Signal processing by the HOG MAP kinase pathway

Pascal Hersen1‡, Megan N. McClean§, L. Mahadevan§, and Sharad Ramanathan†¶

1 FAS Center for Systems Biology and §School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138; ‡Laboratoire Matière et Systèmes Complexes, Centre National de la Recherche Scientifique and Université Paris Diderot, 75205 Paris Cedex 13, France; and ¶Bell Laboratories, Murray Hill, NJ 07974

Edited by Charles F. Stevens, Salk Institute for Biological Studies, La Jolla, CA, and approved February 29, 2008 (received for review November 13, 2007)

Signaling pathways relay information about changes in the external environment so that cells can respond appropriately. How much information a pathway can carry depends on its bandwidth. We designed a microfluidic device to reliably change the environment of single cells over a range of frequencies. Using this device, we measured the bandwidth of the Saccharomyces cerevisiae signaling pathway that responds to high osmolarity. This prototypical pathway, the HOG pathway, is shown to act as a low-pass filter, integrating the signal when it changes rapidly and following it faithfully when it changes more slowly. We study the dependence of the pathway’s bandwidth on its architecture. We measure previously unknown bounds on all of the cascade measured by the levels of the activated enzyme previously unknown bounds on all of the cascade measured by the levels of the activated enzyme.

Information about the environment is then transmitted through the input oscillations. The pathway acts as a low-pass filter with a bandwidth, $\omega_b$, dominating by the lowest time scale in the cascade. Thus, all activation and deactivation rate constants in this cascade must be at least as large as $\omega_b$ for the cascade to respond faithfully to the inputs oscillating at frequencies lower than $\omega_b$. For a branched pathway, the activation rate constant of the slower branch does not affect the bandwidth. However, the deactivation rate constants of both branches have to be at least as fast as the bandwidth.

To measure signaling pathway response in vivo over different input frequencies and, hence, the bandwidth, as it is usually done in engineering, we developed a microfluidic device that allows for rapid periodic changes in media (Fig. 2). Rapid changes in media are exceptionally difficult to achieve in conventional microfluidic devices. Our device has two fluids entering through different inlets of a Y-shaped flow chamber, as shown in Fig. 2A. The region of lateral diffusion and, hence, mixing scales as $\sqrt{D\tau}$, with $D$ representing the diffusion constant of the media, $\tau$ the speed of the laminar flow, and $x$ the distance from the point of union of the two fluids, measured along the direction of the flow. Near the point where the two fluids meet, mixing is minimal. By changing pressure difference between the fluids by using a computer-controlled switch, we can sweep the separation line across the width of the flow cell. This allows us to rapidly switch the conditions to which the cells in the flow chamber are exposed. The media can be changed as frequently as twice a second, i.e., at 2 Hz (Fig. 2) without perturbing cell adhesion. Appropriate alignment is achieved by observing the separation line in real time by using phase contrast microscopy [see supporting information (SI) Text and Movie S1 and Movie S2].

We used this device to measure the bandwidth of the hyper osmolar glycerol (HOG) pathway in S. cerevisiae single cells (Fig. 1A). This MAP kinase pathway (4) is used by S. cerevisiae and other fungi (5, 6) to sense osmolar pressure in the environment and maintain water homeostasis. Although the HOG pathway is well documented, its transduction kinetics and in vivo reaction rates have been only roughly estimated, and its filtering properties are unknown. The SLN1 (7, 8) and SHO1 (9, 10) branches, shown in Fig. 1A, are redundant for cell survival under conditions of moderate osmotic stress. However, the SLN1 branch seems more important for pathway response to low, and very high, osmotic stress (11–13). Additionally, it is known that in an ssk2Δ ssK22A strain, the maximal phosphorylation of Hog1 is delayed from 1 min to ~3–5 min (11, 14). Once phosphorylated, Hog1 localizes to the nucleus (15–17), where it is recruited to target promoters and activates several transcription factors.

**Author contributions:** P.H. and M.N.M. contributed equally to this work; P.H., M.N.M., L.M., and S.R. designed research; P.H. and M.N.M. performed research; P.H. and M.N.M. contributed new reagents/analytic tools; P.H., M.N.M., and S.R. analyzed data; and P.H., M.N.M., and S.R. wrote the paper.

The authors declare no conflict of interest.

This article is PNAS Direct Submission.

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1 To whom correspondence should be addressed: 206 Bauer Center, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138. E-mail: sharad@post.harvard.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0710770105/DCSupplemental.

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To determine the bandwidth of the HOG pathway, we followed the nuclear localization of Hog1 as a reporter of the output of the signaling activity of the cascade (Movie S3). We exposed yeast cells in the flow chamber to an osmolar shock oscillating between 0 and 1 M sorbitol over a range of frequencies. These measurements allowed us to extract dynamical signaling properties of the HOG pathway. We measured frequency-dependent colocalization of Hog1-GFP with the nuclear protein Htb2-mCherry (Fig. 3). Assuming a first-order linear filter, we could obtain the bandwidth of the Hog1-GFP localization response to be $4.6 \times 10^{-3}$ Hz (see Methods and Fig. S1). Thus cytoplasmic activity in the signal-transduction cascade results in the phosphorylation of Hog1 and its subsequent nuclear translocation upon the beginning of the input pulse and nuclear delocalization at the end of the input pulse, all in ~220 seconds. At frequencies slower than $4.6 \times 10^{-3}$ Hz, the nuclear level of Hog1-GFP faithfully follows the input (Fig. 3B). At
frequencies higher than $4.6 \times 10^{-3}$ Hz, the steady-state response of the signaling cascade to the oscillating input drops sharply.

As in our model (Fig. 1B, Box 1), the pathway transiently integrates the signal (Fig. 3C) before reaching an unresponsive steady-state at high frequencies. Integration is possible if the time scale of pathway deactivation is limiting for pathway bandwidth. When the time of the off-phase of the pulse (when there is no sorbitol in the medium) is less than the time for complete pathway deactivation, components of the pathway remain activated. Activated pathway components, and hence activated nuclear Hog1, accumulate during each on-phase of the pulse (when there is 1 M sorbitol in the medium), leading to integration. Deactivation time refers to the time required for the pathway to deactivate all components when confronted with the sorbitol signal turning off. This is not to be confused with the time scale for pathway adaptation, which is much slower. Our experiments show that the deactivation rate of the pathway may be limiting for pathway bandwidth.

To confirm HOG pathway activity and low-pass filtering behavior at the transcriptional level, we used Gpd1-GFP (glycerol-3-phosphate dehydrogenase), a gene that is strongly up-regulated by the inductor 3-Hz input oscillations (filled circles and red lines). At input frequencies above the bandwidth ($\omega = 6.7 \times 10^{-2}$ Hz, $\omega = 3.3 \times 10^{-4}$ Hz, or $\omega = 1.4 \times 10^{-2}$ Hz), the pathway does not respond faithfully to the input oscillations but, rather, integrates the signal. The traces for response to a 1 M sorbitol (black line) and a 0.5 M sorbitol (red line) step shock show that for input at frequencies above the bandwidth, the pathway responds approximately to the mean change in input. At input frequencies above the bandwidth ($\omega = 0.5$ Hz, purple line) the transcriptional response of Gpd1-GFP integrates the signal and responds to the mean change in input. (F) At frequencies higher than the bandwidth ($\omega = 0.33$ Hz, red line) the magnitude of cell-size response drops dramatically. Time has been normalized by the input frequency, and fluorescence intensity variations are shown around their mean intensity.
levels, transcription begins during each osmotic input cycle (Fig. 3D), showing a faithful response to the input signal. At high frequencies, the transcriptional response is activated just once with subsequent adaptation. The transcriptional response at high frequencies mimics the response of the cell to a step shock of lower magnitude. Thus, we again see that at high frequencies, the cell effectively integrates the signal. Although transcription and translation are not fast reporters of HOG pathway activity, these results do allow us to confirm the Hog1 localization results at both very high and very low frequencies.

Pathway bandwidth could be set by the time scale of water export and/or the resulting size change after osmotic pulse. We measured the size response of the cells by again exposing yeast cells in the flow chamber to an osmolar shock oscillating between 0 and 1 M sorbitol over a range of frequencies. We monitored accurately the change in cell size on osmotic shock by measuring the change in the cell’s mean fluorescent intensity in a strain with cytoplasmic fluorescent protein Ura1-GFP (Fig. S2). We also studied changes in cell size in Hog1-GFP strains in which PBS2 was deleted, rendering the HOG pathway inactive. By comparing cell-size response for 1 and 2 M sorbitol input over a range of frequencies, we found that the mechanical response of the cell is linear and acts as a low-pass filter (Fig. 3E) with a bandwidth of 0.033 ± 0.01 Hz. At higher frequencies, the size oscillations drop dramatically. At lower frequencies, the size oscillations follow the input (Fig. 3F). This, in turn, sets the typical time scale for water transport in and out of the cell at 30 ± 1 s, and the porosity of the cell wall to be of the order of 1.75 mPa−1 sec−1 (SI Text). The mechanical response of the cell occurs on time scales much faster than that of Hog1 localization and therefore cannot set the time scale of pathway activation.

Because the HOG pathway bandwidth is not constrained by the cell’s mechanical responses, the 4.6 × 10−3 Hz bandwidth of the HOG signaling pathway must depend on the biochemical reaction rates during signaling. Following our model, the pathway time scale could be set at or below the level of PBS2 where the two branches converge or by the faster of the two branches. To test the role of the PBS2 protein, we studied signaling dynamics under conditions of extreme PBS2 under- and overexpression. Under conditions where PBS2 is underexpressed from the glucose-repressed GAL1 promoter, the response of the HOG pathway is drastically slowed (Fig. S3). When highly overexpressed by induction of the GAL1 promoter, PBS2 does not significantly change the time scale of signaling through the HOG pathway (Fig. S3). This indicates that PBS2 is not in limiting concentrations under wild-type conditions. We also measured the bandwidth in wild-type diploid cells and in pbs2Δ/PBS2 diploid cells. We found that the deletion of a single copy of PBS2 did not change the bandwidth of the diploid cell from that of the wild type (Figs. S4 and S5).

To understand whether either of the input branches set the bandwidth of the pathway, we studied the dynamics of Hog1-GFP in both ssk1Δ and sho1Δ strains where activation of PBS2 through the SHO1 branch is destroyed. The Hog1 localization response in these strains behaved identically to the wild-type strain (Fig. 4A). In striking contrast, blocking signaling from the SLN1 branch by deleting the SSK1 gene caused the Hog1 localization response to slow by almost 2-fold, to a bandwidth of 2.6 × 10−3 ± 0.4 × 10−3 Hz (Fig. 4B).

Additionally, we find that the SHO1 branch does not quickly integrate the high-frequency input signals, whereas the SLN1 branch does (Fig. 4C). The deactivation rate constant of the pathway in mutants without either the SSSK1 or the SHO1 branch are the same (Fig. S6). That the SLN1 branch is faster is consistent with previous results (9). In wild-type cells, it is the SLN1 branch that integrates high-frequency signals leading to the wild-type response.

The SLN1 branch consists of a two-component system, common in prokaryotes, in which there is no enzymatic amplification of the signal. The SHO1 branch is an enzymatic kinase cascade of the type that constitutes most signaling pathways in eukaryotes. We find that signal transduction through the branch with the phospho-relay is more than twice as fast as the SHO1 branch employing an enzymatic cascade. Interestingly in some other yeasts and fungi, the Sho1 protein is not conserved or the SHO1 branch does not signal to the HOG pathway (6, 19, 20). Furthermore, it is the faster SLN1 branch that is conserved in fungi.

The bandwidth of the HOG pathway could be set by the time for Hog1 to be activated and localize to the nucleus or the time for Hog1 to be deactivated and delocalize from the nucleus. The activation time includes the time to activate the SLN1 branch of the pathway, the time for this branch to activate PBS2, or the time for PBS2 to activate Hog1. The pathway deactivation time includes the time to deactivate one of the SLN1 or the SHO1 branch, the time to deactivate PBS2, or the time to deactivate Hog1 and delocalize it from the nucleus. Consistent with the experimental evidence that the pathway is able to integrate the signal at high frequencies, the rate at which the HOG pathway turns off could be limiting for bandwidth. Accurately measuring the deactivation rate constant from nuclear Hog1 traces (such as Fig. S6) is difficult because the initial drop in intensity is due to the cell size suddenly increasing in response to a decreasing osmotic level in the environment. This is followed by nuclear Hog1 export and the nuclear signal decaying into measurement noise, leaving very few data points to fit to an exponential. To carefully measure the deactivation rate constant of the HOG pathway, we used asymmetric pulses of osmolar shock (Fig. 4C). When the off time of the pulse (tOFF) is very short, the cell cannot tell independent pulses apart, responding as if to a step increase in sorbitol. When the pulses are very far apart (tOFF is very large), the pathway responds to each pulse, and Hog1 oscillates in and out of the nucleus each time. The amplitude of this oscillation decreases with tOFF, vanishing as tOFF approaches zero. This amplitude scales as 1 − e−kOFFtOFF, where kOFF is the slowest deactivation rate constant in the pathway. The fit of the amplitude to this single exponential allowed us to extract the rate constant for the slowest deactivation rate to be 4.1 × 10−3 ± 0.5 × 10−3 Hz (Fig. 4E), which is equal to the measured bandwidth of the HOG pathway (Fig. 3) to within error bars. This, coupled with the fact that the deactivation rates in mutants with either a deactivated SHO1 branch or the SLN1 branch are identical to within error bars (Fig. S6), supports the argument that the deactivation rates of components at or downstream of PBS2 must be limiting for pathway bandwidth. It is possible that the bandwidth of the HOG pathway is set by phosphatases responsible for deactivating PBS2 and Hog1 (22). The study of phosphatases in controlling the signaling dynamics of the pathway is left to future work.

Our results indicate that the time scales of Hog1 localization in all strains are set by biochemical reaction rates. Time scales are not set by cell-wall mechanical response and water transport because these processes happen on a much faster time scale. The results from our experiments on wild-type and mutant strains indicate that the activation and deactivation rates of PBS2, Hog1, and all of the components upstream of PBS2 in the SLN1 branch must be ≈ 4.6 × 10−3 Hz. We also show that the deactivation rates of all components in the SHO1 branch must be ≈ 4.6 × 10−3 Hz, whereas the activation rates are significantly slower. As we saw in our model, the bandwidth of a pathway is set by the slowest time scale; thus, if any protein activation or deactivation rate were < 4.6 × 10−3 Hz, the bandwidth of the HOG pathway in wild-type and sho1Δ strains would have to be < 4.6 × 10−3 Hz. Similarly, the difference between the ssk1Δ and sho1Δ strains indicates that the activation rates of all proteins upstream of PBS2 in the Sho1 branch must be ≈ 2.6 × 10−3 Hz. Our systems-level measurements of the HOG pathway put bounds on all of the rate constants in the signaling pathway in vivo.
Our microfluidic device allows for measurement of pathway bandwidth and elucidation of how bandwidth is set. Bandwidth measurement places bounds on all of the rate constants in the pathway. Similar experiments can help compare the design and dynamics of different signaling pathways within the same cell as well as the same pathway in different evolutionarily related species.

**Methods**

**Box1: Model.** The proteins $X_1$ and $X_1^*$ are directly activated by the input $l$ (Fig. 1) to their excited form $X_1^*$ and $X_1^{*\prime}$ at rates given by

$$
\frac{dX_1}{dt} = \frac{k_{f1}l}{1 + K_{M1}X_1} X_1 - \frac{X_1}{T_1}.
$$

$$
\frac{dX_1^*}{dt} = \frac{k_{f1}l}{1 + K_{M1}X_1} X_1 - \frac{X_1}{T_1}.
$$

These proteins in turn excite $X_2$ to $X_2^*$ at a rate

$$
\frac{dX_2^*}{dt} = \left(\frac{k_{f2}X_2^*}{1 + K_{M2}X_2^*} + \frac{k_{f2}X_1^*}{1 + K_{M2}X_1^*}\right)X_2 - \frac{X_2^*}{T_2}.
$$

which in turn excites $X_3$ to $X_3^*$ at a rate

$$
\frac{dX_3^*}{dt} = \frac{k_{f3}X_3^*}{1 + K_{M3}X_3^*} X_3^* - \frac{X_3^*}{T_3}.
$$

$k_{f1}$s are the rate constant, $K_{Ms}$ are the Michaelis–Menten constants for the reactions activated by $X_1$, and $T_i$ is the lifetime of activated protein $X_i^*$. For an input that fluctuates in time about a constant value $I$ by a small $\delta I(t)$, we can linearize the above equations in fluctuations in the concentrations of excited forms of the proteins about the steady-state. The Fourier transform of the fluctuations, $\delta X_i^*(\omega)$ is found to be proportional to

$$
\delta X_i^*(\omega) \sim \left(\frac{1}{1 + i\omega T_1} + \frac{1}{1 + i\omega T_1} + \frac{1}{1 + i\omega T_2} + \frac{1}{1 + i\omega T_3} - \delta I(\omega)\right).
$$

[5]
where $\delta(\omega)$ is the Fourier transform of the input and $\tau_1$, $\tau_2$, and $\tau_3$ are the sum of the time scales of excitation and deexcitation of $X_1$, $X_2$, and $X_3$, respectively. At high frequencies $|\omega| > \max(1/\tau_1, 1/\tau_2, 1/\tau_3)$, the amplitude of the fluctuations in the excited proteins falls rapidly, and the linearization is valid. The bandwidth of the pathway fluctuations in the excited proteins fall rapidly, and the linearization is valid.

### Yeast Strains and Plasmas

Yeast strains and plasmas were constructed by using standard molecular biology techniques. Table S1 contains the list of strains and plasmas used in this study.

### Yeast Growth Conditions

Cells were grown overnight at 30°C in synthetic complete (SC) medium, reincubated into fresh SC medium and grown at 30°C for 4–6 h before microscopy.

### Microfluidic Chamber and Attachment of Yeast Cells

Microfluidic chambers were made in PDMS (polymethylsiloxane) by using classic soft lithography techniques and then adhered to a coverslip (24 mm × 24 mm) by using a plasma oven. Our device used a three-way electrovalve (The Lee Company) controlled by a computer. A saturated solution of Con A (25 mg/ml; Sigma–Aldrich) was made in PDMS (polydimethylsiloxane) by using classic soft lithography techniques and then adhered to a coverslip (24 mm × 24 mm) by using a plasma oven. Our device used a three-way electrovalve (The Lee Company) controlled by a computer. A saturated solution of Con A (25 mg/ml; Sigma–Aldrich) was added into the chamber, and after washing the chamber with fresh SC, yeast cells were injected and allowed 5–10 min to settle down and stick to the coverslip. Once started, the flow removed the unattached cells. The visual basic code for controlling the switch and flow cell design was made freely available upon publication.

### Microscopy and Image Processing

The cells were observed by using a Zeiss 200M fluorescent microscope with an Orca-II-ER camera and a 1.45 N.A. plan α fluor objective. Emission from GFP was visualized at 528 nm (38-nm bandwidth) upon excitation at 490 nm (20-nm bandwidth), and emission of mCherry was visualized at 617 nm (73-nm bandwidth) upon excitation at 555 nm (28-nm bandwidth). Cells were allowed to adapt to the oscillating conditions for up to 20 min before starting acquisition. Subsequently cells were photographed at regular intervals and faster than twice the frequency of the oscillations of the flowing media. Image analysis was done by using Imagej (http://rsb.info.nih.gov/ij/).

### Bandwidth Measurements

Amplitude measurements as in Figs. 3 and 4 were fit to the classic low-pass filter form

$$\chi = \sqrt{\frac{G}{1 + (\omega T)^2} + \delta}, \quad [6]$$

with $\omega$ representing the frequency and $T$ the time scale of the filter. We also fit the measured amplitudes with the response of a model pathway with the slowest activation, $k_{on}$ and the slowest deactivation rate $k_{off}$ to the periodic inputs of our flow cell. This leads to a fit of the amplitude $\chi$ of the form

$$\chi = A \left(1 - e^{-k_{on}T/2}\right) \left(1 - e^{-k_{off}T/2}\right) + \delta,$$  \quad [7]

with $T$ representing the period of oscillation of the stimulus in the flow cell. A representing the gain of the system, and an offset value, $\delta$, that takes into account the fact that, at even high frequencies, the amplitude of nuclear amplitude of the oscillatory signal is non-zero because of the autofluorescence intensity changes due to size variations. Both fits lead to consistent extraction of the slowest rate constant in the pathway.

### ACKNOWLEDGMENTS

We thank Andrew Murray, Michael Laub, and Erin O’Shea for discussions; Derek Bruevich and the Whitesides laboratory for help with microfabrication; and Bodo Stern for comments on the manuscript. This work was supported by a Graduate Research Program for Women Fellowship, Lucent Technologies (to M.N.M.), Programme Jeunes Chercheurs de l’Agence Nationale de la Recherches (P.H.), the Human Frontier Science Program (S.R.), and the FAS Center for Systems Biology (P.H., S.R., and M.N.M.).
Single-Cell Permeability Estimation. From the measurements of size fluctuations (Fig. 3 and Fig. S1), it is possible to extract a precise value for the water permeability of yeast cells in the S288C background. Size fluctuations are due to water exchange between the environment and the cytoplasm that is driven by an osmotic pressure gradient across the cell wall and the plasma membrane. Using a d’Arcy’s law to model the water transport across the cell plasma membrane, the water flux is proportional to the gradient of pressure across the membrane:

$$ \tilde{q} = -\frac{k}{v} \partial_{t}P. \quad [1] $$

Using mass conservation and assuming a spherical shape leads to:

$$ \rho \partial_{t}R = -\frac{k}{v} \partial_{t}P. \quad [2] $$

The driving force is the gradient of osmotic pressure that can be estimated as:

$$ \partial_{t}P \sim \frac{\Pi_{\text{out}} - \Pi_{\text{in}}}{e}. \quad [3] $$

This leads us to write for a dilute solution and after linearization that the size fluctuations follow the osmotic fluctuations with a typical timescale $\tau = evpR_{eq}/3k\Pi_{eq}$, where $e$ is the plasma membrane thickness, $v$ the viscosity of water, and $\rho$ its density. $R_{eq}$ and $\Pi_{eq}$ are the size and internal osmotic pressure at equilibrium. This time scale is related to the water permeability by

$$ L_{p} = \frac{k}{\rho vp} = \frac{R_{eq}}{3\Pi_{eq}\tau}. \quad [4] $$

Using our experimental result for the mechanical response of yeast cells ($\tau = 4.8 \pm 0.9$ s) and assuming reasonable values for $R_{eq} = 2.5$ μm and $\Pi_{eq} = 1$ MPa gives the following water permeability:

$$ L_{p} = 1.75 \times 10^{-13} \text{ m}^{2}\text{ Pa}^{-1}\text{ s}^{-1}. \quad [5] $$

This value is in excellent agreement with data from the literature for various organisms. This shows how this setup can be used to measure precisely the dynamics of water transport.
Fig. S1. Hog1 localization responses of the HOG pathway in *S. cerevisiae* at different osmolar pressures. The response of Hog1 localization to oscillating input is studied for different sorbitol concentrations. Each point represents the average response amplitude as measured by Hog1-GFP colocalization with Htb2-mCherry over, typically, 5–10 cells. Error bars denote one standard deviation from the mean. Yeast cells have bandwidths of $\omega_0 = 4.6 \times 10^{-3} \pm 1 \times 10^{-3}$ Hz (filled squares) for 1 M sorbitol oscillation and (filled triangles) $\omega_0 = 4.6 \times 10^{-3} \pm 2 \times 10^{-3}$ Hz for a 1.5 M sorbitol oscillation.
Fig. S2. The mechanical response of *S. cerevisiae*. (A) The mechanical response of a *S. cerevisiae* cell to oscillating 1 M sorbitol input is measured as amplitude variation in cytoplasmic fluorescence as a function of input frequency. The strain used contains the cytoplasmic Ura1 protein tagged with GFP. Each point represents 5–10 cells. Error bars denote one standard deviation from the mean. Size response is a low-pass filter with a bandwidth $\omega_{\text{eq}} \approx 0.03 \pm 0.02$ Hz. This bandwidth is consistent with the mechanical response results discussed in the main text (Fig. 3). (B) At frequencies slower than $\omega_{\text{eq}}$, the cell size response follows the input faithfully ($\omega \approx 17 \times 10^{-3}$ Hz, red squares, red line). (C) At frequencies higher than the bandwidth ($\omega = 0.2$ Hz, green squares, green line), the magnitude of cell size response drops dramatically.
**A.**

![Graph showing Hog1-GFP contrast versus Frequency [Hz]](image)

**B.**

![Graph showing Hog1-GFP contrast versus Time [sec]](image)

**Fig. S3.** Effect of PBS2 over- and underexpression on HOG pathway bandwidth. (A) Hog1 localization in cells overexpressing Pbs2 from the GAL1 inducible promoter (blue triangles) was compared with wild-type cells grown under the same conditions (filled squares). Hog1 localization was measured as the maximum GFP intensity inside the cell, normalized by the average intensity in the cell: max(GFP) / (GFP). The bandwidth for the wild-type cells was comparable with the bandwidth of the cells overexpressing PBS2. Thus, PBS2 overexpression does not have a significant effect on pathway bandwidth. (B) Hog1 localization is delayed when Pbs2 is underexpressed from the uninduced GAL1 promoter (red circles) as compared with the wild type (filled squares). This can be seen from the time course of Hog1 localization after a 1 M step shock.
Fig. S4. Hog1 localization response for diploid cells. The response of Hog1 localization to 1 M sorbitol oscillating input is studied for haploid and diploid cells. Each point represents the average response amplitude as measured by Hog1-GFP colocalization with Htb2-mCherry over, typically, 5–10 cells. Error bars denote one standard deviation from the mean. Haploid cells (filled squares) are compared with PBS2/PBS2 diploids (blue diamonds, $\omega_0 = 4.6 \times 10^{-3} \pm 1.5 \times 10^{-3}$ Hz), and PBS2/pbs2 diploid cells (red diamonds, $\omega_0 = 4.2 \times 10^{-3} \pm 1.5 \times 10^{-3}$ Hz). Homozygous and heterozygous diploids show the same bandwidth. Interestingly, the diploid bandwidth is very close to the haploid bandwidth (filled squares, $\omega_0 = 4 \times 10^{-3} \pm 1 \times 10^{-3}$ Hz).
Fig. S5. Effect of carbon source and selective media on HOG pathway bandwidth. Comparison of Hog1-GFP nuclear colocalization in wild-type cells grown in 2% galactose selective (−Leu) synthetic complete (SC) media (△) with measurements of cells grow in 2% glucose SC (filled squares) shows that growth conditions have an effect on pathway bandwidth. Thus in Fig. S3, we compare cells grown under the same growth conditions.
Fig. S6. Comparison of activation and deactivation rates of the Ste11 and Ssk1 branches. The time course of Hog1 nuclear GFP levels are shown for ste11Δ (green) and ssk1Δ (gray) cells in response to a 1 M impulse of sorbitol (red) as a function of time. Although the time to accumulate Hog1 in the nucleus is clearly slower in cells lacking the Ssk1 branch of the pathway, the deactivation profiles of both mutants, when the pulse of sorbitol is turned off, is identical to within error bars. This suggests that the deactivation rates of the two branches are set by that of either Pbs2 or Hog1.
Movie 1.
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</tbody>
</table>

All strains are in the S288C background. To titrate the expression of PBS2, we constructed pSR261, which contains a \( P_{\text{GAL1}} \)-PBS2-mCherry fusion inserted between the XhoI and PacI restriction sites on the LEU2 CEN plasmid pRS415 (3).
