Chapter 4

SIGNAL PROCESSING ACROSS THE YEAST PROTEOME

Motivation

In our previous investigation, we observed that the yeast transcription factor Crz1 exhibits frequency-modulated bursts of nuclear localization in response to its stimulus, calcium. This behavior enables proportional regulation of Crz1 target genes in response to calcium. Because this regulatory strategy is very general, it could be employed more generally by cells in order to optimally tune expression levels of groups of promoters (regulons). Moreover, cells may employ other dynamic regulatory strategies to enable other functional behaviors. Based on these considerations, we performed a systematic screen for dynamic regulatory behaviors in yeast.

Screen Methods/Results

Since we were unable to determine which proteins would be most appropriate to observe, we decided to look at as many as we could. Taking advantage of the yeast GFP library,[23] we used multi-well time lapse microscopy to systematically search for dynamic and/or heterogeneous localization behaviors. Cells were imaged in one of 4 media conditions: Standard synthetic complete or wild-type, carbon-limited, nitrogen-limited, or high salt. The screen involved two stages: First, we acquired low time-resolution (~51 min/frame) movies of each protein in each condition. These movies were inspected visually to detect heterogeneous localization patterns in one or more media conditions. This screen suggested a few candidate genes, but, because of its low time resolution, left ambiguity in many of these cases. We classified each protein based on the likelihood that we observed real heterogeneity in any one of four conditions. Interestingly, almost all protein localization patterns were independent of condition.

In the second stage, heterogeneously localized protein strains were re-analyzed at higher time-resolution movies (~3 minutes between frames). If they still showed dynamic or heterogeneous localization patterns we examined them further in several ways:

- Focal drift exclusion: Several strains showed dynamic patterns of non-nuclear localization to regions smaller than a cell nucleus. To test for possible effects due

to 3-D diffusion of a localized spot, we re-acquired movies of these strains with multiple z-slices. In all cases, fluctuations in fluorescence intensity in these strains could be explained by movement of a localized sub-cellular spot, rather than fluctuations in the intensity of the spot (fraction of proteins localized).

- Cell cycle exclusion: We excluded proteins whose localization patterns were principally determined by cell-cycle phase. To investigate cell-cycle dependence, we grew up all the proteins on our list and synchronized the cell cycle with hydroxyurea. After releasing the synchrony , we added the appropriate stimuli to the media and took movies. (In some cases, because the hydroxyurea does not work perfectly, we also took very long movies where we could observe several cellular divisions and checked by eye to see whether there was any correlation between cellular division and burst occurrence). From these results, we were able to erase several proteins off our list.

The remaining proteins exhibited dynamic and/or heterogeneous localization dynamics not principally correlated with cell cycle. These proteins consisted almost entirely of transcription factors.

Based on these results, we undertook a final additional screen, which focused specifically on transcription factors. For each transcription factor, we recorded a series of movies with varying levels of factors or conditions known to induce its transcriptional activity. The results of the final screen are tabulated in table 1.

The validated dynamic/heterogeneous proteins consisted exclusively of transcription factors. Several conclusions emerged from this dataset: First, most transcription factors are constitutively nuclear. Second, except for a two cases that require special media conditions (alpha pheromone), our original proteome-wide screen identified 10 out of the 12 bursting proteins. Third, we observed distinct timescales of localization bursts. We found five rapid-bursting proteins (τ<30 min), and seven with slower dynamics (τ~30 min to 2 hour), most of which appear to be involved in metabolism in some way. Note that these slower proteins were not cell cycle correlated in phase. Fourth, some proteins exhibited heterogeneous patterns of nuclear localization that did not involve dynamic bursts. Rather, stimuli appear to control the fraction of cells which exhibit strong

nuclear localization. In these "fractionally heterogeneous" proteins, the number of cells with fully localized protein increases with the level of stimulus. Finally, we identified "amplitude-modulated (AM)" transcription factors, in which the fraction of total protein localized to the nucleus increased with stimulus level. We identified no additional proteins resembling Hog1, which has been shown to adapt perfectly to osmotic stress.[24]

Localization Behavior	Protein (Media Condition)
I. Fast Bursting	Crz1 (Calcium), Msn2 (salt, rapamycin, calcium, glucose deprivation), Mig1 (glucose deprivation), Dot6 (wild-type), Rtg1 (wild-type)
II. Slow Bursting (no Modulation)	Nrg2 (Glucose Deprivation), Mig2 (Glucose Deprivation), Kar4 (alpha pheromone), Tos1 (alpha pheromone), Arg81 (Arginine), Leu3 (Leucine), Tea1 (Arginine)
III. Statically Heterogeneous	Dig1 (alpha pheromone), Dig2 (alpha pheromone), Pci8 (alpha pheromone), Sch9 (salt), Ino2 (lithium acetate), Sfl1 (salt)
IV. Constitutive but Amplitude Modulated	Abf1 (glucose deprivation), Cin5 (salt), Cti6 (no glucose, galactose titration), Mcm1 (alpha pheromone), Put3 (Nitrogen deprivation) , Ste12 (alpha pheromone), Xbp1 (glucose deprivation),
V. Perfect Adaptation	Hog1 (salt)

Table 1. **Modes of Signal Processing in Yeast**. The different types of localization behavior from the transcription factor screen are summarized.

As a nice positive control, Crz1 came up in our screen; as previously stated, it exhibits FM bursts in response to calcium. Msn2 and Mig1 had also been previously identified as fast bursting proteins.[55] Msn2 is a general stress response protein that undergoes nuclear localization to a wide variety of stresses.[56,57] Our screen results suggest that Msn2 responds to several stresses similarly, with fast bursts. Mig1 is a carbon utilization transcriptional repressor[58,59] that exhibits both FM and pulse width modulation (PWM) in response to glucose deprivation; both burst duration and frequency increases as you decrease the amount of glucose. Dot6 is not a well-characterized protein, but has been implicated in transcriptional silencing.[58,59] Without any stressful stimuli, Dot6 exhibits bursts. Similarly, Rtg1, a transcription factor implicated in nitrogen stress,[58,59] bursts without any provocation.

The second class of localization behavior observed is that of the slow bursting proteins. These proteins exhibit bursts with a timescale of approximately one hour. Interestingly, all these transcription factors are involved in sensing nutrient availability but none of them show any sort of modulation. For example, Nrg2 and Mig2 are both transcriptional repressors that respond to glucose.[58,59] However, their localization appears to remain consistent across all concentrations of glucose. Similarly, Arg81, Leu3, and Tea1 respond to amino acid deprivation and Kar4 and Tos1 respond to alpha pheromone,[58,59] but the localization of all these proteins show slow bursting across various concentration of the stimulus.

At any given time during a movie of a bursting transcription factor, the heterogeneity in localization is quite striking as some cells are nuclear while others are cytoplasmic. Of course, one can imagine a far simpler mechanism to generate such heterogeneity, if only a fraction of the cells respond to the stimulus and localize. This is precisely the behavior we observe in the third class of proteins, the statically heterogeneous ones. The interesting aspect of these proteins is that their localization is modulated by stress also. As the amount of stress increases, the fraction of cells that respond via nuclear

localization also increases. These proteins, Dig1, Dig2, Ino2, Pci8, Sch9, and Sfl1, respond to various stresses including alpha pheromone, salt, and lithium acetate.[58,59]

Finally, we also observe amplitude-modulated (AM) proteins. These proteins are constitutively nuclear, but their intensity increases with stress, yielding an AM phenotype, as described in Figure 4 of Chapter 3. From the movies, it appears that the intensity increase is from direct or indirect transcriptional regulation of the transcription factor and not from increased nuclear import. The transcription factors that behave in this manner are Abf1, Cin5, Cti6, Mcm1, Put3, Ste12 and Xbp1.

In other work,[24] the localization dynamics of Hog1 have been characterized as being perfectly adapted in response to salt, i.e. upon salt stress, cells respond with a quick pulse of localization before redistributing back to their basal pre-stressed cytoplasmic state. We reconfirmed Hog1 behavior but did not find any other proteins that behaved similarly in our screen.

Integration of Multiple Bursting Regulatory Systems

These data show that multiple bursting systems are active simultaneously in the yeast cell. In at least some cases, multiple burst systems co-regulate common target genes. These observations provoke the questions of (a) how multiple burst systems combine to regulate target genes and (b) what additional signal encoding and regulation capabilities are provided by such combinatorial burst systems.

We chose to investigate Msn2 and Mig1, two bursting transcription factors that both respond to carbon deprivation. This pair has several ideal features for this analysis: (a) both factors can be induced to burst in the same conditions, (b) both factors can be induced by the same condition (glucose) allowing analysis of a combinatorial response to a single stimulus, and (c) they regulate both separate and common sets of target genes, allowing analysis of co-regulation. Interestingly, Msn2 also responds to several other stimuli, making it unique amongst the hits in our screen.

Mig1 represses genes involved in metabolism of alternate carbon sources when glucose is present.[56] At high concentrations of glucose it is constitutively nuclear while at lower concentrations, it exhibits bursting behavior. Under similar conditions, Msn2 bursts as it senses insufficient carbon in the media. In the dual-color strain, it is apparent from the

traces that both proteins can burst coincidentally (Fig. 1a- at 50 min) but there do appear to be signs of an anti-correlation (Fig reference).

This is different than what we observed in a previous experiment where we measured the cross-correlation of Msn2 and Crz1 in response to calcium.[60] Here we found two largely uncorrelated bursting systems.[60] With Msn2 and Mig1, we find two timescales of correlation. There is a positive correlation between the bursts of both proteins on the timescale of an hour; however, at shorter timescales a lack of correlation becomes evident (Fig. 1b). This lack of correlation is reproducible in several low-glucose conditions, but it is difficult to quantify its effect due to its subtle nature.

Fig. 1. **Correlations amongst Bursting Transcription Factors**. A strain tagged for both Msn2 (black) and Mig1(red) is imaged at limiting amounts of glucose (.025%). In **a**, the sample trace shows that both proteins bursts on a fast timescale. In **b**, the average cross-correlation of 27 cells shows a positive correlation with a timescale of ~ 1 hour and an anti-correlation with a timescale of ~ 1 minute.

It is unclear whether this correlation function suggests that such an interaction affects localization bursts in any meaningful way. Because there are several similarities between the two proteins: nuclear import of Msn2 and Mig1 are dephosphorylation-dependent and the cytoplasmic redistribution of these proteins is dependent upon phosphorylation by several kinases, one of which is Snf1,[58,59]any interaction between the two may simply be due to utilizing similar enzymes. Furthermore, there are several conditions in which only one of the two proteins exhibits localization bursts. In these cases, the fact that one of the proteins is not bursting does not appear to change the behavior of

the other, showing that neither protein is necessary for the other to burst nor does one protein modulate the other's burst statistics.

One possible explanation for this lack of correlation is that the export machinery used by both Msn2 and Mig1to leave the nucleus can be saturated when both proteins leave the nucleus simultaneously. Although this seems unlikely given the typical load these export enzymes are known to carry, the only way to disprove this hypothesis would be to find conditions in which the lack of correlation disappears from the cross-correlation function. After screening through different carbon sources, we have been unable to find such a condition.

We now have two bursting regulatory systems with common target genes. Without understanding the molecular mechanisms responsible for generating the observed cross-correlation function, we can still generate a toy model to understand the potential consequences this can have on downstream combinatorial target genes. Suppose a target gene promoter has binding sites for an activator and a repressor; but neither protein can bind to the promoter simultaneously. If only a repressor were bound, expression would be downregulated significantly while if only an activator were bound, expression would be increased significantly. However, if both an activator and repressor were bound, the change in expression would be tempered. By inhibiting the binding of both factors, the system enables the target promoter to experience the full range of repression and activation (Fig. 2).

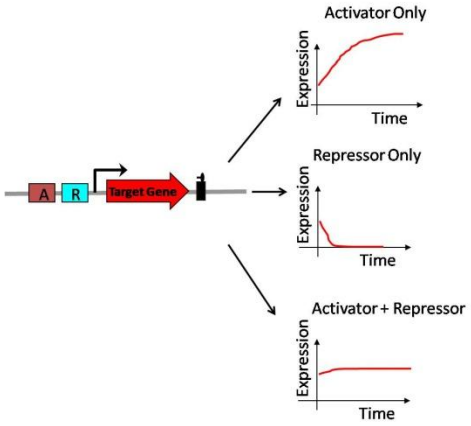

***Fig. 2*. Model of Combinatorial gene Regulation**. A target gene (red) is controlled by an activator (A) and a repressor R. If only the activator were present, expression would increase. If only the repressor were present, expression would be suppressed. If both were present, expression would not change much. By limiting the DNA occupancy on the promoter to only of the two transcription factors, the dynamic range of the combinatorial target gene is increased.

Future Directions

As with any genome-wide experiment, there are always several small-scale follow-up experiments. For the purposes of the screen, our first priority is to take higher time-

resolution movies of the proteins classified as bursters to try to quantify their bursts statistics. Additionally, we would like to do more in-depth bioinformatic analysis to understand what properties, if any, distinguish bursting transcription factors from their non-bursting counterparts.

In terms of our analysis of Msn2 and Mig1, there are three important sets of experiments that we will conduct. First, we will continue taking more low glucose movies to get better statistics and quantify the lack of correlation observed at time lag 0. Next, we will screen pharmacological inhibitors of kinases and phosphatases involved in Msn2 and/or Mig1 nuclear localization to see how they affect the cross-correlation between the proteins. These inhibitors have very specific targets, enabling direct mechanistic hypotheses from any observed effects. Finally, we will experimentally verify the combinatorial target gene expression prediction (Fig. 3). Here we will create synthetic promoters with binding sites for both Mig1 and Msn2, integrate them into yeast cells, grow the cells in low glucose +/- glycerol and measure expression of the target promoters. We expect that the dynamic range of the combinatorial promoters is higher in just glucose when compared to cells grown in glucose *and* glycerol.

Discussion

Examination of dynamic localization behavior reveals that yeast cells can process signals in a variety of ways, including modulating the properties of localization bursts, the intensity of localization events, or the fraction of cells that localize in response to a stimulus. Interestingly, the majority of transcription factors in yeast appear constitutively nuclear. Clearly, in all these cases, signal processing still occurs but cannot be observed through our assay. Perhaps bursting is occurring on promoters as transcription factors are binding and unbinding to DNA binding sites. This is what one would expect in bacteria where nuclei do not exist, but such dynamic regulation can still occur. Emerging optical techniques such as super-resolution may enable characterization of such phenomena.

In-depth analysis of two bursting transcription factors, Msn2 and Mig1, reveals an atypical cross-correlation function in which a mild correlation exists on a long timescale of ~1 hour but is damped at a time-lag of 0 minutes. This lack of correlation is reproducible in several media conditions but disappears in the presence of glycerol. We predict that this lack of correlation enables a higher dynamic range in downstream combinatorial target

promoters and plan to experimentally verify this prediction. Such analysis will yield fundamental insight into combinatorial gene regulation as we connect environmental input (carbon source) to transcription factor interaction (Msn2 and Mig1) to understand combinatorial expression. Not only can environmental inputs influence expression affect the interplay between transcription factors, changing the mode of regulation cells employ to control regulons of combinatorial target genes.

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