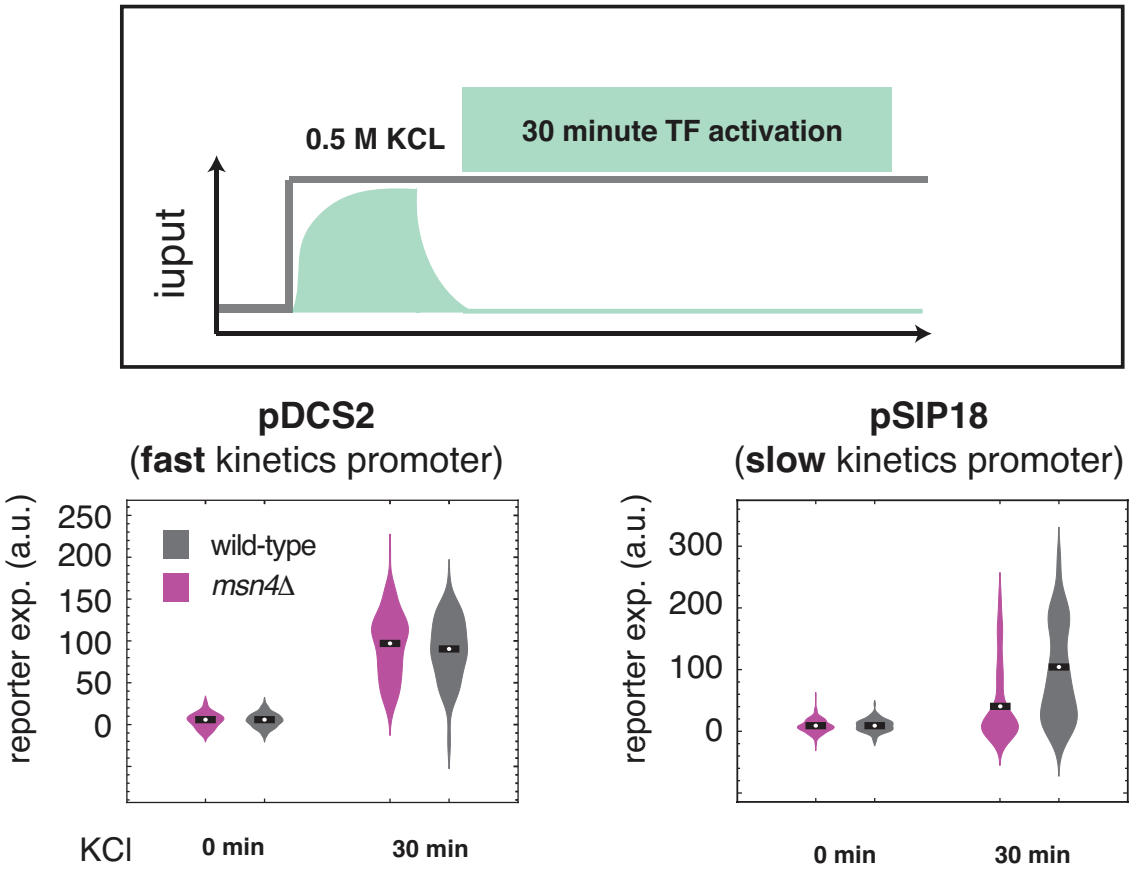


Figure 3: Msn4 is needed for slow kinetic promoter activation during osmotic stress

Violin plots illustrating the distribution of pSIP18 and pDCS2 reporter expression from single cells. Analogous to inhibitor experiments from Figure 4, a continuously administered dose of 0.5M KCL (a duration modulated natural stress) was used to induce the nuclear localization of Msn2 and Msn4 for approximately 30 minutes in both wild type and *MSN4* deleted cells.

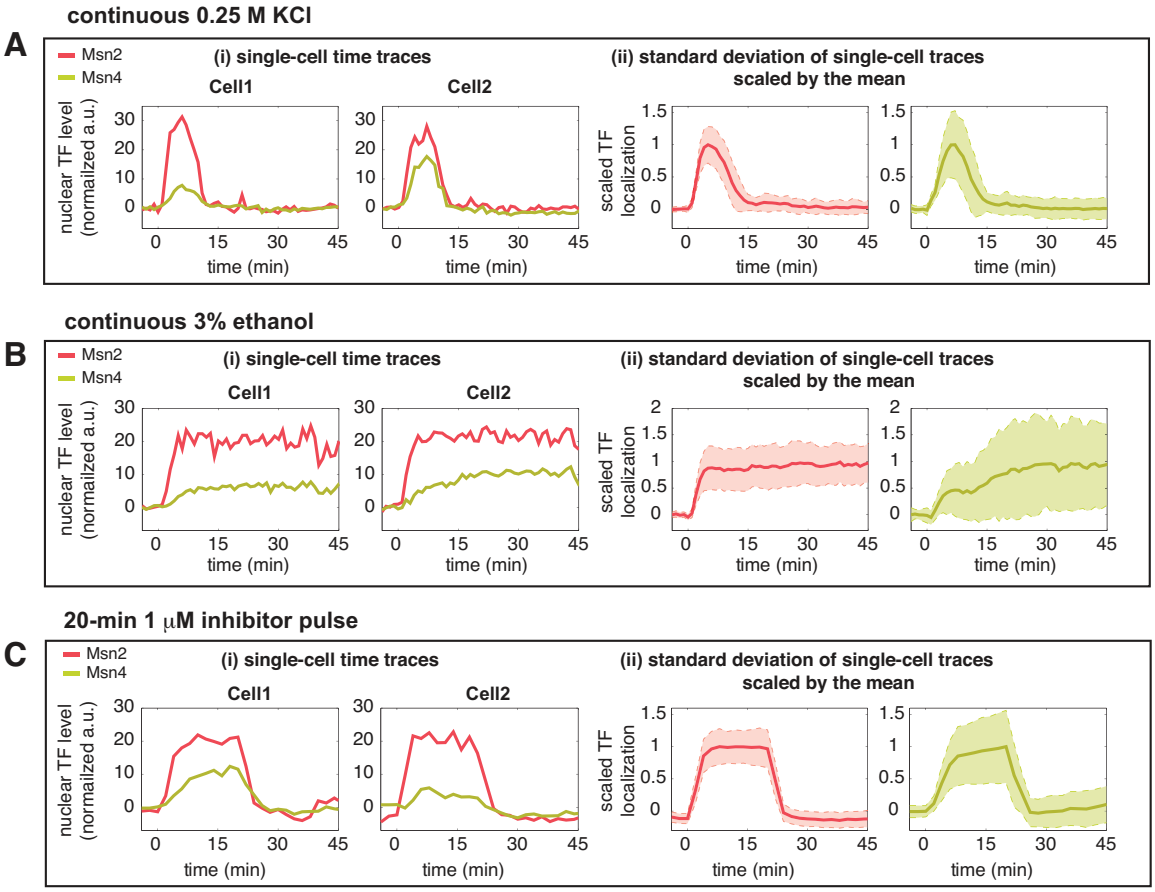


Figure 4: Msn4 translocation exhibits a greater degree of heterogeneity than that of Msn2

Single cell and mean time traces of Msn2 and Msn4 translocation in response to **(A)** 0.25M KCL, **(B)** 3% ethanol and **(C)** 20 min pulse of 1 μM inhibitor. In each panel, **(i)** representative time traces of Msn2 and Msn4 in a single cells; **(ii)** mean of the single-cell time traces scaled to its peak value (% max of mean). In **(ii),** the solid trajectory is an averaged time trace, while the shaded overlay represents the scaled standard deviation of single cell trajectories.

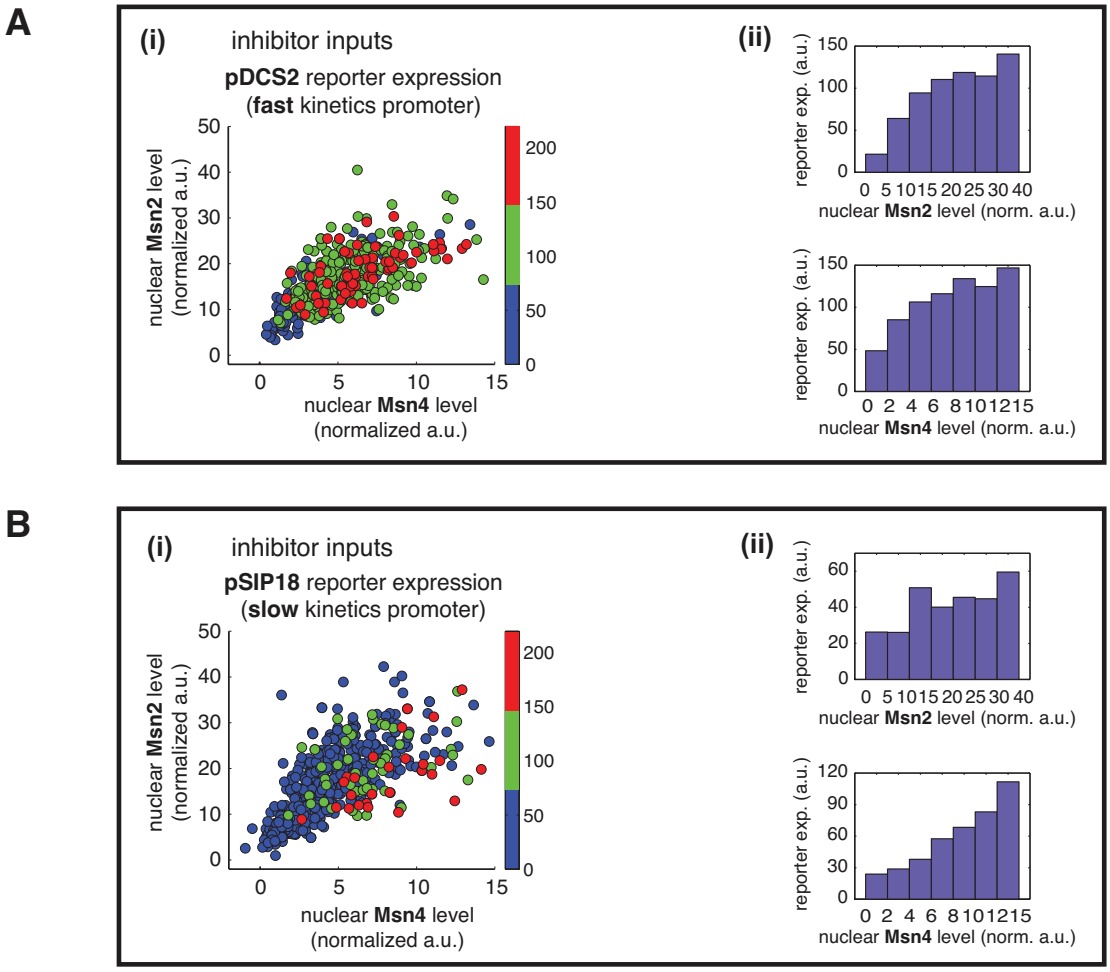


Figure 5: Regulatory role of Msn4 in slow kinetic promoter activation

Scatter plots illustrating maximal single cell Msn2 and Msn4 induction in response to various doses of inhibitor (0μM, 0.25μM, 0.5μM, 0.75μM, μM) from both **(A i)** pSIP18 and **(B i)** pDCS2. Points are color-coded based on the peak level of gene expression for a single cell. **(A ii, B ii)** Single cell data was binned based on relative induction of Msn2 or Msn4 and plotted as a function of reporter expression using bar graphs.

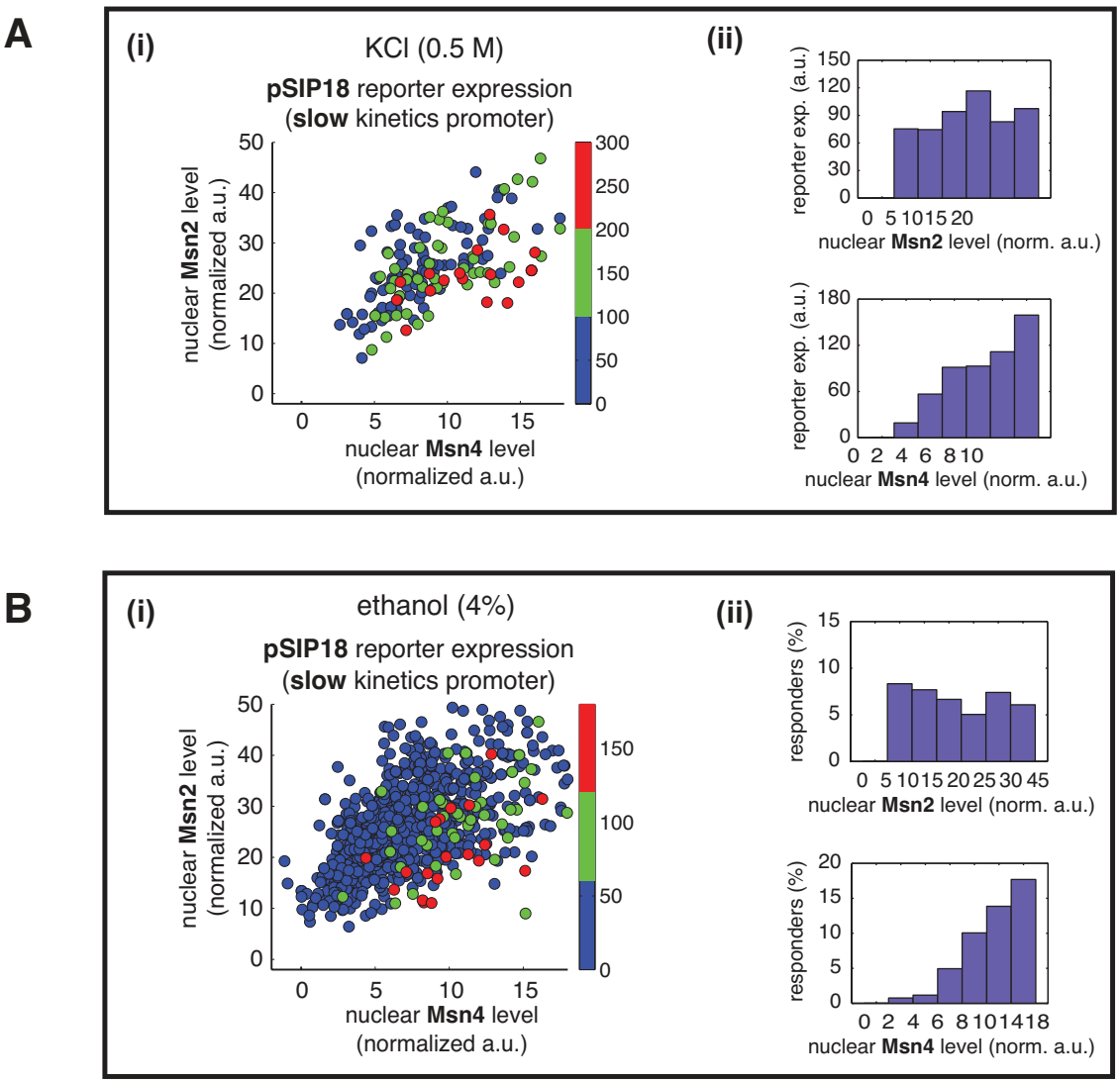


Figure 6: Msn4 activates slow kinetic promoters during natural stress

Scatter plots illustrating maximal single cell Msn2 and Msn4 induction in response to either **(A i)** 0.5M KCL or **(B i)** 4% ethanol. Points are color-coded based on peak level of pSIP18 reporter expression for a single cell in response to either stress. **(i)** Scatter plot of maximal induction of Msn2 and Msn4 on either axis as a function of gene expression. **(ii)** Binned histogram showing the relationship between either Msn2 or Msn4 and reporter genes. Single cell data was binned based on relative induction of Msn2 (upper panel) or Msn4 (lower panel) and plotted as a function of reporter expression.

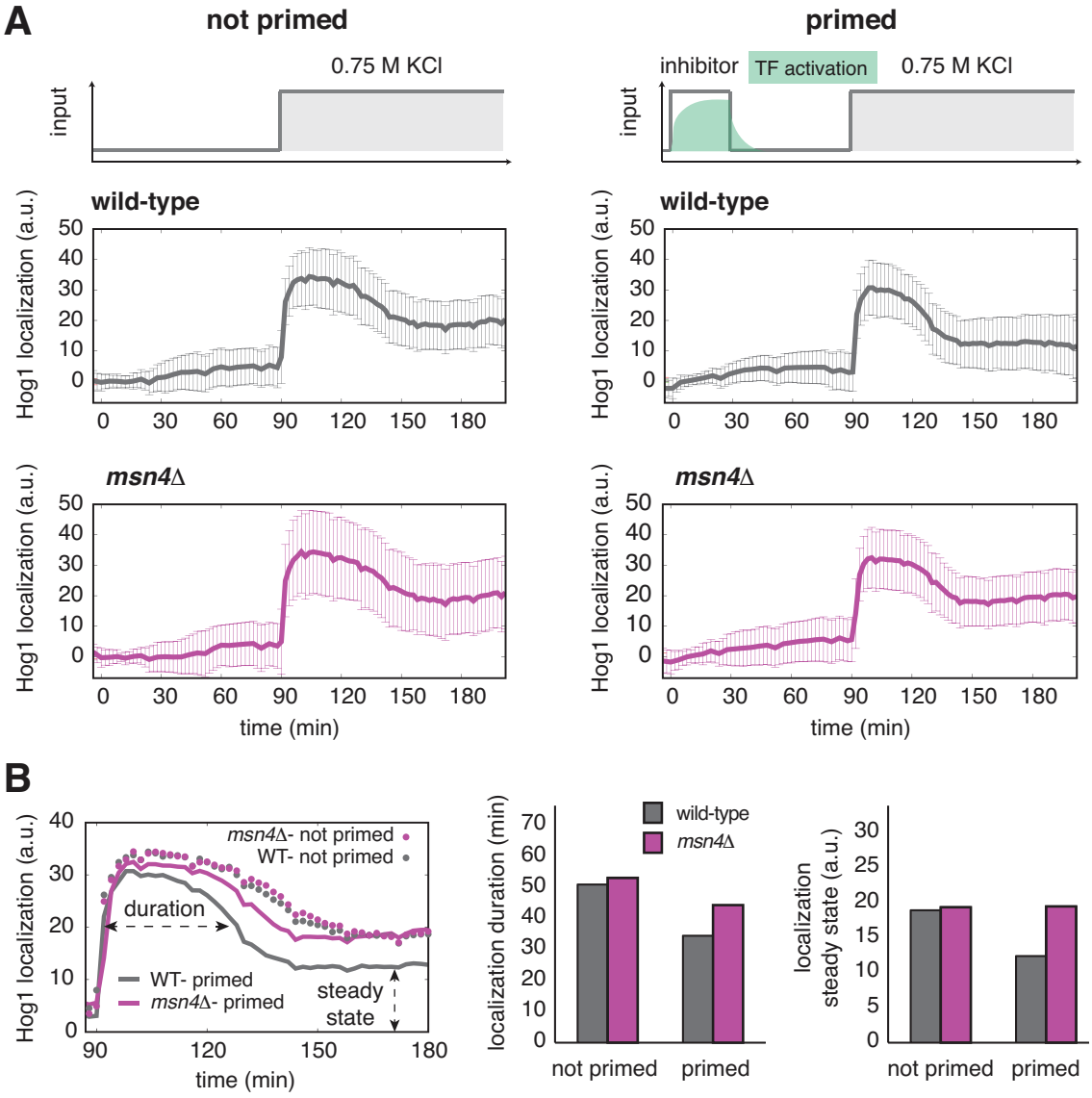


Figure 7: Msn4 plays a role in adaptive stress response

(A) Wildtype and *MSN4* deleted cells are either exposed to regular media (not primed) or pre-conditioned with a 30 minute treatment of 3μM of inhibitor before being treated with a continuous 2 hour osmotic stress of 0.75M KCL leading to the nuclear induction of Hog1 kinase (time traces). A 1-hour break was introduced in between the inhibitor removal and stress treatment to facilitate protein synthesis in cells. **(B)** Recovery from stress or adaptation for cells was quantified by measuring the mean nuclear duration and steady state levels of Hog1 of cells in each group (primed and not primed) after treatment. Results are quantified using bar graphs.

SUPPLEMENTAL FIGURES

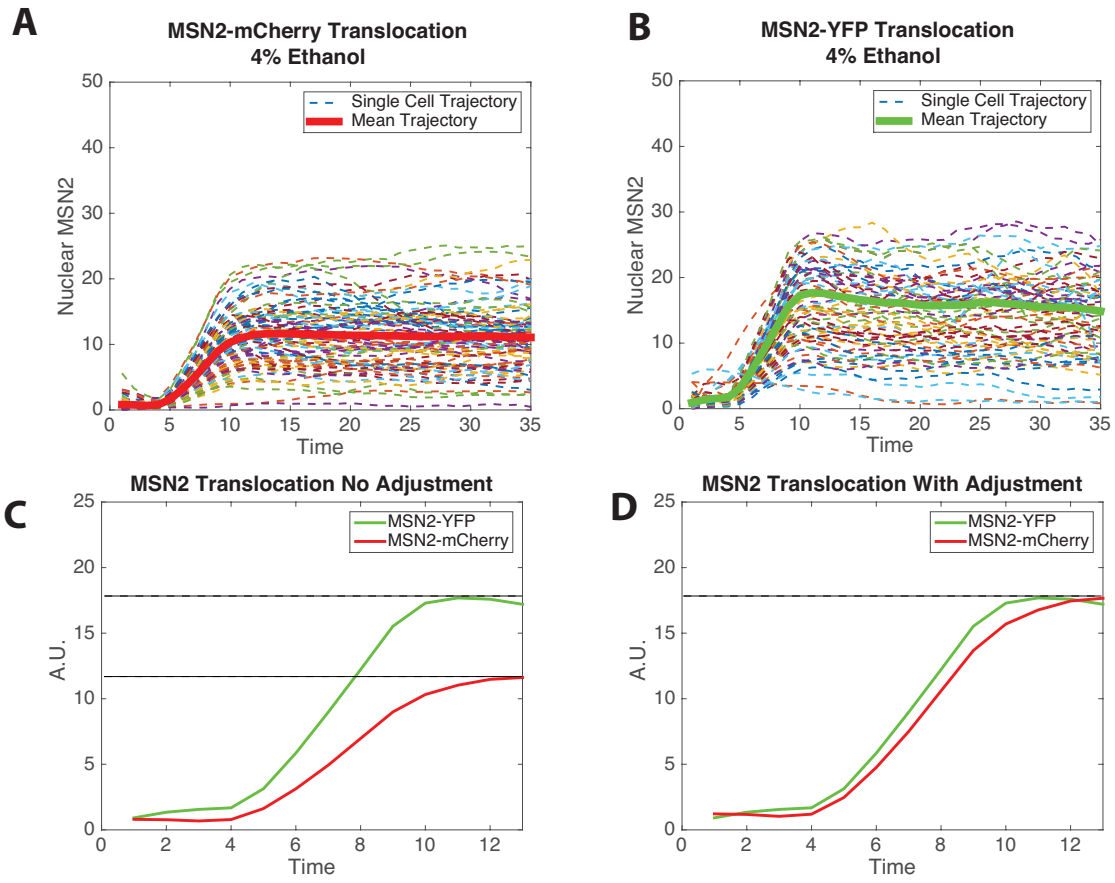


Figure S1: Normalization of fluorescence Intensities

To compare the stoichiometric ratios of Msn2 to Msn4, a conversion factor between mCherry and mCitrine (V163A) was needed to account for unique microscope settings used in each channel as well as inherent emission differences between each fluorophore. This was determined by creating two yeast strains in which Msn2 was C-terminally tagged with either florescent protein mCherry (RFP) or mCitrine-V163A (YFP) respectively. Nuclear translocation of Msn2 was induced in both stains using SD medium containing 4% ethanol, which leads to relatively high levels of sustained nuclear translocation of Msn2. **(A, B)** Cells were imaged in our microfluidics device and single cell traces of Msn2 translocation of both strains were extracted from these experiments by quantifying changes in nuclear fluorescence intensity in both YFP and mCherry channels. **(C)** A mean Msn2 translocation trace was generated for each strain by averaging the single cell nuclear Msn2 intensity at each time point over approximately 100 cells. The conversion factor was determined by taking the ratio between the maximal steady state fluorescence intensity of each mean trace, which was around 1.52. **(D)** Multiplying all fluorescence values of the mCherry channel by our conversion factor converges it with the trace obtained from the YFP channel.

MATERIALS AND METHODS

Medium and solutions

The low Fluorescence Synthetic Dextrose (SD) Media used to culture cells for microfluidics was Made up of 1.71g YNB-Folic Acid-Riboflavin Powder, 0.74 g Complete Supplemental Mixture (CSM), 2% glucose, and 5g Ammonium Sulfate per 1L of medium. Concanavalin A (Con A) (Type IV, Sigma-Aldrich) solution containing 5mM $CaCl_2$, 5mM $MnCl_2$, and 2mg/mL ConA was stored at -20°C and thawed at room temperature just before each experiment. 1-NM-PP1 was stored as a 1000x DMSO stock at -20°C and thawed and added to medium just before an experiment. KCL stress medium was made by diluting SD medium containing 0.5M KCL to the desired concentrations. Stress medium containing ethanol was made fresh just before an experiment by adding an appropriate amount of a stock 100% ethanol to the SD medium.

Microfabrication

The microfluidics device used in this study is modified from a design previously described in (Hersen et al., 2008) so that two independent experiments can be run in parallel on a single chip. The mask was designed to allow for bonding of two antiparallel Y-shaped devices on one microfluidics chip. The SU8 wafer was fabricated using standard photolithography methods as described in (Hansen et al., 2015b) to ensure a channel width of 400μm and a height of 111 μm. Microfluidic chips were made by pouring PDMS (polydimethylsiloxane) onto the wafer, and degassing the mold for one hour to remove air bubbles before being curing in an 80°C oven for a minimum of one hour to ensure proper polymerization. The PDMS was then removed from the wafer and individual chips were cut and hole punched using a Harris Uni-Core 1.00mm puncher. The chips were cleaned using first 70% ethanol and then water to remove debris before being bound to Thermo Scientific Gold Seal Cover Glasses (Rectangles; 24 x 50mm; Thickness 1.5). The cover glasses were pre-treated in sequential order with heptane, methanol, and water to ensure efficient bonding. The cover glass was then added, along with the chips (feature side facing up) into a UVO bonder for three minutes. After UV treatment, the chips were immediately placed on top of coverslips and left in an 80°C oven overnight to secure the bond.

Microfluidics

The microfluidic chip was placed in a vacuum chamber for 20 minutes before starting the experiment to evacuate any air from the channels. Once removed from the vacuum, water drops were then immediately added to each port to prevent re-introduction of any air. A solution of 2 mg/mL concanavalin A (ConA) was then injected into the channels of the chip using a 1 mL syringe inserted into an ~1.5in segment of polyethylene tubing with a 20 gauge connector. The chip was then left to incubate for roughly 15 minutes. Using a similar method, SD from a fresh syringe was then flowed into our channel used to wash off excess ConA after incubation. Yeast cells were then spun down at 3000 rpm for one minute and re-suspended in 5mL of SD. The resuspended cells were then loaded into the microfluidics channel and incubated inside the chip for 10 minutes to allow for sufficient adhesion to the glass.

While cells incubate in ConA coated channels of the device, two inlet tubes (50ml Falcon Tubes) with 20 mL of media or stimulus and one waste outlet tube with 10 mL SD were set up next to our microscope using ring stands. The tubes were set-up to allow for a 10cm height difference between the inlets and the outlet direction flow facilitated by gravity once all our tubing was connecting to the chip. Soft polyethylene tubing (inner diameter, 0.86 mm; outer diameter, 1.27 mm) was then placed into the media and a 1mL syringe was used to prime the lines so a small volume of media was siphoned through ensuring liquid was present along the entire length of tubing. Caution was taken to avoid the introduction of any air bubbles in the process. A binder clip was then used to temporarily stop the flow while a 20-gauge connector was inserted into the end of the tubing for later attachment of the line onto the microfluidics chip.

A small air bubble followed by a small volume of SD was then intentionally introduced into each channel using our syringe to remove any vertically stacked yeast and obtain a monolayer of yeast cells for imaging. The chip was then mounted onto our microscope using a specially designed holder and taped securely to the stage to prevent unwanted movement. The chip was then connected to the two media inlets first. The flow from the inlet with stress media or 1-NM-PP1 was always immediately cut off using a

binder clip allowing flow only from SD to avoid pre-stressing cells. The waste port was then connected, completing the flow circuit in our device. Tubing was subsequently taped to the stage to minimize any mechanical stress induced vibrations on the chip that could lead to spurious stage movement.

Time-lapse microscopy

All microfluidics time-lapse experiments were imaged using a Nikon Ti-E inverted fluorescence microscope coupled to a CCD camera (Andor). Images were taken at 25°C using a 60X oil immersion objective with our microfluidics chip taped to the stage of the microscope using a specially designed holder. For transcription factor dynamic experiments, three positions were chosen for each channel and the microscope was programmed to acquire Phase, YFP, mCherry, and Cy5 images every two minutes. The cells were exposed to SD media for the first five minutes to obtain a baseline fluorescence background before the media was switched to stress or 1-NM-PP1. This was done by clipping the SD media inlet and un-clipping the stress or 1-NM-PP1 media with a binder clip. For gene expression studies, six positions were chosen for each experiment and the microscope was programmed to take Cy5 and YFP or mCherry images every two minutes and both Phase and CFP images every 14 minutes for a total of three hours. Again the cells were exposed to SD media for the first five minutes and then exposed to natural stress or 1-NM-PP1 for the appropriate amount of time to obtain an appropriate baseline of fluorescence on each channel. Consistent microscope settings were used for each channel throughout all experiments. The CFP exposure time was 300ms at 9% laser, YFP exposure time was 400ms at 20% laser, mCherry exposure time was 300ms at 10% laser, and Cy5 exposure time was 200ms at 15% laser. The camera settings were EM Gain 1MHZ @16 bit and 300 gain for all four channels.

Quantitative Image Analysis

Fluorescence microscopy image stacks were pre-processed to subtract background in ImageJ. Images were then processed with a custom MATLAB code. A nuclear marker was used to track the cells by tagging Nhp6a with an infrared fluorescent protein. Cells were manually picked based on the nuclear marker profile (Cy5) for each single cell plot. All figures were generated using a combination of MATLAB

(Mathworks), Mathematica (Wolfram), and Illustrator (Adobe) software packages.

Yeast Transformation

The *Saccharomyces cerevisiae* strains used in this study are all derived from W303 ADE+ MATa trp1 leu2 ura3 his3 can1 GAL+ psi+. A standard lithium acetate based transformation method was used as described in (Geitz et al. 2007) to generate our synthetic yeast strains. DNA fragments for genomic insertion were amplified using Phusion High Fidelity DNA Polymerase and purified using Invitrogen PureLink PCR purification kit.

Fluorescent Protein Tagging

The nuclear marker for cell tracking was generated by C-terminally tagging Nhp6a with JCat yeast optimized infrared fluorescent protein (iRFP) (Grote et al., 2005). The linker-iRFP::KAN MX fragment was generated by PCR amplification from a pKT vector (pKT-iRFP-KAN MX) containing a linker sequence of GDGAGLIN (Sheff and Thorn 2004). Msn2 was C-terminally tagged with a yeast codon-optimized mCherry by replacing the endogenous stop codon of the *MSN2* locus with *URA3* and then replacing the *URA3* with a linker-mCherry PCR fragment from a pKT vector using 5-FOA. Msn4 was C-terminally tagged with a linker-mCitrineV163A PCR fragment generated from a pKT vector containing JCat optimized mCitrine with the V163A mutation to allow for fast maturation. The endogenous *MSN2* and *MSN4* terminators were left unchanged. *MSN2* and *MSN4* deletion strains (*msn2Δ* and *msn4Δ*, respectively) were made by replacing the endogenous *MSN2* or *MSN4* open reading frame (ORF) with a TRP1 nutritional marker.

Reporter gene construction

To create reporters for promoter activation, the entire endogenous ORF of the gene of interest was replaced with a PCR fragment containing JCat optimized mTurqouise2 followed by the ADH1 terminator and HIS5 selection marker generated by PCR amplification. This was performed as described previously in (Hansen and O'Shea, 2013).

Material in this thesis in this thesis, in part, is currently being prepared for submission for publication. AkhavanAghdam, Zohreh; Sinha, Joydeb; Tabbaa, Omar P.; Hao, Nan. Joydeb Sinha was a primary co-author of this work.

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