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Genetic and Biochemical Analysis of the SLN1 Pathway in *Saccharomyces cerevisiae*

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Abstract

The histidine kinase based signal transduction pathway was first uncovered in bacteria and is a prominent form of regulation in prokaryotes. However, this type of signal transduction is not unique to prokaryotes; over the last decade two-component signal transduction pathways have been identified and characterized in diverse eukaryotes, from unicellular yeasts to multicellular land plants. A number of small but important differences have been noted in the architecture and function of eukaryotic pathways. Because of the powerful genetic approaches and facile molecular analysis associated with the yeast system, the SLN1 osmotic response pathway in *S. cerevisiae* is particularly useful as a eukaryotic pathway model. This chapter provides an overview of genetic and biochemical methods that have been important in elucidating the stimulus-response events that underlie this pathway and in understanding the details of a eukaryotic His-Asp phosphorelay.

Introduction

The SLN1 two-component signaling pathway of *S. cerevisiae* is a branched multistep phosphorelay containing a single hybrid histidine kinase, a histidine-containing phosphotransfer protein, and two downstream response regulators (Fig. 1A). Like the well-studied bacterial two-component pathways, the activity of the SLN1 pathway responds to environmental conditions. Phosphorelay from the Sln1 histidine kinase to the cytoplasmic Ssk1 response regulator is important in dampening the activity of the HOG1 MAP kinase pathway under normal osmotic conditions (Maeda *et al.*, 1994; Posas and Saito, 1997; Reiser *et al.*, 2003) while phosphorelay from the Sln1 histidine kinase to the nuclear Skn7 response regulator appears to play an important role in the cellular response to cell wall perturbations (Shankarnarayan *et al.*, 2008).

Like most eukaryotic histidine kinases, Sln1 is a hybrid protein containing both kinase and receiver domains (Ota and Varshavsky, 1993) (Fig. 1B). In this configuration the first phosphotransfer step is between H576, the phosphorylatable histidine within the kinase domain of Sln1 to D1144, the phosphoaccepting aspartate within the receiver domain of Sln1. The second phosphotransfer step is between Sln1 D1144 and histidine H64 in the phosphotransfer protein, Ypd1. The final phosphotransfer steps in the pathway occur between H64 of Ypd1 and the phosphoaccepting aspartate D554 in the receiver domain of the cytoplasmic response

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regulator, Ssk1 and between H64 of Ypd1 and aspartate D427 in the receiver domain of the nuclear response regulator, Skn7 (Fassler *et al.*, 1997; Li *et al.*, 1998; Posas *et al.*, 1996)(Fig. 1A).

The kinase activity of Sln1 is regulated in response to the environment. Under normal growth conditions, a modest level of kinase activity appears to be crucial for viability (Fig. 1A). Deletion of *SLN1* or mutation in any of the phosphorylatable residues in Sln1, Ypd1 or Ssk1 leads to inviability that is suppressible by inactivating mutations in components of the HOG1 MAP Kinase pathway and by over-expression of HOG1 pathway phosphatases (Maeda *et al.*, 1994; Ota and Varshavsky, 1992; Wurgler-Murphy *et al.*, 1997). This type of evidence led to our current understanding of the *SLN1*-*YPD1*-*SSK1* pathway as important in negatively regulating the activity of the HOG1 pathway in the absence of stress.

SLN1 pathway activity is diminished in the presence of elevated osmotic conditions (Ostrander and Gorman, 1999) leading to decreased turgor (Reiser *et al.*, 2003)(Fig. 1A, C). These conditions lead to the accumulation of *SLN1* pathway components in the dephosphorylated form. Dephosphorylated Ssk1 interacts with and activates the kinase activity of the Ssk2 and Ssk22 MAPKKs (Maeda *et al.*, 1995; Posas and Saito, 1998) of the HOG1 pathway thus setting in motion the signaling cascade that ultimately results in changes in expression of osmotic response genes including those involved in biosynthesis of the compatible osmolyte, glycerol (Albertyn, Hohmann, Thevelein *et al.*, 1994; Albertyn, Hohmann, and Prior, 1994).

SLN1 pathway activity can also be increased (Fig. 1A, C). This was first shown by the isolation of mutations exhibiting elevated expression of *SKN7* target genes. These have been dubbed *sln1** activating mutants since they increase the activity of the Sln1 phosphorelay (Fassler *et al.*, 1997; Ault *et al.*, 2002; Tao *et al.*, 2002). Both the Skn7 and the Ssk1 response regulators appear to be more highly phosphorylated in these mutants. The elevated phosphorylation of Skn7 is responsible for the increase in expression of Skn7 dependent target genes (Li *et al.*, 1998; Li *et al.*, 2002) and the elevated phosphorylation of Ssk1 causes defects in the cellular response to hyper-osmotic conditions due to changes in the kinetics of Hog1 phosphorylation (Fassler *et al.*, 1997). In principle, *sln1** activating mutations leading to increased phosphorylation of the Ssk1 and Skn7 response regulators could be attributable to a variety of mechanisms, including, for example, increased rate of Sln1 autophosphorylation or phosphotransfer activity, or diminished Sln1 directed phosphatase activity. In practice, *sln1** activating mutations map to both the Sln1 receiver domain and to the coiled-coil domain located between the second transmembrane domain and the kinase domain (Fassler *et al.*, 1997; Ault *et al.*, 2002; Tao *et al.*, 2002). The *sln1** P1148S activating allele is a Pro to Ser mutation of a helix capping proline that is conserved in the receiver domain of most response regulators and is equivalent to P61 of *E. coli* CheY (Fassler *et al.*, 1997; Ault *et al.*, 2002). In vitro phosphotransfer analysis of the P1148S mutant revealed a shift in the phosphorelay equilibrium from Sln1 to Ypd1 but no change in the rate of hydrolysis of the aspartyl phosphate on the Sln1 receiver domain. Consistent with the increase in expression of *SLN1*-*SKN7* dependent genes observed in the *sln1** P1148 mutant, in vitro phosphorelay assays revealed a two-fold increase in accumulation of phosphorylated Skn7 with the mutant Sln1* receiver domain as the phosphodonor compared to the wild type receiver (Fig. 5B) (Ault *et al.*, 2002).

The *sln1** T550I activating mutation located just upstream of H576 led to the identification of a functional coiled-coil (CC) domain in the linker region of Sln1 between the extracellular domain and the kinase domain (Fig. 1B). This region was recognizable as a coiled-coil using the Learn Coil algorithm (Singh *et al.*, 1998) that was developed to detect weak coiled-coils thought to be a general feature of histidine kinases occurring just upstream of the H box. The impact of the *sln1* T550I mutation and of the coiled-coil domain was studied using a functional kinase domain construct consisting of aa 537–950. Derivatives of that construct that lack aa

537–570 showed no evidence of autophosphorylation at 40 minutes, a period of time sufficient for autophosphorylation of a coiled-coil containing construct to plateau (Tao *et al.*, 2002). At three hours, the mutant was phosphorylated to about 20% the extent of wild type kinase (Ault, 2001). These observations are consistent with the inviable phenotype of the *sln1ΔCC* mutant (Tao *et al.*, 2002). The activation phenotype of the *sln1* T550I mutant correlates with the increased hydrophobicity afforded by the isoleucine residue at position 550. Additional mutations in the region that increased hydrophobicity at the “a” and “d” positions of the helix were likewise activating while mutations at other positions had no effect (Tao *et al.*, 2002). The absence of an effect of CC domain activating mutations on Sln1 homodimerization led to the hypothesis that the rotational flexibility of the coiled-coil in Sln1 is important for normal Sln1 activity (Tao *et al.*, 2002) (Fig. 1B). Consistent with this hypothesis, the coiled-coil domain of the strongly dimerizing mammalian C/EBP transcription factor rescued viability of the inviable *sln1ΔCC* mutant (Tao *et al.*, 2002), but like *sln1** mutants, this chimeric *SLN1* allele caused a salt-sensitive phenotype reflecting a defect in the ability of the kinase to be down-regulated in response to osmotic stress (Tao *et al.*, 2002).

Although the isolation of activating mutations in *SLN1* suggest that Sln1 kinase activity can be stimulated, it has been difficult to define the environmental conditions that trigger an increase in Sln1 kinase activity. The study of *fps1* mutants led to the hypothesis that Sln1 kinase activity and/or signaling is stimulated by osmotic imbalance (Tao *et al.*, 1999). The *FPS1* gene encodes the major glycerol channel responsible for glycerol efflux (Tao *et al.*, 1999) and *fps1* mutants accumulate intracellular glycerol even in the absence of osmotic stress (Luyten *et al.*, 1995). Interestingly, although *SLN1* pathway activity is elevated in the *fps1* mutant, transient osmotic imbalance caused, for example, by shifting an *FPS1*⁺ strain from high to low osmotic environments does not activate the *SLN1* pathway (Li, 2001; Shankarnarayan, 2007). Efforts to clarify the difference in physiology between transient and prolonged hypo-osmotic stress led to the identification of a cell wall protein, encoded by the *CCW12* gene. Strains lacking the *CCW12* gene exhibit constitutive activation of the Sln1 kinase as do strains in which the Ccw12 protein is enzymatically removed from the wall (Shankarnarayan *et al.*, 2008). Interestingly, the *fps1* mutant is deficient in wall-associated Ccw12 protein suggesting that the activity of the Sln1 kinase is regulated by aspects of the environment that trigger specific wall perturbations (Shankarnarayan *et al.*, 2008).

Increased Sln1 kinase activity causes changes in gene expression via changes in the activity of the Skn7 response regulator. Skn7 is a transcription factor that binds to Skn7 response elements regardless of its phosphorylation state, but stimulates expression of *SLN1* dependent genes only in response to phosphorylation of D427 in the Skn7 receiver domain. The non-phosphorylatable *skn7D427N* mutant fails to respond to elevated Sln1 signaling and the constitutive *skn7D427E* mutant increases expression of *SLN1* dependent genes independent of *SLN1* (Li *et al.*, 1998; Li *et al.*, 2002).

The branched architecture of the pathway (Fig. 1A) in which a single histidine kinase signals to the Skn7 as well as the Ssk1 response regulator was suggested by genetic analysis and confirmed by biochemical analysis (Li *et al.*, 1998; Ketela *et al.*, 1998). Sln1 and Ypd1 dependent phosphorylation of the Skn7 response regulator was confirmed by reconstitution of the phosphorelay pathway using purified recombinant proteins consisting of single domains of each protein (Li *et al.*, 1998; Ault *et al.*, 2002). The radiolabeled phosphoryl group on the Sln1 kinase domain could be distributed in turn to the Sln1 receiver domain, the Ypd1 phosphorelay protein and to the receiver domain of Skn7. Furthermore, the distribution of the phosphoryl groups to each of these domains was dependent on the presence of domains participating in earlier steps in the pathway and on the phospho-accepting residues in each domain (Li *et al.*, 1998). *In vitro* reconstitution of the Sln1 phosphorelay pathway allowed for biochemical characterization of *sln1* and *ypd1* mutants (Ault *et al.*, 2002; Tao *et al.*,

2002;Janiak-Spens *et al.*, 2005;Janiak-Spens *et al.*, 2000;Janiak-Spens and West, 2000)and for measurements of the phosphorylated lifetime of receiver domains (Janiak-Spens *et al.*, 2000;Janiak-Spens *et al.*, 1999). It also paved the way for structural analysis of pathway components (Xu and West, 1999;Xu *et al.*, 2003;Zhao *et al.*, 2008). In this chapter we describe genetic and biochemical methods for analysis of SLN1 pathway activity in response to relevant mutational and environmental factors.

Materials and Methods

A. *In vivo* methods to investigate Sln1 pathway activation

1. EMS¹ Mutagenesis and Deletion Collection Screen for Pathway Activating Mutants—The screen in which the *sln1** and *fps1* mutants were isolated involved random mutagenesis of the genome using the chemical mutagen, EMS, to identify mutations that elevate expression of a *SLN1* dependent *lacZ* reporter gene (Tao *et al.*, 1999; Yu *et al.*, 1995). EMS mutagenesis is conducted according to the method of Lawrence *et al.* (Lawrence, 1991)with minor modifications. Grow a 20 ml culture of the starting strain² to saturation in dropout media to select for the continued presence of the plasmid carrying the *lacZ* reporter gene. Wash the cells twice with potassium phosphate buffer (0.0195 M KH₂PO₄, 0.0305 M K₂HPO₄, pH 7.0) and resuspend them at 1×10^8 cells/ml in the same buffer. Divide the cells into 5 ml aliquots and add 180 μ l EMS (Sigma) to each. Incubate the treated cells at 30°C for various times, stopping each reaction by addition of an equal volume of 10% sodium thiosulfate. Collect the cells by centrifugation, wash them twice with water, and resuspend the cells at a concentration of 1×10^8 cells/ml. Perform serial dilutions and spread various dilutions on selective plates to give a projected yield of 300–400 cells/plate.

Select the EMS treatment time resulting in ~ 65% killing and spread these cells on 150–200 selective plates to generate between 50,000 and 100,000 colonies for screening³. Replica-plate colonies to selective media containing X-Gal⁴. Approximately 1% of colonies screened on X-Gal plates under these conditions exhibit a blue phenotype on X-Galplates consistent with elevated expression of the *lacZ* reporter gene (Tao *et al.*, 1999). *KEX2* mutations account for more than 50% of the mutants in this type of screen (Tao *et al.*, 1999). Mutations in *KEX2* (encoding a serine protease) cause a non-specific increase in the color of reporter bearing strains on X-Gal plates. Eliminate *kex2* mutants from further analysis by introducing a *KEX2* plasmid into the candidates via mass mating with a karyogamy deficient strain (*kar1*) of the opposite mating type (Georgieva and Rothstein, 2002). Using the recessive drug resistance alleles present in the starting strain, cytoductants retaining the desired nucleus that have acquired the (*LEU2* marked) *KEX2* plasmid can be selected⁵ Select only those candidates whose blue phenotype is not complemented by the *KEX2* plasmid. Tests of the remaining mutants to ensure that the phenotype is specific for *SLN1* dependent reporter genes are described later in this section.

¹Abbreviations used in this chapter include β ME, β -mercaptoethanol; BSA, bovine serum albumin; CBD, chitin binding domain; DTT, dithiothreitol; EDTA, ethylenediaminetetracetic acid; EMS, ethyl methane sulfonate; GST, glutathione-S-transferase; HK, histidine kinase; IPTG, isopropyl- β -D-thiogalactopyranoside; MOPS, 3-morpholinopropanesulfonic acid, ONPG, ortho-nitrophenyl- β -galactoside; PMSF, phenylmethanesulfonyl fluoride; Rec, receiver domain of a response regulator; SC, synthetic complete; SC-aa (synthetic complete media lacking specific amino acids); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; X-Gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

²JF1567, the strain used in Tao *et al.* (Tao *et al.*, 1999)was transformed with a plasmid bearing a *SLN1* responsive *lacZ* reporter gene. JF1567 has recessive canavanine and cycloheximide resistance mutations (*can*^R and *cyh*^R) useful in the later screening steps of this protocol.

³A final plating density of between 300 and 400 colonies per plate is best for subsequent screening on X-Gal plates.

⁴X-Gal plates are prepared by addition of $1 \times M9$ salts (Miller, 1972)(Miller, 1972)and 50 mg/ml X-Gal dissolved in N, N-dimethylformamide to standard synthetic complete (SC or SC-aa) media. X-Gal is added to media cooled to 65°C immediately prior to pouring. The X-Gal concentration can be increased or decreased but should be titrated such that the starting strain is white or very pale blue.

⁵Selection is on SC media plates lacking the amino acid encoded by the *KEX2* plasmid and containing the appropriate drugs.

Because mutations in *KEX2* represent such a large fraction of the X-Gal positive mutants isolated by mutagenesis, it is useful to repeat the screen by examining individual mutants of the haploid deletion collection (Research Genetics). Conducting both types of screens is especially important when the deletion is lethal or when the deletion phenotype is not the same as the phenotype of point mutants. For example, deletion of the *SLN1* gene is lethal and would not be recovered in the deletion screen, but several different *sln1** mutations were identified in the EMS screen. The deletion collection is transformed with a *SLN1* responsive *lacZ* reporter plasmid such as *P_{OCHI}-lacZ* plasmid (pZL1320 (Li *et al.*, 2002)) using an adaptation of the micro-scale protocol of the Frozen-EZ yeast transformation kit (Zymo Research). Inoculate wells of a microtiter dish containing 0.25 ml of YPD medium plus 50 mg/liter G418 using a sterile 96 pronged device. Incubate the microtiter dishes at 30°C for 24–48 hours without aeration. Remove the media using a multichannel pipettor being careful not to disturb the cells that have settled to the bottom of the wells. Wash cells in 0.1 ml EZ1 solution and then pellet the cells using a microtiter plate microfuge at 1300 rpm for 5 minutes. Resuspend the pellet then in 10 µl of EZ2 and 0.1 ml of EZ3 to which the plasmid DNA (5–6 µg/well) has been added. Incubate the dishes at 30°C for 90 minutes and spread cells over ¼ of a selective (SC-Ura) plate using a small sterile glass spreader. Incubate the plates several days at 30°C until transformants became visible. An alternative approach in which pools of deletion strains are transformed can also be employed. In this approach, individual deletions are grown in the wells of a microtiter dish as before. However, prior to transformation, all the strains in a single microtiter dish are transferred using a sterile 96-prong device into a large petri dish containing YPD. Use the Frozen-EZ yeast transformation kit (Zymo Research) to prepare competent cells and transform them with a *SLN1* responsive *lacZ* reporter plasmid. Spread the transformation mix on selective media.

Replica plate transformants from the deletion collection obtained using either method to X-Gal plates. Compare the color of transformants on X-Gal plates to the parental strain corresponding to the deletion collection in use. For example, transformants of strain BY4742 (Kelly *et al.*, 2001), the parent strain of the MATa haploid deletion collection, carrying the *SLN1* responsive *lacZ* reporter, are white on plates containing X-Gal (50 mg/l). Transformants that are blue on X-Gal plates should be chosen for further analysis. Conduct liquid β-galactosidase assays to confirm the phenotype and to establish the magnitude of the effect.

Quantitative β-galactosidase assays are performed using glass bead-lysed extracts. Grow a small culture in synthetic complete dropout media (e.g. SC-uracil to select for the presence of plasmids with a *URA3* marker) to saturation. Make a 1:50 dilution into 5 ml of fresh media and grow to a density of $1-2 \times 10^7$ cells/ml. Collect the cells by centrifugation and resuspend the pellets in 0.2 ml breaking buffer (0.1 M Tris pH 8.0, 20% glycerol, 1 mM DTT; stored at –20°C) plus 13 µl of 40 mM PMSF (PMSF is prepared in 95% ethanol and stored at –20°C). Transfer the cell suspension to a 1.5 ml microtube and add 200 mg of glass beads. Store frozen at –80°C for at least 15 minutes⁶. Thaw cells on ice, vortex briefly to mix and then alternate vortexing (15 seconds) with incubation on ice (15 seconds) between 8 and 15 times until cells are broken⁷. Pellet cell debris by microfugation on high at 4°C for 15 minutes. Keep extracts on ice while measuring protein concentration using a Bradford protein assay (Biorad). The β-gal assay is conducted as described by Miller ((Miller, 1972)p. 352–359) with minor modifications. Add 900–1000 µl of Z buffer (0.06 M Na₂HPO₄(7H₂O), 0.04M NaH₂PO₄(H₂O), 0.01M KCl, 0.001M MgSO₄(7H₂O) pH 7.0 with 0.05 M βME added fresh for each use) to reaction tubes and add protein extract to generate one ml reaction mixtures. Incubate these tubes at 28°C. Add 200 µl ONPG (4 mg/ml prepared in Z buffer, stored at 4°C

⁶Incubation for 15 minutes at –80°C facilitates breakage of the cells.

⁷The exact number of vortexing cycles will depend on the vortex and the strain. Cell breakage can be monitored by phase contrast microscopy. Ideally, cell breakage should exceed 80%.

and prepared fresh weekly) to each tube and vortex briefly to begin the reaction, noting the time. When the reaction is just visibly yellow⁸, add 500 µl of 1M Na₂CO₃ to each tube to stop the reaction. Note the time at which each reaction is stopped. Read the A₄₂₀. Calculate the activity using the formula: $A_{420} \times 378/\text{time (minutes)} \times \text{volume (ml)} \times \text{protein (mg/ml)}$. Activities are reported as the average of 4–6 assay values using at least three independent transformants.

The gene of interest in deletion candidates chosen for their blue phenotype from among transformants of pooled deletion strains is identified by sequencing. Prepare genomic DNA and amplify the unique tag sequence built into each deletion cassette using a forward primer complementary to the Up Tag sequence (5' CTACAGGTGCTCCAGAGA-3') (<http://chemogenomics.stanford.edu/supplements/04tag/protocols.html>) and the Kan 250 R reverse primer complementary to sequences within the KanMX4 cassette (5'-CTGCAGCGAGGAGCCGTAAT-3'). Examine the PCR product by agarose gel electrophoresis and purify it using the QIA quick Gel Extraction Kit (Qiagen). Obtain the sequence using the Kan 200 R primer (5' CGTGCGGCCATCAAAATG-3'). The deletion tag sequence is located just downstream of the forward primer and upstream of the Kan MX4 sequence and is identified using the tag database (http://www.sequence.stanford.edu/group/yeast_deletion_project/downloads.html)

The specificity of the elevated reporter gene expression is examined by comparing the effect of the strain background on the activity of the *SLN1*-dependent pZL1320 (*P_{OCH1}-lacZ*) reporter versus the activity of control reporters such as pSL1156 in which *lacZ* is driven by the basal (–154 to –1) *OCH1* promoter which lacks *SLN1-SKN7* response elements and pZL1369 (Li *et al.*, 2002) which contains a single mutated Skn7 binding site.

2. Activation of the SLN1 pathway with zymolyase—In addition to pathway activation in *sln1**, *fps1* and *ccw12* genetic backgrounds, the SLN1 pathway can be activated with the cell wall digesting enzyme zymolyase. Zymolyase is a complex of enzymes isolated from *Arthrobacter luteus* with strong lytic activity for yeast cell walls (U.S. Biological 10⁵ units/g). The complex consists of zymolyase A, β-1,3-glucan laminaripentaohydrolase and zymolyase B, an alkaline protease thought to be required for the action of zymolyase A by changing the structure of the wall. Zymolyase is routinely used at high concentration to digest the walls from yeast cells. At this enzyme concentration cells must be resuspended in sorbitol to provide osmotic support for the fragile spheroplasts that result from removal of the cell wall. However, the very low zymolyase dose of 0.5–1 unit/ml that is sufficient for activation of the SLN1 pathway causes no loss in optical density or viability of cells. Grow cells to saturation in YPD⁹ and subculture using a 1:50 dilution into fresh YPD medium using a 1:50 dilution. Incubate cells at 30°C with aeration until they reach early log phase (~10⁷ cells/ml). Adjust the pH of all cultures by adding Na₂HPO₄ to a final concentration of 20 mM (Reiser *et al.*, 2003). Add zymolyase to 0.5 or 1 unit/ml and incubate cultures at 30°C for 30–60 minutes. Harvest cells by centrifugation and store pellets at –80°C until needed.

3. SLN1 pathway activation assays

a. Northern analysis of SLN1 dependent genes: The effect of zymolyase treatment on SLN1 pathway activity can be examined by Northern analysis. Grow small cultures to saturation and make a 1:50 dilution into 10–50 ml of fresh medium. Incubate at 30°C with aeration until early

⁸The yellow color should be monitored closely and the reaction stopped promptly so that the A₄₂₀ reading remains within the linear range of the spectrophotometer. Reaction times should not be less than 5 minutes, as these times will tend to be inaccurate. If necessary, repeat the reaction with less extract.

⁹Where plasmid retention is an issue, grow cultures in selective medium overnight and then subculture (1:50) into YPD for two generations prior to harvesting at log phase.

log phase ($\sim 10^7$ cells/ml). Harvest cells by centrifugation and store pellets at -80°C . Isolate total RNA using the acid phenol method (Ausubel *et al.*, 1989) with minor modifications. Resuspend the pellet in 0.4 ml TES (10 mM Tris, pH 7.5, 10 mM EDTA, 0.5% SDS) and add 0.4 ml acid phenol. Vortex 10 seconds. Incubate at 65°C for 1 hour, vortexing 5 seconds every 10 minutes. Move the tubes to ice and add 0.1 ml of TE (10 mM Tris, 1 mM EDTA), pH 7.5. Microfuge at high speed for 5 minutes and transfer the upper phase to a fresh microtube on ice. Add 0.4 ml hot acid phenol and vortex 20 seconds. Microfuge for 5 minutes and transfer the upper phase to new tubes on ice. Add 0.4 ml chloroform and vortex for 20 seconds. Microfuge again for 5 minutes and transfer the upper phase to new tubes. Add 1/10 volume of 3 M NaOAc and 1.0 ml of cold ethanol and allow the RNA to precipitate at -80°C overnight. Microfuge for 5 minutes, aspirate, wash the pellet in 75% ethanol, repeat the spin and aspirate the ethanol. Dry the pellet and resuspend the RNA in 100 μl RNase free water by incubating at 65°C for 5 minutes and vortexing. Store at -80°C until needed. Thaw the samples on ice. Ensure complete resuspension of the samples by incubating samples at 65°C for 2 minutes. Use 1–2 μl to measure the absorbance at 260 nm. An OD of 1 is equivalent to 40 $\mu\text{g/ml}$ RNA. 10 mls of cells at $1 \times 10^7/\text{ml}$ yields $\sim 250 \mu\text{g}$ of RNA. Check the quality of the RNA by running a sample on a standard ($1 \times \text{TAE}$) 1% agarose gel. Sharp ribosomal RNA bands of 1.8 kb and 2.5 kb and a more diffuse tRNA band at 0.2 kb should be visible. Samples with reduced amounts of one rRNA species relative to the other, general smeariness or an accumulation of smaller molecular weight species should not be used further. Mix 10–20 μg of each total RNA sample with $1.3 \times$ RNA loading buffer ($1 \times$ MOPS (0.2 M MOPS, 0.5 M sodium acetate, 0.01 M EDTA), 20% formaldehyde, 50% deionized formamide, 0.04 mg/ml ethidium bromide plus xylene cyanol and bromophenol blue loading dyes), heat samples at 65°C for 10 minutes, move to ice and load a 10% (v/v) formaldehyde 1% agarose gel prepared in $1 \times$ MOPS buffer. Run the gel at 75V for several hours. Photograph the gel, wash it twice 5 minutes each in water and then set up a capillary blot (Ausubel *et al.*, 1989) to transfer the RNA to a nytran filter. UV crosslink the RNA to the blot by exposing the blot (RNA side up) to a germicidal lamp¹⁰. Prepare a ^{32}P labeled probe using random primers and the Prime-It random priming labeling kit (Stratagene) with appropriate PCR fragments as templates¹¹. Store the probe at -20°C . Prehybridize the blot in a small rotisserie bottle (Bellco Glass Inc.) using 4–6 ml of Perfect Hyb (Sigma) hybridization buffer rotating for at least one hour at 68°C . Incubate the probe at 100°C for 10 minutes, and then chill it on ice. Add the probe ($\sim 10 \mu\text{l}$) to the rotisserie bottle and hybridize overnight at 68°C . Wash the blots twice, 5 minutes each, in 30 ml of $2 \times \text{SSC}$ -0.1% SDS¹². Wash the blots twice more in $0.5 \times \text{SSC}$ -0.1% SDS at 68°C . Remove the blots from the rotisserie bottle, wrap it (RNA side down) in plastic film and place it in a phosphorimager cassette (or X-ray film) for 6–30 hours. Visualize radiolabeled bands with a phosphorimager. Analyze bands using Quantity One software (Biorad). Strip the probe from the blot by incubating the blot in $0.2 \times \text{SSC}$ -1% SDS at 75°C for 1 hour followed by a rinse in 10 mM Tris (pH 7.5). Repeat prehybridization and hybridization procedures using a probe suitable for normalization¹³.

b. Osmotic activation of the HOG1 pathway: SLN1 pathway activation leads to curtailment of HOG1 pathway signaling in response to salt (Fassler *et al.*, 1997). Hence, another assay for SLN1 pathway activation is to examine the effect of osmotic shock on Hog1 phosphorylation

¹⁰The appropriate time of exposure for optimal retention of RNA on the blot will depend on the age of the bulb and the distance from the bulb to the blot and should be determined empirically.

¹¹Expression of the *SLN1* responsive *NCA3* gene is frequently used to monitor SLN1-SKN7 pathway expression because it is more abundant than *OCH1*. The *NCA3* probe is 389 bp PCR product generated using *NCA3* 9F (5'-TTCCGACGCTTTAATATTGTCTTCC-3') and *NCA3* 398R (5'-TCACATGCATAAGAACAGTAGTAGCC-3').

¹²SSC is diluted from a $20 \times$ stock (3M NaCl, 0.3 M sodium citrate pH 7).

¹³Normalization in zymolyase treatment experiments is performed using a probe corresponding to *CDC33*. The *CDC33* probe is a 636 bp PCR product generated using *CDC33* 3F (5'-GTCCGTTGAAGAAGTTAGCAAG-3') and *CDC33* 639R (5'-CAAGGTGATTGATGGTTGAGGG-3').

over time. To monitor Hog1 phosphorylation, grow cultures selectively overnight, dilute 1:50 into YPD and aerate at 30°C to a cell density of 1×10^7 /ml. Use a 4M solution of NaCl prepared in YPD to adjust cultures to a final concentration of 0.4M NaCl. Take ten ml aliquots at 0, 2, 4 and 10 minutes after addition of NaCl. Harvest cells by filtration through a Millipore 0.45 μ HA filter (using full house vacuum). Quickly place filters into a microtube and rapidly freeze in a dry-ice ethanol bath. Store samples at -80°C until preparation of the extract. Remove cells from filter into 1 ml of water and pellet the cells by microfugation. Remove the filter. To the pellet, add 100 μ l 16% TCA and ~ 200 mg of glass beads. Vortex approximately 4–5 minutes. Add 400 μ l of ice cold 5% TCA and vortex to mix. Transfer liquid to a fresh microtube and microfuge at high speed in the cold for 10 minutes. Aspirate the supernatant and resuspend the pellet in 50–100 μ l loading buffer (150 mM Tris Base, 10 mM EDTA, 3% SDS, 5% β ME, 15% glycerol, 0.01 mg/ml bromphenol blue). Boil samples for 5 minutes, microfuge samples at high speed for 5 minutes and load (~15 μ l) onto two identical 10% SDS PAGE gels (29:1 acrylamide:bisacrylamide, 10% running gel, 6% stacking gel). Transfer proteins to nitrocellulose membranes (0.45 micron; Schleicher & Schuell). Stain with Ponceau S staining solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid), wash briefly in distilled water and scan or photograph for a record of relative loading of each lane and the quality of the protein extracts. Block the blot with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and 4% BSA. Wash twice for 10 minutes in TBST and incubate for 2 hours at 4°C with RC20 recombinant anti-phosphotyrosine antibody conjugated to horseradish peroxidase (Transduction Laboratories) diluted 1:2500 in TBST + 4% BSA. Wash twice with TBST. For Hog1 protein analysis, block the membrane in TBST with 5% skim milk for one hour. Incubate with yC-20 anti-Hog1 antibody (Santa Cruz Biotechnology) diluted 1:2000 in TBST + 5% BSA overnight at 4°C. Wash twice in TBST and then incubate with peroxidase conjugated bovine anti-goat IgG (Santacruz Biotech sc2378) diluted 1:1000 in TBST. Wash the blot three times with 10 ml of TBST for 5 minutes each. Treat both blots with Super Signal West Pico chemiluminescent substrate (Pierce) and exposed to film to visualize the proteins.

B. *In vitro* Sln1 kinase and Sln1 pathway phosphorelay assays

1. Expression and purification of SLN1 pathway components from *E. coli*

a. GST-Sln1-HK and GST-Sln1-HK-Rec domains: The Sln1 histidine kinase (HK) domain (aa 537–950) is expressed as a glutathione-S-transferase fusion protein in DH5 α cells and is routinely used as the initial phosphodonor to downstream phosphorelay components. This protocol is a slightly modified version of the published procedure (Li *et al.*, 1998; Ault *et al.*, 2002). Grow 1 liter of cells in LB medium containing 100 μ g/ml ampicillin at 37°C until late log phase (an optical density at 600 nm of ~0.9). Add IPTG to a final concentration of 0.2 mM and grow cells at a lower temperature (16°C) for an additional 24 hours. Pellet cells by centrifuging at $4500 \times g$ for 10 minutes and resuspend in 4–5 ml/g (wet weight of cell pellet) in lysis buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% β ME, 1 mM PMSF] supplemented with a 1X protease inhibitor cocktail¹⁴ (0.1 μ g/ml chymostatin, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin, 1.1 μ g/ml phosphoramidon, 7.2 μ g/ml E-64 protease inhibitor, 0.5 μ g/ml leupeptin, 2.5 μ g/ml antipain, 100 μ M benzamidin, 100 μ M sodium metabisulfite). If desired, the cell paste can be stored frozen at -20°C. Lyse (thawed) cells by sonication¹⁵ and clarify the cell lysate by centrifugation at $27,200 \times g \times 30$ minutes. Apply the supernatant to a 2 ml glutathione-Sepharose 4B (GE Healthcare) column¹⁶ that has been pre-equilibrated in lysis buffer. Wash sequentially with 10 column volumes of lysis buffer, then 5 column volumes of wash buffer [50 mM Tris-HCl pH 8.0, 2 mM DTT, 1 mM EDTA], and finally with 5 column volumes of storage buffer¹⁷ (50 mM Tris-HCl pH 8.0, 100 mM KCl,

¹⁴Any commercial PIC cocktail appropriate for bacterial cells can be substituted here.

¹⁵Lysis can be achieved using a French press in place of sonication.

¹⁶Alternatively, batch purification method can work as well using a 50% slurry as described for full-length Skn7 (Section B.1b)

2 mM DTT, 1 mM EDTA, 10% glycerol). Leave about 2 ml of storage buffer above the resin bed and gently mix the slurry. Bead-bound Sln1-HK protein can be stored in aliquots at -20°C and is fully active for subsequent autophosphorylation and phosphotransfer experiments. By retaining GST-Sln1-HK on the glutathione-Sepharose beads, one can conveniently remove this phosphodonor from phosphorecipient proteins by low speed centrifugation¹⁸.

Another plasmid was constructed that expresses the Sln1 HK domain as well as the C-terminal receiver domain, Sln1-HK-Rec (aa 537–1220) (Ault, 2001; Janiak-Spens *et al.*, 2005). Purification is the same as described above for the Sln1-HK domain and is useful in preparing phosphorylated Ypd1 (see section B3. c). [Note: the GST-SLN1-HK-Rec domain protein is susceptible to proteolytic degradation, thus it is important to include protease inhibitor cocktails for all purification and storage conditions.]

b. Full length Ssk1 and Skn7: Full length Ssk1 and Skn7 are expressed as GST fusions in pGEX vectors (Li *et al.*, 1998; Ault, 2001) in DH5 α cells. Grow one liter of culture to an OD₆₀₀ between 0.6–1.0 in LB medium containing 100 $\mu\text{g/ml}$ ampicillin. Add 0.2 mM IPTG¹⁹. Optimal induction of GST-Ssk1 protein occurs after two hours at 37°C and optimal induction of GST-Skn7 protein occurs after 20 hours at 16°C . The low temperature incubation reduces the concentration of inclusion bodies and improves the yield of active protein²⁰. Harvest cells by centrifugation and resuspend the pellet in 5 ml lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1% βME , 1X protease inhibitor cocktail (Sigma)) and store frozen²¹. Thaw the cell pellets and add 1 mM PMSF. Lyse cells using a French press or by sonication²². Centrifuge for 10 minutes at $7000 \times g$ and to the supernatant add 3 mls of a 50% slurry of glutathione agarose beads. Incubate for one hour at 4°C . Wash the beads four times in lysis buffer and four times in storage buffer (50 mM Tris pH 7.6, 50 mM KCl, 5 mM MgCl_2 , 0.1% βME , 50% glycerol). Store bead-bound proteins at -20°C . If desired, proteins can be eluted from the beads by incubation in elution buffer (100 mM glutathione, 50 mM Tris pH 7.6, 50 mM KCl, 5 mM MgCl_2 , 0.1% βME) with shaking at room temperature for one hour²³. Remove beads from the eluted protein by brief centrifugation in a microfiltration unit. Protein concentration can be determined based on analysis of band intensities on Coomassie stained SDS-polyacrylamide gels compared to stained gels with BSA concentration standards using the public domain NIH Image program (<http://rsb.info.nih.gov/nih-image/>).

c. Sln1, Ssk1, and Skn7 receiver domains: The C-terminal response regulator domains from Sln1, Ssk1, and Skn7 can be expressed as cleavable tripartite fusion proteins consisting of an N-terminal receiver (Rec) domain, followed by the yeast VMA1 protein-splicing intein domain

¹⁷GST-Sln1-HK can also be stored in reaction buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM MgCl_2 , 2 mM DTT with the addition of 10–50% glycerol).

¹⁸For experiments in which the presence of GST might complicate analysis, use thrombin to cleave the protein from the GST moiety. Wash the bead bound protein four times in thrombin cleavage buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl_2), resuspend in the same buffer and add 5 units of biotinylated thrombin. Incubate at 25°C for times from 1–6 hours depending on the protein. Add 25 μl of streptavidin beads during the final 15 minutes of the reaction. Recover the protein by transferring the reactions to microfuge filtration units and centrifuging briefly.

¹⁹This relatively low concentration of IPTG is important in minimizing the number of truncation products in GST-Skn7 cultures. Since D427 is at the C terminus of the protein and the GST tag at the N terminus, truncation products are inactive.

²⁰Induction of GST-Skn7 and GST-Ssk1 at 37°C results in a low yield of full-length protein with many truncation products. GST-Skn7 that is induced at 37°C is inactive in phosphotransfer assays. Performing the induction at 16°C does not improve the yield of full-length protein but does improve the activity of the protein in the phosphotransfer assay (Ault *et al.*, 2002)

²¹Freezer storage can be at -20°C or at -80°C .

²²Add 1.25 mg lysozyme, 50 units of DNase (Boehringer Mannheim) and 10 μg of RNase to lysis buffer prior to sonication. Set tubes in an ice water bath and do multiple rounds of sonication for 30 seconds each at 60 seconds intervals

²³A higher concentration of Skn7 is achieved by replacing batch-wise elution of GST-Skn7 with elution of the protein in a chromatography column using 50 mM glutathione. For a column consisting of three ml bead bound GST-Skn7, discard one ml of void volume and collect 0.5 ml aliquots for analysis by SDS-PAGE. The GST-Skn7 protein is concentrated in aliquots 3 and 4 (Ault *et al.*, 2002).

and a chitin binding domain (CBD) at the C-terminus. The CBD facilitates affinity purification, while the self-splicing activity of the intein domain can be induced at high DTT or β ME concentrations to cleave the polypeptide at the junction between the receiver and intein domains. All protein purification steps described below should be carried out at 4°C.

The Sln1 receiver domain (aa 1084–1220) is expressed from an inducible *tac* promoter (pCYB2 vector, IMPACT system, New England Biolabs)(Janiak-Spens *et al.*, 1999) in DH5 α cells. Grow 1 liter of cells in LB medium containing 100 μ g/mL ampicillin at 37°C until an optical density at 600 nm reaches 0.6. Add IPTG to a final concentration of 0.4 mM and grow cells an additional 8 hours at room temperature. Harvest cells by centrifugation at 4500 $\times$ g for 10 minutes and resuspend the cell pellet in wash buffer (0.1 M sodium phosphate, pH 7.0, 1 mM EDTA). Centrifuge again and resuspend the cells in lysis buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) at ~5 ml/g wet weight of the cell pellet. Lyse cells by sonication and clarify the cell lysate by centrifugation at 27,200 $\times$ g $\times$ 30 minutes. Load the supernatant onto a 3 ml chitin bead column (New England Biolabs) equilibrated in lysis buffer at 4°C using a peristaltic pump at a flow rate of about 0.5 ml/minutes. Wash with 20 column volumes of lysis buffer at a flow rate of about 1 ml/minutes. Then wash sequentially with 4–5 column volumes each of cleavage buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA), cleavage buffer plus 5 mM ATP, 5 mM MgCl₂, then cleavage buffer plus 30 mM β ME. Turn off column flow and incubate overnight at 4°C to allow thiol-induced cleavage of the fusion protein. Elute with 3–4 column volumes of cleavage buffer and identify Sln1-Rec containing fractions by 15 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Pool fractions containing Sln1-Rec domain and apply to a Sephadex G50 (Sigma) gel filtration column (300 mL bed volume) equilibrated in 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT. Protein concentration can be determined by absorbance at 280 nm (ϵ = 4,020 M⁻¹ cm⁻¹). The purified protein can be stored frozen (–80°C) in small aliquots containing 10% glycerol. Typical yields are 0.5 – 1.0 mg pure protein per liter cell culture.

Expression of the Ssk1 receiver domain (aa 495–712) from the pCYB2 vector in DH5 α cells was low. Thus, we excised the Ssk1-Rec-intein-CBD fusion gene from the pCYB2 vector and sub-cloned the fragment into pET11a (Novagen) to give an inducible T7-based vector as described (Janiak-Spens *et al.*, 1999). The expression plasmid was transformed into BL21 (DE3) cells. Grow 1 liter of cells in LB medium containing 100 μ g/ml ampicillin at 37°C until an optical density at 600 nm of about 0.7. Cool the culture to 16°C, then add IPTG to a final concentration of 1 mM and grow cells an additional 16 hours at 16°C. Harvest cells by centrifuging at 4500 $\times$ g for 10 minutes and resuspend the cell pellet in wash buffer. Centrifuge again and resuspend the cells in lysis buffer containing 10% glycerol at ~6 ml/g wet weight of the cell pellet. A pinch of RNase (Sigma) and DNase (Sigma) is added, then lyse cells by French press operated at a cell pressure of 14,000 lb/in². Clarify the cell lysate by centrifugation at 27,200 $\times$ g $\times$ 30 minutes. Load the supernatant onto a 3 ml chitin bead column (New England Biolabs) equilibrated in lysis buffer containing 10% glycerol. Chitin affinity column chromatography is carried out as described above for the Sln1-Rec domain except that 10% glycerol is included in the cleavage buffer and Sephadex G75 resin (Sigma; 300 ml bed volume) equilibrated in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM β ME is used for the gel filtration chromatography step. Protein concentration can be determined by absorbance at 280 nm (ϵ = 25,440 M⁻¹ cm⁻¹). The purified protein can be stored frozen (–20°C) in small aliquots containing 10% glycerol. Typical yields are 1.0 – 1.5 mg pure protein per liter cell culture.

The vector expressing the Skn7 receiver domain (aa 361–622) was prepared as described above for the Ssk1-Rec domain (Janiak-Spens and West, 2000) and transformed into BL21(DE3) cells. Purification of the Skn7-Rec domain is similar to the Sln1-Rec domain as described above except an S-200 gel filtration column (300 mL bed volume; GE Healthcare) is used,

equilibrated in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM β ME. Protein concentration can be determined by absorbance at 280 nm ($\epsilon = 6580 \text{ M}^{-1} \text{ cm}^{-1}$). The purified protein can be stored frozen (-20°C) in small aliquots containing 10% glycerol. Typical yields are 1–3 mg pure protein per liter cell culture.

d. Ypd1 phosphorelay protein: The full length Ypd1 protein (aa1–167) can be constitutively expressed from a pUC12-derived vector in DH5 α cells (Xu *et al.*, 1999). Grow a 1 liter culture to saturation overnight at 37°C in LB medium supplemented with 50 $\mu\text{g}/\text{ml}$ ampicillin. Harvest cells by centrifuging at $4500 \times g$ for 10 minutes and resuspend the cell pellet in lysis buffer (0.1 M sodium phosphate pH 7.0, 1 mM EDTA, 1.4 mM β ME). Centrifuge again and resuspend the cells in lysis buffer at $\sim 5 \text{ ml}/\text{gram}$ wet weight of the cell pellet. Lyse cells by sonication and clarify the cell lysate by centrifugation at $27,200 \times g \times 30 \text{ minutes}$. Measure the volume of the supernatant. In a small glass beaker at 4°C on a stir plate, while gently stirring, slowly add saturated ammonium sulfate to a final concentration of 55%. Collect the protein precipitate by centrifuging at $12,000 \times g \times 10 \text{ minutes}$. Resuspend the protein pellet in 6–7 ml of dialysis buffer (20 mM BisTris pH 6.5, 1.4 mM β ME) and dialyze versus 2 liters of the same buffer for 24 hr. with one change of buffer to remove the ammonium sulfate. Recover the protein solution from the dialysis bag and pellet any precipitated material. Filter the supernatant through a $0.2 \mu\text{m}$ syringe filter and apply to a 5 ml HiTrap Q Sepharose Fast Flow (Pharmacia) anion-exchange column pre-equilibrated in 50 mM BisTris pH 6.5. Elute bound protein from the column with a linear salt gradient of 0–500 mM NaCl in 50 mM BisTris pH 6.5. Identify Ypd1-containing fractions by running aliquots (12–15 μl) from fractions on 15% SDS-polyacrylamide gels. Pool fractions containing Ypd1, concentrate using Centricon 10 filter units (Amicon) to a volume $\leq 10 \text{ ml}$. Apply sample to a Sephadex G50 (Sigma) gel filtration column (350 ml bed volume) equilibrated in 50 mM sodium phosphate pH 7.0, 1 mM EDTA, 1.4 mM β ME. Identify fractions containing pure Ypd1 protein by SDS-PAGE and combine. Protein concentration can be determined by absorbance at 280 nm ($\epsilon = 15,280 \text{ M}^{-1} \text{ cm}^{-1}$). Ypd1 can be stored frozen (-20°C or -80°C) in small aliquots containing 15% glycerol. Yields can be as high as 40 mg pure protein per liter cell culture.

Note: When we embarked on reconstituting the phosphorelay system *in vitro*, it was desirable to express and purify these domains without His-or other affinity tags that potentially could interfere with phosphotransfer assays. We have since discovered that most of these proteins can in fact be expressed as GST or His-tagged proteins with little or no loss in phosphotransfer activity. The Sln1-Rec domain and full-length Skn7 and YPD1 can also be expressed and purified as GST-fusion proteins using the procedure described in Section 3.1 a (Li *et al.*, 1998; Ault *et al.*, 2002). Full length Skn7, Ssk1-Rec, and Skn7-Rec domains can be also be expressed and purified as His-tagged proteins (Janiak-Spens, F., Sparling, D., Zhao, X., and West, A.H., unpublished) (Dean, 2004; Mulford, 2009).

3. Preparation of phosphorylated proteins and phosphotransfer assays—The phosphotransfer reactions that comprise the full phosphorelay (Sln1-HK to Sln1-Rec to Ypd1 to Ssk1-Rec or Skn7-Rec) occur very rapidly, within the earliest 8 second time point that can be taken manually at the bench top (Janiak-Spens and West, 2000)(Fig. 3B). Thus, a rapid quench flow instrument is required to carry out initial velocity studies and to measure rate constants for the individual steps (Janiak-Spens *et al.*, 2005). This approach is the subject of a separate chapter in this volume (Kaserer, A., Andi, B., Cook, P.F. and West, A.H. “Kinetic measurements for studying phosphorelay signaling pathways”). Here, we provide methods and strategies for obtaining phosphorylated proteins and for *in vitro* phosphotransfer assays, in which distribution of radiolabeled phosphoryl groups can be monitored over a time course of 5–60 minutes. These procedures have been particularly useful for characterizing mutant proteins (Li *et al.*, 1998; Ault *et al.*, 2002; Tao *et al.*, 2002; Ault, 2001; Janiak-Spens *et al.*,

2005; Janiak-Spens and West, 2000) and for examining half-lives of phosphorylated receiver domains (Janiak-Spens *et al.*, 2000; Janiak-Spens *et al.*, 1999).

a. Autophosphorylation of GST-tagged Sln1-HK bound to glutathione-coupled resin:

Incubate approximately 3 μM of bead-bound protein with 7 μM [γ - ^{32}P]-ATP (30 Ci/mmol) in 50 mM Tris-HCl pH 8.0, 100 mM KCl, 10 mM MgCl_2 , 2 mM DTT, 20% glycerol for 30–60 minutes at room temperature in a total reaction volume of 100 μl . Gently pellet the resin-bound GST-Sln1-HK~P (100 $\times g$ for 1 minutes), wash three times with 100 μl wash buffer (50 mM Tris-HCl pH 8.0, 50 mM KCl, 10 mM MgCl_2 , 1 mM DTT) to remove unincorporated radiolabel (Fig. 2A). Resuspend final pellet in 100 μl wash buffer. A typical autophosphorylation time course is shown in Fig. 2B. The phosphorylated kinase is stable for 8 hours at room temperature. This preparation can be used immediately for phosphotransfer assays (see for example Fig. 2C) or stored frozen in aliquots at -20°C for about a week. For some applications (e.g. mutant studies of the HK domain itself), it may be useful to elute the GST-Sln1-HK protein from the resin or remove the GST tag using thrombin (Fig. 2A).

To examine levels of autophosphorylation or to evaluate phosphotransfer from component to component in the pathway as described in subsequent sections, radiolabeled reaction products are typically separated on 10–15% SDS-polyacrylamide gels²⁴. The wet gel should be wrapped in plastic wrap and exposed to a phosphorimager screen for quantification of radiolabeled band intensity.

b. Phosphorylation of Sln1 receiver domain for half-life studies, mutant studies or phosphotransfer assays:

Phosphorylation of Sln1-Rec can be achieved by direct phosphotransfer from Sln1-HK~P (Fig. 3A). Incubate 7 μM GST-Sln1-HK bound to glutathione-Sepharose beads (as described in B. 2a) with 7 μM [γ - ^{32}P]-ATP (30 Ci/mmol), 60 μM unlabeled ATP²⁵, and 30 μM Sln1-Rec in 50 mM Tris-HCl pH 8.0, 100 mM KCl, 15 mM MgCl_2 , 2 mM DTT, and 20% glycerol for 30 minutes at room temperature in a total volume of 100 μl . Centrifuge the mixture (1 minute at 100 $\times g$) to pellet the resin-bound GST-Sln1-HK and recover P~Sln1-Rec in supernatant. This preparation should be used immediately for half-life studies (see Section B. 3e) or, for example, in vitro phosphotransfer reactions involving Ypd1 (Fig. 3B).

c. Phosphorylation of Ypd1 for half-life studies, mutant studies or phosphotransfer assays:

Incubate GST-Sln1-HK-Rec (1 μM) bound to glutathione-Sepharose beads (as described in B. 1a) with 7 μM [γ - ^{32}P]-ATP (30 Ci/mmol), 100 μM unlabeled ATP, and 40 μM YPD1 in 50 mM Tris-HCl pH 8.0, 100 mM KCl, 15 mM MgCl_2 , 2 mM DTT, and 20% glycerol for 90 minutes at room temperature in a total volume of 100 μl (Fig. 4). Centrifuge the mixture (1 minutes at 100 $\times g$) to pellet the resin-bound GST-Sln1-HK-Rec and recover P~Ypd1 in the supernatant. This preparation can then be used immediately for half-life studies or phosphotransfer experiments involving receiver domains (see Ypd1 mutant study, for example, in Fig. 4B).

d. Phosphorylation of Ssk1 and Skn7 receiver domains for half-life studies, mutant studies or phosphotransfer assays:

The most efficient manner of phosphorylating the Ssk1 and Skn7 receiver domains is through the full phosphorelay (Sln1-HK to Sln1-R1 to Ypd1). However, one can phosphorylate Ssk1-Rec and Skn7-Rec directly using bead-bound Sln1-HK~P as a phosphodonor (as described for Sln1-Rec), but a longer incubation time is necessary (up to 90 minutes) (Fig. 3). This route reduces the number of phosphotransfer and intermediary

²⁴Do not boil samples prior to gel loading as this increases the rate of phosphate hydrolysis.

²⁵Addition of unlabeled ATP is included to drive stoichiometric labeling of Sln1-Rec.

purification steps. The phosphorylated receiver domain(s) should be used immediately due to its short half-life (Janiak-Spens *et al.*, 2000).

e. Measurement of the phosphorylated lifetime of SLN1 pathway components: Response regulator proteins are activated and deactivated by phosphorylation, and the lifetime of the phosphorylated state often correlates with the duration of the output response. Thus, it is important to measure the half-life of the phosphorylated protein domains and to keep the half-life in mind when setting up in vitro time-dependent phosphotransfer experiments. One can obtain phosphorylated Sln1, Ssk1 or Skn7 receiver domains by incubation with ^{32}P -labeled GST-Sln1-HK as described in Sections B. 3b and B. 3d (Fig. 3A). The phosphodonor should be removed by centrifugation and the isolated phosphorylated receiver domain is then recovered in the supernatant. Incubate approximately 5 μM of P~receiver in 50 mM Tris-HCl pH 8.0, 10 mM MgCl_2 , 1 mM DTT in a total volume of 50 μl at room temperature. Remove aliquots (4.5 μl) at various time points and mix with 4X stop buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 60 mM EDTA, 40% glycerol, 0.0008% bromophenol blue) to terminate the reaction. Freeze the aliquots at -20°C until SDS-PAGE analysis. Determine the relative amounts of phosphorylated protein remaining at each time point by phosphorimager quantitation. Plot $\ln$ (% P~receiver remaining) vs. time. The rate constant (k) for the dephosphorylation reaction is determined by least-squares fitting of the natural logarithm of the data to a linear relationship assuming first-order kinetics. The half-life of the phosphorylated receiver domain²⁶ is calculated accordingly ($t_{1/2} = \ln 2/k$). Phosphorylated Ypd1 can be obtained as described in Section B.3.c and the rate constant for dephosphorylation²⁷ is measured in the same manner as for receiver domains.

f. Phosphotransfer or phosphorelay experiments: To monitor Sln1-Rec to Ypd1 phosphotransfer (see Fig. 3B for example), incubate P~Sln1-Rec with Ypd1 (approximately equimolar amounts) in a total volume of 15 μl in reaction buffer (50 mM Tris pH 8.0, 30 mM KCl, 10 mM MgCl_2 , 1 mM DTT) for 5 minutes at room temperature. Stop the reaction by adding 5 μl of 4X stop buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 60 mM EDTA, 40% glycerol, 0.0008% bromophenol blue). For time-course experiments, the reactions are prepared similarly except that the initial volume is 100 μl and aliquots (9 μl) can be removed at various time points, mixed with 4X stop buffer and keep on ice until gel analysis.

To assay phosphotransfer from P~Ypd1 to Ssk1-Rec, Skn7-Rec (or the reverse phosphotransfer to Sln1-Rec), follow the same procedure as above, except P~Ypd1 (1–2 μM) is typically incubated with slight molar excess of receiver domain (see for example Fig. 4B).

Phosphorelay experiments involving all protein components (Fig. 5A) in the reaction mix are typically carried out by incubating equimolar amounts ($\sim 2 \mu\text{M}$) of either P~GST-Sln1-HK or P~Sln1-Rec as the initial phosphodonor, and Ypd1 and Ssk1-Rec (or Skn7-Rec) for 5 min in reaction buffer at room temperature. Representative phosphorelay experiments are shown in Fig. 5B and 5C.

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²⁶The half-lives of P~Sln1-Rec, P~Ssk1-Rec and P~Skn7-Rec are 13 ± 2 minutes, 13 ± 3 minutes, and 144 ± 6 minutes, respectively (Janiak-Spens *et al.*, 2000). However, in the presence of Ypd1, the lifetime of P~Ssk1-Rec is dramatically extended to 2280 ± 240 minutes presumably through complex formation that shields the phospho-aspartate from hydrolysis. This stabilization effect is specific to the Ssk1-Rec domain and is not observed for Sln1-Rec or Skn7-Rec.

²⁷The half-life for P~Ypd1 is 4.1 ± 0.8 hr (Janiak-Spens and West, 2000).

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Figure 1A Fassler and West

Figure 1B Fassler and West

Regulation	Molecular Circuit
1. Kinase Regulation	$\text{Sln1 Kinase} \xrightleftharpoons[\text{osmotic stress}]{\text{wall stress}} \text{Sln1 Kinase-P}$
2. Hog1 Activation	$\text{Sln1 Kinase} / \text{Ypd1} / \text{Ssk1} \longrightarrow \text{Ssk2/22-P} / \text{Pbs2-P} / \text{Hog1-P}$
3. Skn7 Activation & Hog1 Inactivation	$\begin{aligned} \text{Sln1 Kinase-P} / \text{Ypd1-P} / \text{Skn7-P} &\longrightarrow \uparrow \text{Skn7 target genes} \\ \text{Ssk1-P} &\longrightarrow \downarrow \text{Hog1} \end{aligned}$

Figure 1C Fassler and West

Figure 1.

SLN1 pathway organization and regulation. (a) Schematic showing flow of phosphoryl groups through the SLN1 pathway. Plasma membrane associated Sln1 kinase is autophosphorylated on the H576 residue under normal growth conditions. Hyper-activation occurs under conditions causing weakening of the cell wall (wall stress). Hyperosmolarity and other conditions causing reduced turgor lead to a reduction in Sln1 kinase activity and accumulation of Sln1 in the dephosphorylated form. The phosphoryl group on H576 (H) is transferred to D1144 (D) in the receiver domain of Sln1 and then to H64 of the phosphotransferase, Ypd1, and finally to D554 and D427 in the receiver domains of the Ssk1 and Skn7 response regulators, respectively. When Ssk1 is unphosphorylated it interacts with and stimulates the activity of the Ssk2 and Ssk22 MAPKKKs of the HOG1 pathway. Phosphorylation of Ssk1 renders it inactive with respect to the HOG1 pathway. Phosphorylation of Skn7 leads to activation of *SKN7* dependent genes such as the mannosyltransferase, *OCH1*. (b) Schematic depicting the domain structure of Sln1. Two molecules of a Sln1 dimer are shown associated with the plasma membrane via two transmembrane domains (TMDs). An extensive extracellular domain (ECD) protrudes into the periplasmic space. This protein configuration is conducive to detection of changes in pressure against the membrane (turgor pressure) and changes in composition of the cell wall which may lead to altered rigidity. The stimulus is transduced through several juxtamembrane domains including a coiled-coil domain (CC) which appears to be contiguous with the phosphoaccepting H576 residue. Rotation of the coiled-coil domain in response to a stimulus is thought to regulate access of H576 to the kinase domain (HK) and hence the level of kinase phosphorylation. The structure of the *Thermotoga maritima* TM0853 HK domain (PDB ID 2C2A) (Marina *et al.*, 2005) is depicted here. Transfer of the H576 phosphoryl group to the receiver domain (Rec) is a prerequisite for subsequent phosphotransfer to Ypd1 and the response regulators Ssk1 and Skn7 (not shown). The structure of the Sln1-Rec domain is shown here (PDB ID 2R25) (Zhao *et al.*, 2008) and the dashed lines represent linker regions of unknown structure. (c) Several types of regulation are embodied in the Sln1 pathway. (1) The Sln1 kinase itself is positively regulated by changes in the cell wall structure, and negatively regulated by hyper-osmolarity and other situations leading to reduced turgor. (2) The HOG1 pathway is positively regulated by an inactive SLN1 pathway. Only the dephosphorylated form of the Ssk1 response regulator stimulates the Ssk2 and Ssk22 MAPKKKs of the HOG1 pathway. Finally, (3) the HOG1 pathway is inactivated and Skn7 is activated by the phosphorylated SLN1 pathway intermediates.

Figure 2.

Autophosphorylation of Sln1-HK. (a) Schematic showing two alternate routes for working with resin-bound GST-Sln1-HK. The Sln1-HK domain can be cleaved from the GST tag and eluted from the resin using thrombin. The isolated Sln1-HK domain can then be autophosphorylated using radiolabeled ATP. This strategy is best for working with Sln1-HK mutants. A convenient alternative is to autophosphorylate GST-Sln1-HK while bound to resin. Bead-bound phospho-GST-Sln1-HK can then be used directly as a phosphodonor for in vitro phosphotransfer assays. (b) A representative autophosphorylation time-course for soluble Sln1-HK (reproduced from Tao *et al.*, 2002). Sln1-HK (2 μg) was incubated with [γ - ^{32}P]ATP in reaction buffer and aliquots were removed at the times indicated and quenched in SDS loading buffer. Maximal levels of autophosphorylation are typically reached in 30–40 minutes (c) An example of phosphotransfer from bead-bound GST-Sln1-HK to the Sln1-Rec domain (reproduced from Ault *et al.*, 2002). ^{32}P -labeled GST-Sln1-HK (2.5 μg) was incubated alone (lane 1) or with 1 μg Sln1-Rec for 5, 15, 30, or 60 minutes (lanes 2–5, respectively). Most of

the radiolabel is transferred within 5 minutes and the gradual loss of radiolabel from Sln1-Rec is due to its intrinsic rate of dephosphorylation.

Figure 3.

Phosphorylation of receiver domains. (a) Schematic showing strategy for co-incubation of bead-bound GST-Sln1-HK and receiver domains in the presence of [γ - ^{32}P]ATP for a period of time (~30 minutes) that allows for autophosphorylation of GST-Sln1-HK and rapid phosphotransfer to the receiver domain. The phosphorylated receiver domain can then be recovered in the supernatant by pelleting the bead-bound GST-Sln1-HK. The isolated P~Rec can then be used for various in vitro biochemical studies. (b) An example phosphotransfer experiment using Sln1-Rec~P as a phosphodonor to Ypd1 (reproduced from Janiak-Spens *et al.*, 2000). Isolated ^{32}P -labeled Sln1-Rec (1.6 μM) was incubated with 2 μM Ypd1 and aliquots were removed at the indicated time points. This time course experiment showed that with respect to distribution of radiolabel for WT proteins, equilibrium is reached at the earliest time point taken (8 sec.). For mutant studies, it is useful to establish a time frame for optimal phosphotransfer.

Figure 4.

Phosphorylation of Ypd1. (a) Schematic depicting strategy for obtaining phosphorylated Ypd1 for subsequent in vitro biochemical studies. Bead-bound GST-Sln1-HK-Rec is co-incubated with [γ - ^{32}P]ATP and Ypd1 and the products separated by centrifugation. (b) An example phosphotransfer experiment in which isolated ^{32}P -labeled Ypd1 (WT) and a number of Ypd1 mutants were examined for their ability to transfer phosphoryl groups to the Ssk1-Rec domain (reproduced from Janiak-Spens *et al.*, 2000).

Figure 5.

Phosphorelay experiments. (a) Schematic diagram representing the purified components of the SLN1 pathway. For phosphorelay experiments, one can start with ^{32}P -labeled GST-Sln1-HK or ^{32}P -labeled Sln1-Rec as the initial phosphodonor. Experiments are conducted with all phosphorelay components in the reaction mix in order to determine the total amount of radiolabel transmitted to the terminal phosphoreceiver (either Ssk1 or Skn7). (b) An example of a phosphorelay assay to examine phosphorylation of GST-Skn7 comparing WT Sln1-Rec to the Sln1* receiver mutant (P1148S). Equimolar concentrations of GST-Skn7, GST-Sln1-HK, Ypd1 and ^{32}P -labeled Sln1-Rec (WT) or Sln1*-Rec were incubated together for 15 minutes (lanes 2 and 4, respectively). Lanes 1 and 3 are the ^{32}P -labeled Sln1-Rec (WT) and Sln1*-Rec, respectively, incubated alone in reaction buffer. This in vitro experiment supported the in vivo genetic results that indicated that the P1148S mutation in the Sln1 receiver domain results in a hyperactive phenotype, *i.e.* an increase in phosphorelay activity leading to a higher level of Skn7 phosphorylation. No reverse phosphotransfer from Sln1-Rec to GST-Sln1-HK was observed (lanes 2 and 4) (reproduced from Ault *et al.*, 2000). (c) An example phosphorelay experiment examining Ypd1-mediated phosphotransfer from Sln1-Rec to Ssk1-Rec. Equimolar concentrations of WT Ypd1 (lane 2) or a H64Q mutant Ypd1 (lane 3) was incubated with ^{32}P -labeled Sln1-Rec and Ssk1-Rec for 5 minutes. Lane 1 contains radiolabeled Sln1-Rec incubated alone. Using WT Ypd1, virtually all the radiolabel is transferred to Ssk1-Rec within 5 minutes (lane 2). In contrast, when the H64 residue within Ypd1 is mutated, little or no phosphotransfer is observed (reproduced from Janiak-Spens *et al.*, 2000).