

Pulsatile Dynamics in the Yeast Proteome

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Summary

The activation of transcription factors in response to environmental conditions is fundamental to cellular regulation. Recent work has revealed that some transcription factors are activated in stochastic pulses of nuclear localization, rather than at a constant level, even in a constant environment [1–12]. In such cases, signals control the mean activity of the transcription factor by modulating the frequency, duration, or amplitude of these pulses. Although specific pulsatile transcription factors have been identified in diverse cell types, it has remained unclear how prevalent pulsing is within the cell, how variable pulsing behaviors are between genes, and whether pulsing is specific to transcriptional regulators or is employed more broadly. To address these issues, we performed a proteome-wide movie-based screen to systematically identify localization-based pulsing behaviors in *Saccharomyces cerevisiae*. The screen examined all genes in a previously developed fluorescent protein fusion library of 4,159 strains [13] in multiple media conditions. This approach revealed stochastic pulsing in ten proteins, all transcription factors. In each case, pulse dynamics were heterogeneous and unsynchronized among cells in clonal populations. Pulsing is the only dynamic localization behavior that we observed, and it tends to occur in pairs of paralogous and redundant proteins. Taken together, these results suggest that pulsatile dynamics play a pervasive role in yeast and may be similarly prevalent in other eukaryotic species.

Results

A Four-Phase Screen Enables the Identification of Proteins that Exhibit Dynamic Localization Pulsing

We designed a sequential screening strategy to identify genes from the GFP protein fusion library [13] that showed pulses of localization under constant media conditions (Figure 1). The screen was conducted in four phases. First, we performed an initial low-time-resolution movie-based screen to identify candidate genes that showed heterogeneous localization patterns across a population. Second, we performed a higher-time-resolution movie-based screen to confirm or reject candidate proteins from the first phase. Third, we performed an additional screening step to discriminate pulsing from cell-cycle-correlated localization. Fourth, we used a final set of more detailed 3D (z stack) movies of the remaining candidates to exclude proteins in which apparent pulsing was only an artifact of fluctuations in the z position of the localized protein.

Phase I of the screen produced filmstrips for 4,159 strains, under four different media conditions, with a time resolution of ~51 min between frames (Figure 1B and Figure S1 available online). Visual inspection revealed that most proteins exhibit relatively homogeneous localization patterns, with cells showing similar types of localization across time (7–12 hr) and condition (3,989 strains; Figure 1C). In contrast, 170 strains showed apparently heterogeneous localization patterns (see STB1, YOX1, and GLN1 in Figure 1B; Table S1).

In phase II, we rescreened candidate proteins that were positively identified as heterogeneous in phase I. We imaged them at a higher time resolution of ≤ 4 min between frames, choosing a single media condition for each protein. Because we did not observe condition dependence of heterogeneity in phase I, we screened for pulsatility in a single condition in subsequent phases. Of the 170 candidates, 64 appeared to behave in a pulsatile fashion in phase II, as judged by manual inspection of all movies (e.g., DOT6 and NRG2 in Figure 1D; Table S1). For nonpulsatile proteins, localization patterns were observed to be stable over the ~4 hr duration of the movie (Figure 1D, ISM1 and SPT7).

In phase III, our goal was to exclude proteins whose pulsatile dynamics were driven by the cell cycle, which is known to regulate the nuclear localization of many proteins, such as Msa1 and Whi5 [14]. We used two strategies to exclude cell-cycle-driven pulsing. First, we synchronized cell cycles using a transient hydroxyurea DNA replication block prior to the start of movie acquisition [15] and imaged them for ~6 hr at a time resolution of ≤ 4 min between frames. Second, we acquired 12–14 hr movies, also at a time resolution of ≤ 4 min, that included multiple cell divisions, and we visually inspected the correlation of nuclear localization pulses with cell-cycle phase, as measured by the time between successive cell division events. Together, these results were used to eliminate 25 proteins from our visual analysis (Figure 1E and Table S1).

Thirty-nine proteins remained after phase III, all of which showed pulsing apparently uncorrelated with the cell cycle (Table S1). Many of these proteins, such as Glc3 and Gln1, were localized to smaller organelles rather than to the nucleus [13]. We reasoned that proteins localized to small organelles could appear to pulse due to small drifts in z position relative to the focal plane. To eliminate such artifacts, we performed a fourth phase of screening to specifically test for this issue with non-nuclear-localized proteins. We acquired 3D movies of these proteins across a set of three focal planes, spaced 0.5 μm apart, with a time resolution of 3 min for 4–6 hr (Figure 1F). This visual analysis revealed that for all non-nuclear-localized proteins, apparent localization pulses could be attributed to z position fluctuations. By contrast, the 3D analysis did not exclude nuclear localized proteins such as Crz1, which showed clear pulsatile behavior.

The entire four-part screen identified nine proteins that showed pulsatile localization dynamics not explained by cell cycle or positional fluctuations. All previously known pulsatile proteins (Msn2, Crz1, and Mig1) were recovered in the screen [2–8], validating its ability to detect pulsing.

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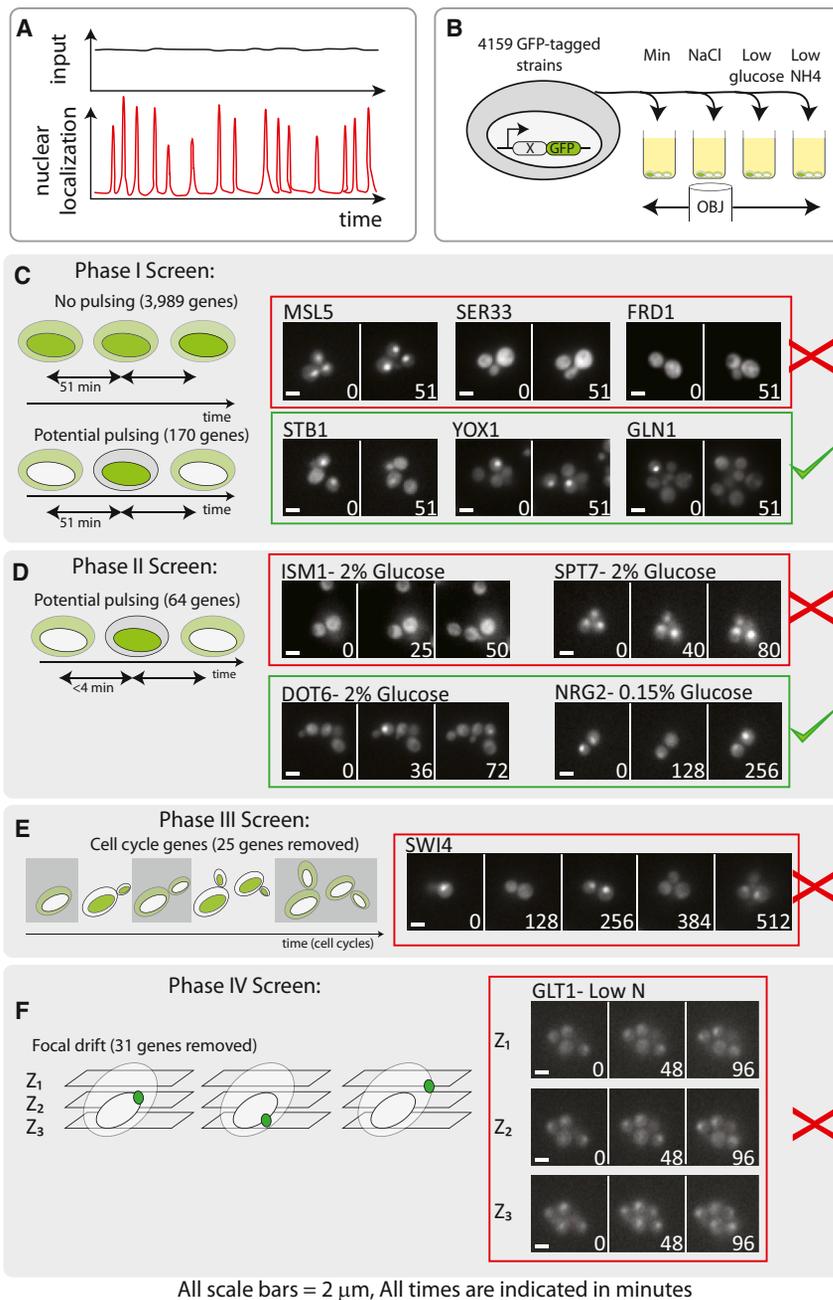


Figure 1. A Four-Phase Screen Identifies Nine Pulsing Proteins, All of which Are Transcriptional Regulators

(A) Pulsing is defined as the coherent translocation of many molecules of a protein in and out of an organelle in response to a constant input.

(B) Time-lapse movies of the GFP library were acquired at low time resolution (~51 min) under four environmental conditions.

(C) Examination of these low time-resolution movies revealed 170 potential pulsing proteins.

(D) Repeats of movies of these 170 proteins at higher time resolution showed 64 pulsing proteins.

(E) Twenty-five of these 64 pulsing proteins were cell-cycle related (E), whereas 31 were due to focal drift (F), leaving nine pulsing proteins remaining. Seven pulsing proteins are sequence-specific transcriptional regulators, and two are histone deacetylase complex members.

Scale bars represent 2 μm . Times are indicated in minutes. See also [Figure S1](#) and [Table S1](#).

conditions examined. Therefore, we set up a transcription factor screen to explore the dynamic single-cell behavior of each transcription factor in the library under conditions expected to activate it ([Figure 2](#)) [16]. We examined 121 transcription factors out of 143 in the GFP library [13] ([Table S2](#)), omitting the 22 transcription factors for which we could not find readily available inducers [16]. An additional 90 transcription factors were not analyzed because they are not included in the yeast GFP library [13, 16, 17].

For each transcription factor, we selected a corresponding inducer known to modulate its activity [16] and titrated the inducer concentration over a broad and physiologically relevant range (at least 10-fold). At each inducer concentration, we acquired a 4–6 hr time-lapse movie, with intervals between frames ranging from 30 s to 4 min. We then visually analyzed nuclear localization dynamics in each of these movies ([Table S2](#); [Figures 2](#) and [3](#); [Movie S1](#)).

Based on these movies, we confirmed the pulsatile behavior of the nine transcription factors identified in the original four-phase screen and further identified the glucose-dependent regulator Mig2 as an additional pulsatile gene regulatory protein ([Figures 2I](#) and [3I](#)), bringing the total number of pulsatile gene regulatory proteins to ten ([Table 1](#)). This validated the original proteome-wide screen and suggested that its false-negative rate was quite low; only Mig2, or one out of 112 additional pulsing transcription factors, was identified. Traces from these movies ([Figure 3](#)) revealed a wide range of pulse frequencies and durations, suggesting that pulsatility operates on multiple timescales.

These data also permit detection of other dynamic behaviors such as exact adaptation to a step change in input. For example, Hog1 nuclear localization increases in response to

Screen of Transcription Factor Nuclear Localization Dynamics

A striking feature of the proteins identified in the screen is that they were all directly involved in transcriptional regulation, either as sequence-specific gene regulatory proteins (seven) or as general transcription factors, i.e., histone deacetylase complex members (two), strongly suggesting that pulsatile spatial regulation of localization is predominantly, or even exclusively, used in the control of transcription ($p < 10^{-13}$, Fisher's exact test; see [Transcription Factor Over-Representation in the Supplemental Experimental Procedures](#)).

Because all proteins identified in the original screen were involved in transcriptional regulation, we reasoned that there might be additional pulsatile transcription factors in the library that were not activated or not pulsatile under the media

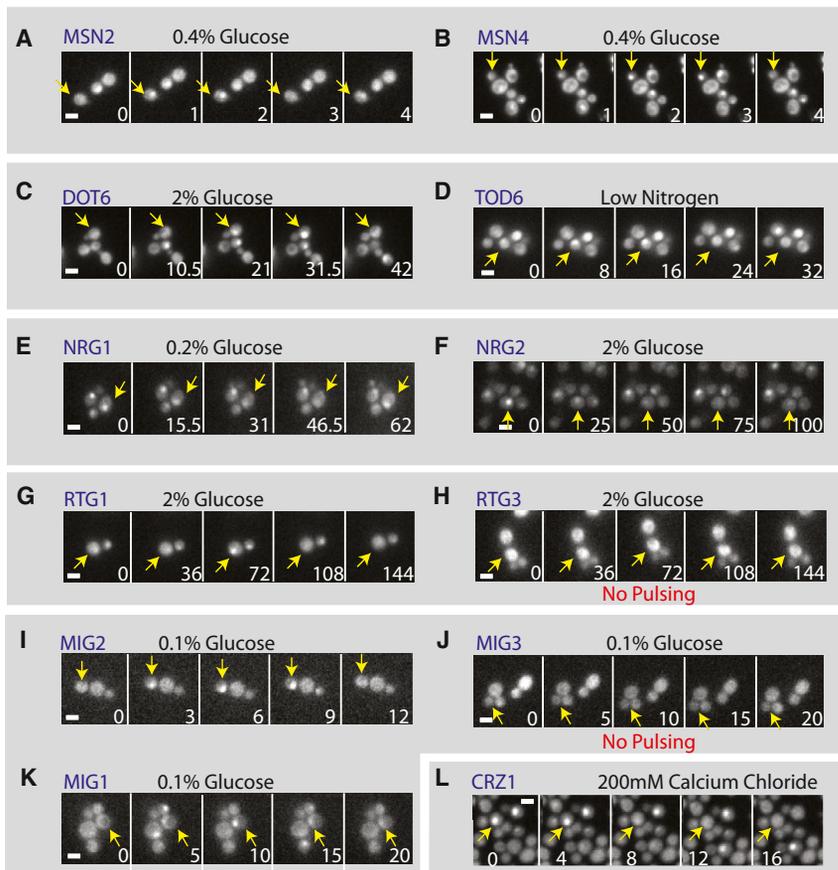


Figure 2. A Transcription Factor Screen Confirms Ten Pulsing Transcription Factors

Filmstrips of 12 transcription factors reveal pulses in nuclear localization across varying timescales and conditions (A–L). Many, but not all, of the proteins that pulse have a duplicate or redundant protein that also pulses. These proteins are grouped accordingly in gray boxes. Filmstrips are labeled with the protein name and condition. Scale bars represent 2 μm . Times are indicated in minutes. See also [Tables S2](#) and [S3](#).

Two pulsatile transcription factors had functionally redundant, but nonparalogous, partners. Among these, Mig1 and Mig2 both pulsed in low glucose ([Figures 3J](#) and [3K](#)), and Mig2 pulsed, whereas its redundant counterpart Rtg3 did not ([Figures 3G](#) and [3H](#)).

Although the sample sizes are small, these results suggest that pulsatility is generally correlated between paralogs and redundant partners. To examine whether pulse dynamics across paralogs are also correlated, we constructed a two-color strain in which the localization of both paralogs Msn2 and Msn4 can be examined in individual cells. We took movies of this strain in low glucose and found that Msn2 and Msn4 pulses were generally correlated ([Figure S2](#)); both proteins pulse together in most cases, though Msn2 sometimes pulses

whereas Msn4 does not (for example, see [Figure S2H](#) at ~ 200 min and [Figure S2I](#) at ~ 350 min).

a step increase in osmotic stress and then returns to its prestimulus level [18, 19]. Although the movies acquired here confirmed this previously characterized behavior in Hog1, they did not identify additional examples of it. Finally, we observed that most transcription factors (92 out of 121) are constitutively nuclear and that, with the exception of cell-cycle transcription factors, pulsing was the only type of sustained dynamic behavior we observed. It should be noted that in principle, other transcription factors could be regulated in a dynamic fashion through mechanisms (e.g., phosphorylation) that do not influence their nuclear localization. This type of dynamic regulation, lacking observable changes in localization, would not be detectable using this screen.

Regulatory Dynamics of Paralogs

Yeast has maintained many paralogous protein pairs produced by a whole-genome duplication event [20], enabling us to ask whether the pulsatility of one transcription factor is informative about the pulsatility of its paralog.

With the exception of Crz1, all pulsatile transcription factors are members of a pair of either functionally redundant or paralogous transcription factors [16]. Paralogs Msn2 and Msn4 both pulsed in response to glucose deprivation and other stress conditions, as did Dot6 and Tod6 ([Figures 3A–3D](#)). The Nrg1 and Nrg2 glucose repressor paralogs both pulsed, as well ([Figures 3E](#) and [3F](#)). In contrast, Mig3 did not pulse in the conditions investigated [21], although its paralog Mig2 did ([Figures 3I](#) and [3J](#)). Thus, the property of pulsatility is conserved in at least three out of four paralog pairs.

Discussion

Pulsing appears in diverse contexts but has not been examined systematically at a genomic scale. Hence, it had remained unclear how prevalent pulsatile dynamics are in the eukaryotic proteome. Our systematic approach identified ten proteins that show nuclear localization pulsing, all transcription factors, suggesting that this regulatory mode is used predominantly to regulate transcription. From our results, the fraction of transcription factors that utilize pulsatile nuclear localization is $\sim 8\%$ (ten out of 121 examined). The yeast GFP library is incomplete [13], but extrapolation from the frequency of pulsatile proteins among those examined to the remaining ~ 110 transcription factors *not* examined here suggests that we might expect approximately nine additional pulsatile proteins yet to be discovered. Moreover, since most transcription factors appear to be constitutively nuclear, it remains possible that these proteins are also activated in pulses that do not involve changes in spatial localization and therefore could not be detected by this screen. Thus, this study provides only a lower limit on the full extent of pulsatile dynamics in the cell.

Since most pulsing proteins are members of a pair of paralogous or functionally redundant transcription factors, one explanation for the evolution of pulsing is one in which pulsing is ancient and existed prior to the whole-genome duplication (estimated to be ~ 80 million years ago [20]). Since then,

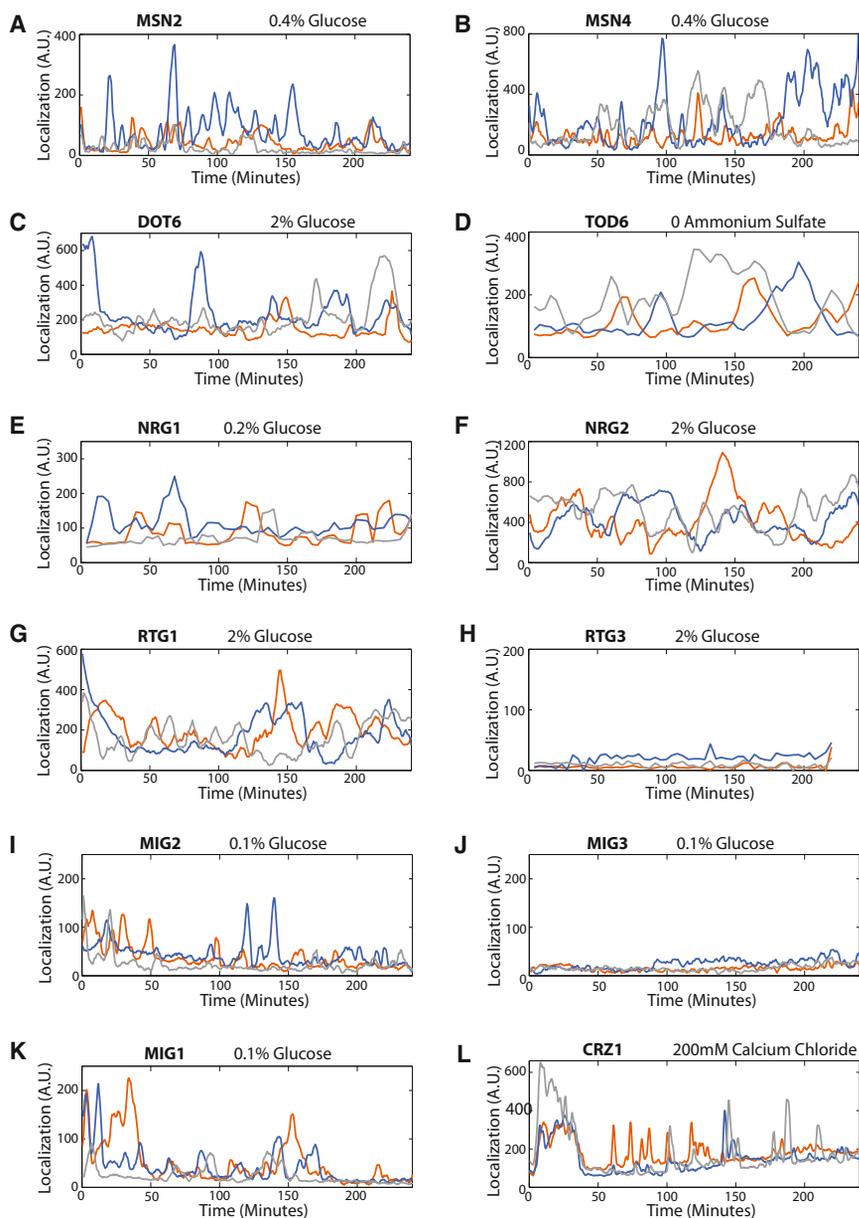


Figure 3. Pulsing Is Variable

Single-cell traces show that pulses vary from cell to cell (different colors on the same trace), from paralog to paralog (across columns) and from protein to protein (A–L). All traces are from the same movie that generated corresponding filmstrips in Figure 2. All traces have been smoothed. See also Figure S2 and Movie S1.

pulsing may be a common solution to many biological problems. For example, pulsing has already been shown to proportionally regulate entire regulons of target genes [2, 7], implement transient differentiation [26, 29], enable a multi-cell-cycle timer [27], and promote bet-hedging [25]. Pulsing may provide a time-based mode of regulation that facilitates these and other functions [1].

Taken together, these observations reveal that pulsatility is surprisingly pervasive in cells. It will now be critical to determine its mechanisms and functions and understand how these dynamics are integrated into the core functions of living cells. Although recent work has provided new insights into Msn2 pulsing [3, 4, 7, 8, 30, 31] and other work has provided a mechanism for pulsatile activation of a sigma factor in bacteria [28], we still lack a full understanding of the mechanisms of pulse generation and modulation for any yeast transcription factor. Do different pulsing systems use a common type of mechanism for pulsing, or are there many distinct mechanisms that can generate similar pulse dynamics? Pulsatility appears to be a core regulatory mechanism in yeast and most likely in other cell types as well [9]. The pulsatile proteins identified here should provide a starting point for understanding the

pulsing appears to have been lost only in some proteins (Mig3 and Rtg3), and the paralogs that have retained the ability to pulse have changed in their dynamics (Figure 3). Alternatively, paralogs that both pulse could have acquired pulsatile regulation through shared regulatory inputs that later became pulsatile. Further work analyzing whether proteins orthologous to the pulsing transcription factors described here also pulse, specifically in species that diverged prior to the whole-genome duplication, will distinguish between these hypotheses.

Recent work shows that pulsatile regulation occurs in diverse mammalian systems including NF-AT [9], p53 [10], Erk signaling [11], TGF- β signaling [12], and NF- κ B [22–24]. Moreover, many bacterial systems, such as persistence in *Mycobacterium smegmatis* [25] and bacterial competence [26], sporulation [27], and stress response in *Bacillus subtilis* [28], employ pulsing. The presence of pulsing in so many systems across a wide range of species suggests that

roles that this dynamic regulatory mechanism plays in diverse cell types.

Experimental Procedures

Strains and Media

All GFP strains were obtained from the GFP C-terminal protein fusion library, available from Invitrogen [13].

Saccharomyces cerevisiae were grown in synthetic complete or the appropriate dropout media made using low-fluorescence yeast nitrogen base, adapted from previous work [2, 32]. This media is yeast nitrogen base without riboflavin or folic acid: 5 g/l (NH₄)₂SO₄, 1 g/l KH₂PO₄, 0.5 g/l MgCl₂, 0.05 g/l NaCl, 0.5 mg/l H₃BO₃, 0.04 mg/l CuSO₄, 0.1 mg/l KI, 0.2 mg/l FeCl₃, 0.4 mg/l MnCl₂, 0.2 mg/l Na₂MoO₄, 0.4 mg/l ZnSO₄, 2 μ g/l biotin, 0.4 mg/l calcium pantothenate, 2 mg/l inositol, 0.4 mg/l niacin, 0.2 mg/l PABA, 0.4 mg/l pyridoxine HCl, 0.4 mg/l thiamine, 0.1 g/l CaCl₂, and 20 g/l dextrose.

Just prior to imaging (~10–20 min before movie acquisition), various permutations were made to the media, including changing the concentration or identity of the sugar source, changing the concentration or identity of the nitrogen source, and/or adding various chemicals or stressors.

Table 1. Summary of Observed Pulsing Behavior

Pulsing Protein	Conditions in which Pulsing Observed
Crz1	calcium
Msn2	all stresses
Msn4	all stresses
Mig1	low glucose
Mig2	low glucose
Nrg1	low glucose
Nrg2	low glucose
Rtg1	wild-type media only
Dot6	all conditions tested
Tod6	all conditions tested

Time-Lapse Microscopy

Cells were attached to 96-well or 384-well plates with glass-bottom dishes (Matrical) that were functionalized with 0.1 mg/ml Concanavalin-A (Sigma #C275). Fluorescence images were taken at room temperature on an Olympus IX81 with the ZDC autofocus option and an Andor Ikon (DU-934) camera. Automation was controlled by Andor IQ software. The time resolution of movies varied from 15 s to 51 min.

Analysis

For generation of traces in **Figures 3** and **S2**, fluorescence cell images were segmented using a Hough transformation algorithm in MATLAB, provided by Sharad Ramanathan [33]. The localization score was determined by the difference between the mean intensity of the five brightest pixels in the cell and mean intensity of the rest of the pixels in the cell [2]. These localization scores were smoothed and plotted using MATLAB.

Target genes of pulsing transcription factors were downloaded from Yeasttract [17, 34, 35]. Both direct and indirect target genes were included in tabulating total target genes in **Table S3**. Interaction partners of pulsing transcription factors were downloaded from the BioGrid [36] and tabulated in **Table S3**. Gene Ontology (GO) analysis of transcription factors was conducted using the GO Slim Mapper Process tool at the *Saccharomyces* Genome Database (<http://www.yeastgenome.org>) [16]. These results are tabulated in **Table S2**.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, two figures, three tables, and one movie and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2014.07.076>.

Author Contributions

C.K.D., L.C., and M.B.E. conceived and designed the screen. C.K.D., L.C., and K.R. conducted and analyzed the screen. Y.L. conducted and analyzed the two-color experiments. C.K.D., L.C., and M.B.E. wrote the manuscript with help from Y.L. and K.R.

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