

Supporting Online Materials for Park *et al.*

MATERIALS AND METHODS

Constructs and Strains

Yeast cells were grown in YEPD medium or synthetic complete medium for maintaining plasmids. Glucose (2%, w/v) was used as a carbon source, unless otherwise indicated. Expression of all scaffold and kinase fusions was driven by their respective endogenous promoters in CEN/ARS plasmids, unless otherwise indicated.

Alternative recruitment constructs. For Ste5-Ste11 recruitment, the Ste5 I504T (S1) mutant (Ste5*) was fused to the syntrophin (syn) PDZ domain (residues 77-171) (S2). For the Ste5-Ste7 recruitment, the Ste5 V763A/S861P (S1) mutant (Ste5**) was fused to the syntrophin PDZ domain. The corresponding partner fusions, Ste7-nNOS and Ste11-nNOS, were constructed by fusing the fulllength kinases to the nNOS PDZ domain (1-130) (S2). For coimmunoprecipitation of Ste5 recruitment interactions, three tandem copies of HA epitope were fused to C-termini of Ste5*, Ste5*-syn PDZ, Ste5**, Ste5**-syn PDZ and wild-type Ste5. The resulting epitope tagged proteins were expressed from 2 μ based plasmids using the endogenous promoter of Ste5. Ste11-nNOS PDZ and Ste7-nNOS PDZ fusions were expressed from *gal1* promoter. The strain IH2731 was used for scaffold-kinase recruitment experiments and the strain RB105 was used for kinase-kinase recruitment experiments.

Diverter scaffold constructs. For the diverter scaffold, a C-terminal deletion allele of Ste5 (1-744) lacking the Ste7 docking site was fused to a Pbs2 variant carrying P96A/P99A mutation, which disrupts the interaction with the Sho1 SH3 domain (A. Zarrinpar, unpublished data) using a Gly-Ser linker. A series of mutations were incorporated into the diverter scaffold to assess the role of individual scaffold functions in signal diversion. The following mutations were made in the Ste5 portion of the diverter scaffold to disrupt component functions: Ste11 binding ~~I~~504T (S1); Fus3 binding ~~I~~2 Δ 241-336; the nuclear localization

signal (NLS) Δ 49-66 (S3). The following mutations were made in the Pbs2 portion of the diverter scaffold: Sho1 binding Δ P96A/P99A; kinase activity Δ K389M (S4). For non-covalent expression of Ste5 and Pbs2 fragments of the diverter, Ste5 (1-744) and Pbs2 (P96A/P99A) were coexpressed. For co-immunoprecipitation of diverter scaffold interactions, three tandem copies of HA epitope were fused to C-termini of diverter, wild-type Pbs2 and wild-type Ste5, and the resulting epitope tagged proteins (diverter-HA, Pbs2-HA and Ste5-HA) were expressed from 2 μ based plasmids. Thirteen tandem copies of c-Myc epitope were fused to the C-termini of Ste11 and Hog1. Hog1-Myc and untagged Ste7 proteins were expressed from a CEN/ARS plasmid and 2 μ -based plasmid, respectively, using their endogeneous promoters, whereas Ste11-Myc fusion was expressed from *gal1* promoter. SH003 and SH002 strains were used for osmoresistance growth assays of the diverter. SH005 was used for western blot analysis of Hog1 phosphorylation. Hog1 kinase dead allele was made by introducing K52R mutation.

Mating Assays

For mating growth assays, IH2731 or RB105 transformants were grown as patches on agar plates and were mated by replica plating onto the lawn of a mating tester strain IH1793 as described (S5). For quantitative mating assays, IH2731 or RB105 transformants harboring appropriate plasmids was grown at 30 $^{\circ}$ C to mid-log phase (OD₆₀₀ = 0.5). 2×10^6 cells of IH2731 or RB105 transformants were mixed with 10^7 cells of IH1793 strain and mating assay was performed as described (S5).

Co-immunoprecipitation

Preparation of lysates. Cells bearing 2 μ plasmids were grown at 30 $^{\circ}$ C to mid-log phase (OD₆₀₀ = 0.5) in a selective medium containing glucose (2%, w/v) before harvesting. Cells bearing *gal1* promoter plasmids were grown in a selective medium containing raffinose (2%, w/v) as a carbon source at 30 $^{\circ}$ C. At mid-log phase (OD₆₀₀ = 0.5), galactose was added to 2% (w/v) for induction and

cells were further grown for 6 hours at 30 °C before harvesting. 50 mL of cells were harvested and resuspended in Y-PER lysis solution (Pierce) containing protease inhibitors (leupeptin 5 µg/mL, chymostatin 5 µg/mL, pepstatin 5 µg/mL, PMSF 1 µg/mL) at 3 mL Y-PER/g cell pellet, and incubated at 25 °C for 20 minutes by gentle shaking. After incubation, lysates were cleared by centrifugation at 18,000 g for 10 minutes.

Immunoprecipitation and detection. The cleared lysate (300 µL) were mixed with 20 µL of anti-HA agarose beads (Santa Cruz Biotechnology) and incubated at 4 °C for 1 hour by gentle rocking motion. Beads were washed three to five times with 0.6 mL of ice-cold TBST buffer. The washed beads were resuspended in SDS sample buffer (50 µL) and 10 µL of each sample were used for immunoblotting. For Ste5 recruitment experiments, Ste11-nNOS and Ste7-nNOS were detected by an anti-nNOS antibody (Zymed). For diverter scaffold experiments, Myc-tagged Ste11 and Myc-tagged Hog1 were detected using an anti-Myc antibody. Ste7 was detected by an anti-Ste7 antibody and endogenous Fus3 was detected by an anti-Fus3 antibody. The West Pico Chemiluminescent Substrate (Pierce) was used for detection according to the manufacturer's instruction. All antibodies were purchased from Santa Cruz Biotechnology, unless otherwise indicated.

Osmolarity response assay

For high osmolarity growth (osmoresistance) assay, 3 µL of fresh overnight culture of appropriate yeast strains were streaked onto synthetic medium plates containing 1M KCl. For diverter scaffolds, 12 µL of 1mM α -factor was top spread onto the identical salt plates prior to streaking cells. Plates were incubated at 30 °C for 2 to 3 days.

α -factor disc assay for diverter scaffold activity

10^7 cells of yeast strain to be tested were mixed with 2.5mL of synthetic medium containing 1M KCl and top agar (0.7% w/v) and poured onto an agar plate of synthetic medium containing 1M KCl. After top agar dried, filter discs (1/4" dia.)

were placed and 5 μ L of 1 mM α -factor was applied onto discs. Plates were incubated at 30 $^{\circ}$ C for 2 to 3 days. Growth surrounding the disc indicates α -factor dependent osmoresistance (i.e. diverter function).

Fus3 phosphorylation assay

Cells in mid-log phase (OD600 = 0.5) were treated with α -factor (10 μ M) and 50 mL of cultures were harvested at each time point. Lysates preparation and immunoblotting were performed as described in co-immunoprecipitation except that Y-PER solution additionally contained sodium orthovanadate (1 mM). The phosphorylation of the endogenous Fus3 was detected using an anti-phospho p44/42 antibody (Cell Signaling Technology) as described (S6). This antibody also detects the phosphorylated Kss1.

Microarray experiments

To assess signal diversion by the diverter scaffold, SH003 cells containing the diverter expression plasmid were treated with α -factor (10 μ M) at mid-log phase and 250mL of cells was harvested at various time points. To assess wild-type responses of osmo and mating pathways, SH003 strain containing Pbs2 expression plasmid was treated with NaCl (0.4M) and IH2731 strain containing Ste5 expression plasmid was treated with α -factor (10 μ M). Preparation of total RNA and mRNA, labeling of cDNA, hybridization, and data analysis were performed as described (S7).

