The yeast histidine protein kinase, Sln1p, mediates phosphotransfer to two response regulators, Ssk1p and Skn7p

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The *Saccharomyces cerevisiae* Sln1 protein is a ‘two-component’ regulator involved in osmotolerance. Two-component regulators are a family of signal-transduction molecules with histidine kinase activity common in prokaryotes and recently identified in eukaryotes. Phosphorylation of Sln1p inhibits the HOG1 MAP kinase osmosensing pathway via a phosphorelay mechanism including Ypd1p and the response regulator, Ssk1p. *SLN1* also activates an *MCM1*-dependent reporter gene, *P-lacZ*, but this function is independent of Ssk1p. We present genetic and biochemical evidence that Skn7p is the response regulator for this alternative Sln1p signaling pathway. Thus, the yeast Sln1 phosphorelay is actually more complex than appreciated previously; the Sln1 kinase and Ypd1 phosphorelay intermediate regulate the activity of two distinct response regulators, Ssk1p and Skn7p. The established role of Skn7p in oxidative stress is independent of the conserved receiver domain aspartate, D427. In contrast, we show that Sln1p activation of Skn7p requires phosphorylation of D427. The expression of *TRX2*, previously shown to exhibit Skn7p-dependent oxidative-stress activation, is also regulated by the *SLN1* phosphorelay functions of Skn7p. The identification of genes responsive to both classes of Skn7p function suggests a central role for Skn7p and the *SLN1*–*SKN7* pathway in integrating and coordinating cellular response to various types of environmental stress.

Keywords: histidine kinase/osmotic stress/oxidative stress/Skn7/Sln1

Introduction

Saccharomyces cerevisiae Sln1p is a histidine kinase belonging to the family of ‘two-component’ regulators which play a prominent role in the response of prokaryotic organisms to the extracellular environment (Ota and Varshavsky, 1993; Alex and Simon, 1994). The term ‘two-component’ refers to the prototype in which component one is a sensor/kinase, consisting of an extracellular domain and a histidine autokinase activity (transmitter),

and component two is a response regulator, consisting of a receiver domain with a conserved aspartate residue. In many cases the response regulator also carries a domain (e.g. DNA-binding domain) responsible for pathway output. Sln1p itself contains both kinase and receiver domains and is thus classified as a hybrid kinase.

One function of Sln1p is to regulate the osmotic response MAP kinase pathway (Maeda *et al.*, 1994, 1995). It does so via a phosphorelay system involving Ypd1p and the response regulator, Ssk1p (Posas *et al.*, 1996). The initial event in *SLN1* signaling is autophosphorylation of histidine 576 (Posas *et al.*, 1996). This phosphate is transferred to a conserved aspartate (D1144) in the Sln1p-associated receiver domain. Sln1p D-P is subsequently transferred to a histidine on the small phosphorelay molecule, Ypd1p. Finally Ypd1p-P is transferred to an aspartate in the receiver domain of the response regulator, Ssk1p. Unphosphorylated Ssk1p interacts physically with Ssk2p and Ssk22p (Posas and Saito, 1998), two MEK kinases whose activation ultimately leads to Hog1p phosphorylation and the changes in gene expression required for survival in a hyperosmotic environment (Brewster *et al.*, 1993). Under normal conditions, phosphorylation of Sln1p and the downstream (effector) proteins, Ypd1p and Ssk1p, prevent activation of the HOG1 MAP kinase pathway. In contrast, hyperosmotic conditions promote dephosphorylation of Sln1p and consequently activate the HOG1 MAP kinase pathway (Posas *et al.*, 1996).

In addition to regulating the HOG1 osmotic-response pathway, a separate role for *SLN1* was revealed using the *MCM1*-dependent reporter gene, *P-lacZ*. Deletion of the *SLN1* gene reduces reporter gene expression 10-fold, while activating alleles of *SLN1* (*sln1**) increase reporter gene expression between three- and fivefold (Yu *et al.*, 1995; Fassler *et al.*, 1997; Tao *et al.*, 1998). *SLN1* regulation of the HOG1 and *P-lacZ* pathways appears to be reciprocal since *sln1** mutations not only increase the activity of the *P-lacZ* reporter but also decrease Hog1p phosphorylation and enhance cellular sensitivity to hyperosmotic conditions (Fassler *et al.*, 1997). Neither Hog1p nor Ssk1p is directly involved in *SLN1*-mediated *P-lacZ* regulation since *HOG1* and *SSK1* deletions do not affect reporter activity (Yu *et al.*, 1995; Fassler *et al.*, 1997). These observations, together with the mapping of *sln1** mutations to residues in *SLN1* that possibly affect rates of phosphotransfer (Fassler *et al.*, 1997), led to a model for Sln1p function in which the HOG1 MAP kinase cascade represents one branch of a bifurcated pathway and the *P-lacZ* reporter represents a second branch. The HOG1 branch is activated by the accumulation of unphosphorylated Sln1p while the *P-lacZ* branch is postulated to be activated by the accumulation of phosphorylated Sln1p. The effect of *sln1** mutations was hypothesized to shift the equilibrium

between unphosphorylated (Sln1p-0) and phosphorylated Sln1p (Sln1p-P) to favor Sln1p-P. In the context of this hypothesis, Sln1p activation of the *P-lacZ* reporter was presumed to involve the transfer of phosphate from Sln1p to intermediate signaling molecules. Proteins with homology to two-component regulators, including the phosphorelay protein, Ypd1p required for SLN1–HOG1 signaling (Posas *et al.*, 1996) and the response regulator homolog, Skn7p (Brown *et al.*, 1993; Krems *et al.*, 1996), were obvious candidates.

Although *SKN7* was previously ruled out as a potential intermediate in the SLN1–HOG1 pathway (Brown *et al.*, 1994), our current studies indicate that *SKN7* plays an important role in SLN1 signaling. Specifically, *SKN7* was uncovered in our genetic screen to define genes which, when overexpressed, result in activation of the *P-lacZ* reporter gene, as seen in *sln1** mutants (Yu *et al.*, 1995). Involvement of the Skn7p response regulator in a SLN1-regulated phosphotransfer pathway is supported by two additional lines of evidence presented in this paper: (i) deletion of the *SKN7* gene or mutation of the conserved receiver domain aspartate blocked the activating phenotype of *sln1** mutations; and (ii) *in vitro* phosphotransfer experiments demonstrating Ypd1p-dependent phosphorylation of Skn7p by Sln1 kinase using recombinant proteins. The existence of a Sln1–Skn7 pathway was also the recent conclusion of Ketela *et al.* (1998) to explain genetic data which suggest that Skn7p functions downstream of Sln1p.

Skn7 was identified previously in two high-copy genetic screens. One screen was for high-copy suppressors of a cell-wall defect caused by deletion of the *KRE9* gene (Brown *et al.*, 1993). The second screen was for genes that could bypass the usual requirement for the Swi6 protein in directing the G₁-specific cell-cycle expression of a reporter gene normally driven by a protein complex including the Mbp1 and Swi6 proteins (Morgan *et al.*, 1995). In addition, mutations in *SKN7* were identified in a screen for mutants hypersensitive to oxidative stress (Krems *et al.*, 1996).

The results in this paper establish that the Sln1-regulated osmotic-stress pathway is a bifurcated pathway in which Sln1 kinase signals via Ypd1p to two distinct response regulators, Ssk1p and Skn7p. Several lines of evidence indicate that activation of Skn7p by Sln1p is distinct from the previously described activation of Skn7p by oxidative stress (Morgan *et al.*, 1997). Although the two pathways are distinct, we have found at least one gene, *TRX2* that is responsive to both pathways. We therefore conclude that the SLN1–SKN7 branch of the osmotic-response pathway and the SKN7 oxidative-stress pathway may converge on a common set of target genes. The participation of Skn7p in both the osmotic and oxidative-stress pathways may coordinate patterns of gene expression in response to distinct types of environmental stress.

Results

Multicopy genes stimulate expression of the MCM1-dependent reporter gene, *P-lacZ*

To identify potential signaling intermediates that function between Sln1p and Mcm1p, we introduced a high-copy yeast genomic library into wild-type strain JF1493 which

carries a single integrated copy of the *P-lacZ* reporter gene (Yu *et al.*, 1995) in which a palindromic Mcm1-binding site was substituted for the UAS of a *CYC1-lacZ* fusion gene (see Materials and methods). The starting strain exhibited a white phenotype when plated on media containing 150 µg/ml X-gal due to low expression of the reporter. Eleven thousand transformants were replica plated onto X-gal media and colonies with increased blue color were selected for testing by liquid β-galactosidase assays. Plasmids isolated from the best candidates were retransformed into the starting strain to verify that the X-gal phenotype was plasmid associated.

Eight clones were examined by sequence analysis of the insert ends and found to represent six regions of the genome (Table I). Subcloning and deletion experiments have identified the genes responsible for *P-lacZ* activation in several of the high-copy clones, including *BCK2*, a gene encoding a serine/threonine-rich protein with a role in the regulation of G₁ cyclin genes (Lee *et al.*, 1993; Epstein and Cross, 1994), *MSS11*, a gene involved in starch regulation (Webber *et al.*, 1997), *RCK2*, encoding a Ca²⁺/calmodulin-kinase-like protein kinase (Dahlkvist and Sunnerhagen, 1994; Melcher and Thorner, 1996) and *SKN7*. Since the Skn7 protein includes a receiver domain homologous to those of two-component response regulators (Brown *et al.*, 1993), we sought to further examine its involvement in SLN1-signal transduction. Both library plasmid, p49-1 and an independent *SKN7*-containing plasmid (gift of H. Bussey) were introduced into reporter strain, JF1493. Each caused a fivefold increase in *P-lacZ* activity when compared with the same strain transformed with the corresponding empty vector (data not shown).

***skn7* deletion suppresses the effect of *sln1*-activating mutations**

We reasoned that genes actually involved in SLN1 signaling should also exhibit a change in *P-lacZ* activity when deleted. To examine the requirement for *SKN7* in SLN1 signaling we deleted the *SKN7* gene in strains bearing the 2 µ *P-lacZ* reporter and containing either wild-type or activated alleles of *SLN1* (*sln1**). Strains containing activated *sln1* alleles (formerly designated *nnp2*) exhibit three to five times the *P-lacZ* reporter activity of strains with the wild-type *SLN1* allele (Yu *et al.*, 1995; Fassler *et al.*, 1997; Tao *et al.*, 1998). Deletion of *SKN7* abolished the increase usually seen in *sln1** mutants (Table II). To verify that the reduction in reporter activity was in fact due to the absence of *SKN7*, a CEN-based *SKN7* plasmid was introduced into each *skn7-Δ* strain. Introduction of the *SKN7* plasmid into a *sln1** strain increased reporter activity 14-fold, demonstrating that the reduction in reporter activity in the *sln1* skn7-Δ* strain was due to the absence of *SKN7*. We postulate that the absence of an effect of the *skn7-Δ* on *P-lacZ* activity in the *SLN1* background reflects the existence of *SKN7*-independent activators of the *P-lacZ* reporter gene. The change in the *sln1** phenotype due to loss of *SKN7* indicates that Skn7p may play an important role in signaling to *P-lacZ* and that *SKN7* is likely to function downstream of *SLN1*.

Interestingly, levels of reporter activity were significantly higher in strains carrying the centromere-based *SKN7* plasmid than in those carrying a single *SKN7* gene (compare JF1787 with JF1331 and JF1795 with JF1359).

Table I. High copy activators of the P-*lacZ* reporter gene

Candidate clone name	Chromosome No.	Coordinates	Complete ORFs ^a
15-1	5	517031–524655	BCK2 , CCA1
49-1	8	512484–515400	SKN7
7-1	12	439956–445969	STM1, YLR151C, YLR152C
N54-1	2	95340–102411	AST1, PRS45 UBP13 SEF1, KIP1
N50-2	13	586619–595000	MSS11 , SMP2, YMR166C
N62-1	12	633470–642000	RCK2 , YEF3, SSP120, YLR251W, YLR252W

^aBoldface type is used in cases where the ORF responsible for the X-gal phenotype has been definitively determined by subcloning or deletion experiments.

Table II. Changes in *SKN7* dosage affect *SLN1* signaling to the P-*lacZ* reporter

Strain ^a	Relevant genotype ^b	CEN plasmids ^c	Normalized β -galactosidase activity ^d
JF1331	<i>SLN1 SKN7</i>	none	120
JF1738	<i>SLN1 skn7-Δ</i>	none	100
JF1359	<i>sln1* SKN7</i>	none	457
JF1739	<i>sln1*skn7-Δ</i>	none	114
JF1787	<i>SLN1 skn7-Δ</i>	<i>SKN7</i>	236
JF1790	<i>SLN1 skn7-Δ</i>	vector	100
JF1795	<i>sln1* skn7-Δ</i>	<i>SKN7</i>	1554
JF1798	<i>sln1* skn7-Δ</i>	vector	108

^aAll strains carry pGY48, the 2μ P-*lacZ* reporter plasmid (Yu *et al.*, 1995).

^b*sln1** refers to the *sln1-21* allele (Fassler *et al.*, 1997).

^cPlasmids: CEN *SKN7*, pSL232; vector, pRS315.

^d β -galactosidase values are reported as the average of between three and six trials involving at least three individual colonies or transformants. Average values were normalized to the *SLN1 skn7-Δ* strain in each experimental set. Standard deviations were less than 25% of the average. The actual average value for a *SLN1 skn7Δ* strain was 84 Miller units (Miller, 1972).

Thus, even a small increase in *SKN7* dosage increased activity of the P-*lacZ* reporter. The threefold increase in P-*lacZ* reporter expression in a *sln1** strain carrying the CEN-*SKN7* plasmid versus the *sln1* SKN7* strain suggests that Skn7p may normally be limiting in the *sln1** mutant.

Skn7p D427 is required for SLN1 signaling to the P-lacZ reporter

The Skn7 protein contains a typical receiver domain characterized by a conserved aspartate. Previous studies have shown that some, but not all, of the functions of Skn7p are dependent on this aspartate (Brown *et al.*, 1994; Morgan *et al.*, 1995, 1997). To examine the role of D427 in *sln1** activation of the P-*lacZ* reporter, we introduced a plasmid-borne *skn7* D427N mutant allele into the *sln1* skn7-Δ* strain and measured β -galactosidase activity from the P-*lacZ* reporter gene. The *skn7-Δ* P-*lacZ* phenotype was unchanged in the presence of the *skn7* D427N allele (Table III). Thus P-*lacZ* activation requires residue D427 of Skn7p. The role of D427 in the *SKN7* overexpression phenotype was also investigated. Activation of the P-*lacZ* reporter by elevated *SKN7* dosage also required the receiver domain aspartate (data not shown). Hence Sln1p activation requires the conserved aspartate in the Skn7p receiver domain. The D dependence of the deletion and overexpression phenotypes is consistent with a role for Skn7p as a response regulator protein in a two-component signaling cascade involving the Sln1p kinase and Skn7p

Table III. Effect of *SKN7* mutations on *sln1** activation of the P-*lacZ* reporter activity

Strain ^a	Relevant genotype ^b	CEN plasmid ^c	Normalized β -galactosidase activity ^d
JF1790	<i>SLN1 skn7-Δ</i>	vector	100
JF1787	<i>SLN1 skn7-Δ</i>	<i>SKN7</i>	174
JF1788	<i>SLN1 skn7-Δ</i>	<i>skn7</i> D427N	91
JF1789	<i>SLN1 skn7-Δ</i>	<i>skn7</i> D427E	639
JF1798	<i>sln1* skn7-Δ</i>	vector	100
JF1795	<i>sln1* skn7-Δ</i>	<i>SKN7</i>	1039
JF1796	<i>sln1* skn7-Δ</i>	<i>skn7</i> D427N	103
JF1797	<i>sln1* skn7-Δ</i>	<i>skn7</i> D427E	475

^aAll strains carry the 2μ P-*lacZ* reporter, pGY48 (Yu *et al.*, 1995).

^bIn this table the *sln1** mutation refers to the *sln1-21* allele (Fassler *et al.*, 1997).

^c*SKN7* plasmids are centromere based. *SKN7*, pSL232; *skn7* D427N, pCLM699; *skn7* D427E, pCLM700; vector, pRS315.

^d β -galactosidase values are as described in the legend to Table II. The standard deviations in these experiments were <15%.

response regulator. To further investigate the importance of D427, a *skn7* D427E mutant was introduced into *SLN1 skn7-Δ* and *sln1* skn7-Δ* strains. A change from aspartic acid to glutamic acid was shown previously to force the receiver domain of Skn7p into an active conformation (Brown *et al.*, 1994). The presence of the *skn7* D427E allele elevated P-*lacZ* levels 3.7-fold in the *SLN1⁺ skn7-Δ* strain and this level was not stimulated further by the presence of the *sln1** mutation (Table III). Thus, the D427E mutation partially mimics the effect of a *sln1** mutation.

YPD1 is required for SLN1 signaling to P-lacZ

In the *SLN1*–HOG1 pathway, Sln1p histidine phosphate is transferred first to a conserved aspartate in the Sln1p-associated receiver domain, then to a histidine in Ypd1p and finally to a conserved aspartate in the receiver domain of the response regulator, Ssk1p (Posas *et al.*, 1996). If the *SLN1* pathway that leads to P-*lacZ* activation is organized analogously, phosphate from the Sln1p-associated receiver domain would be transferred to the receiver domain of the response regulator, Skn7p via a phosphorelay intermediate. To evaluate the possibility that Ypd1p, the phosphorelay intermediate in the *SLN1*–HOG1 pathway, might also be involved in the *SLN1*–*SKN7* signal transmission, we constructed a *ypd1* deletion mutant. Like *SLN1*, *YPD1* negatively regulates the activity of the HOG1 pathway by maintaining Ssk1p in the phosphorylated state. Since the presence of *SSK1* (in the unphosphorylated form) is required to activate the MEK kinases in the

Table IV. *YPD1* is required for *SLN1* signaling to the *P-lacZ* reporter

Strain ^a	Relevant genotype ^b	CEN plasmid ^c	Normalized β -galactosidase activity ^d
RJY1209	<i>SLN1 ssk1-Δ ypd1-Δ</i>	vector	100
RJY1212	<i>sln1* ssk1-Δ ypd1-Δ</i>	vector	103
RJY1209	<i>SLN1 ssk1-Δ ypd1-Δ</i>	<i>YPD1</i>	158
RJY1212	<i>sln1* ssk1-Δ ypd1-Δ</i>	<i>YPD1</i>	812

^aAll strains carry the 2 μ *P-lacZ* reporter, pGY48 (Yu *et al.*, 1995).

^bIn this table, *sln1** refers to the *sln1-21* allele (Fassler *et al.*, 1997).

^cCEN*YPD1* plasmid, pCLM659.

^d β -galactosidase values were calculated as described in the legend to Table II. In this experiment, standard deviations were <20% of the average with the exception of RJY1209 carrying the *YPD1* plasmid for which the standard deviation was 38% of the average value. Average values were normalized to the *ssk1Δ ypd1Δ* strain. The actual average value of this strain was 66 Miller units (Miller, 1972).

HOG1 pathway, *SSK1* deletion suppresses the inviability of *sln1* and *ypd1* deletions (Maeda *et al.*, 1994; Posas *et al.*, 1996). We have shown previously that the *ssk1* mutation has no significant effect on *P-lacZ* expression on its own (Fassler *et al.*, 1997), and hence we were able to evaluate the effect of a *YPD1* deletion on *P-lacZ* expression in *SLN1* and *sln1** strains deleted for *SSK1*. The results (Table IV) show that the *YPD1* deletion prevents the activation conferred by the *sln1** mutation. Thus, the Ypd1p phosphorelay intermediate is shared between the HOG1 and *P-lacZ* pathways.

Phosphotransfer from *Sln1p* to *Ypd1p* to *Skn7p*

Our genetic analysis suggested that activation of the *P-lacZ* reporter depends on a phosphorelay involving *Sln1p*, *Ypd1p* and *Skn7p*. To gain biochemical support for this hypothesis we constructed and separately purified recombinant glutathione *S*-transferase (GST)-*Sln1p* kinase domain and GST-*Sln1p*-associated receiver domain, as well as GST-*Ypd1p* and GST-*Skn7p* with which to reconstitute the proposed phosphorelay. We have shown previously that the *Sln1* kinase domain and *Sln1* receiver domain can complement a *sln1-Δ* mutation when expressed as separate molecules in the same yeast cell (Fassler *et al.*, 1997). *Sln1p* kinase was first phosphorylated by incubation with [γ -³²P]ATP and then mixed with *Sln1p* receiver, *Ypd1p* and *Skn7p*. In mixtures containing all components, the labeled *Sln1p* phosphate was distributed rapidly between the *Sln1p* kinase, *Sln1p* receiver, *Ypd1p* and *Skn7p* (Figure 1, lane 4). The phosphorelay required the presence of all four components. Omission of either the *Sln*-associated receiver (Figure 1, lane 6) or GST-*Ypd1p* (lane 5) blocked phosphorylation of the *Skn7p* response regulator. Finally, substitution of the presumed phosphorylated aspartate of *Skn7p* with asparagine (D427N) completely abolished GST-*Skn7p* phosphorylation (Figure 1, lane 7). Taken together, the genetic and biochemical data strongly suggest that *Sln1p* regulation of *P-lacZ* reporter activity involves the histidine autokinase activity of *Sln1p* and phosphorelay to *Ypd1p* and *Skn7p*.

Sln1p, *Ypd1p* and D427 of *Skn7p* are dispensable for the oxidative-stress response

In contrast to the proposed dependence of *Skn7p* phosphorylation for *P-lacZ* regulation, the function of *Skn7p*

Fig. 1. *In vitro* phosphorylation of GST-*Sln1p* and *Ypd1p*-dependent phosphotransfer to GST-*Skn7p*. The *Sln1p*-associated histidine kinase domain, receiver domain, *Ypd1p*, and *Skn7p* were purified as GST fusion proteins from *E.coli* as described in Material and methods. Incubation of [γ -³²P]ATP with GST-*Sln1p* kinase resulted in phosphorylation of a protein of the expected molecular weight (lane 1). Subsequent addition of *Sln1p* receiver alone (lane 2) or together with GST-*Ypd1p* (lane 3) and GST-*Skn7p* (lane 4) resulted in phosphorylation of proteins of the expected sizes. Omission of *Sln1p* receiver (lane 5) or GST-*Ypd1p* (lane 6) abolished the phosphorylation and subsequent phosphorelay. Substitution of GST-*Skn7p* with GST-*Skn7p*(D427N) prevented phosphorylation (lane 7). The migration position of each fusion protein determined by Coomassie Blue staining is indicated to the right and the relative mobilities of pre-stained molecular weight standards (Amersham) to the left of the autoradiogram.

in the oxidative-stress response is independent of D427 since both peroxide resistance and inducibility of the oxidative-stress gene, *TRX2*, are normal in a *skn7* D427N mutant (Krems *et al.*, 1996; Morgan *et al.*, 1997). Therefore, we did not anticipate that the *P-lacZ* reporter would respond to oxidative stress. Unexpectedly, however, control experiments in which the activity of the *P-lacZ* reporter was assessed after exposure of cells to *t*-butyl hydrogen peroxide revealed that expression of this reporter is activated by oxidative stress. The observation that the *P-lacZ* reporter could be activated both by oxidative stress and by *Sln1p* prompted us to consider the possibility that in the context of this reporter gene the *Sln1p* and oxidative-stress responses might be related. However, tests of the D-dependence of the oxidative-stress response of the *P-lacZ* reporter ruled out this possibility. The oxidative-stress responsiveness of the *P-lacZ* reporter was independent of D427 (Table V, rows 1–3). Hence the *P-lacZ* reporter is regulated by both the D-dependent and the D-independent aspects of *Skn7p* function.

The lack of a requirement for D427 in oxidative stress implies that *Sln1p* and *Ypd1p* are unlikely to be involved in this pathway. Nevertheless, since the osmotic- and oxidative-stress signals both apparently involve *Skn7p*, the possibility that certain *Sln1p* and *Ypd1p* activities might be important in the oxidative-stress response was considered. To evaluate possible roles for *Sln1p* and *Ypd1p* in oxidative stress, the oxidative-stress activation of the *P-lacZ* reporter in double *sln1 ssk1* and *ypd1 ssk1* mutants was tested. In this experiment, the *P-lacZ* reporter in the wild-type strain was induced 5-fold by treatment with *t*-butyl hydrogen peroxide. This induction was eliminated completely in the *skn7-Δ* strain. This indicates that the oxidative-stress response of the *P-lacZ* reporter is entirely *Skn7p* dependent. In contrast, neither the *sln1 ssk1* nor

Table V. Role of Skn7 D427, *SLN1* and *YPD1* in oxidative-stress activation of the *P-lacZ* reporter

Strain	Relevant genotype (plasmid)	<i>P-lacZ</i> ^a		
		β-galactosidase ^b activity (SD) (untreated)	β-galactosidase ^b activity (SD) (t-bH ₂ O ₂)	Induction factor ^c
JF1790	<i>SLN1 skn7</i> -Δ (vector)	83 (8.8)	80 (11.7)	0.96
JF1787	<i>SLN1 skn7</i> -Δ (<i>SKN7</i>)	145 (17.9)	1046 (68.7)	7.2
JF1788	<i>SLN1 skn7</i> -Δ (<i>skn7</i> D427N)	76 (10.8)	695 (86.1)	9.1
JF1331	<i>SLN1 YPD1 SSK1 SKN7</i>	85 (28.3)	436 (114.4)	5.1
JF1432	<i>SLN1 YPD1 ssk1</i> -Δ <i>SKN7</i>	137 (24.0)	616 (131.5)	4.5
JF1735	<i>sln1</i> -Δ <i>YPD1 ssk1</i> -Δ <i>SKN7</i>	116 (15.7)	668 (117.1)	5.8
RJY1209	<i>SLN1 ypd1</i> -Δ <i>ssk1</i> -Δ <i>SKN7</i>	106 (34.7)	851 (260.4)	8.0
JF1738	<i>SLN1 YPD1 SSK1 skn7</i> -Δ	66 (28.0)	63 (21.7)	0.95

^aReporter plasmid: *P-lacZ*, pGY48.

^bβ-galactosidase values are reported in Miller units (Miller, 1972) as the average of at least three and as many as six trials. SD, standard deviation.

^cThe induction factor was calculated by dividing the average value of the stress treated culture by that of the untreated culture.

Table VI. Analysis of the *sln1** effect on *P-lacZ* reporters

Reporter description (UAS)	Reporter plasmid name ^c	β-galactosidase activity (SD) ^a		
		<i>SLN1 skn7</i> -Δ ^b pSKN7 ^c	<i>sln1</i> * <i>skn7</i> -Δ ^b pSKN7 ^c	<i>sln1</i> */ <i>SLN1</i> ratio
CYC1-lacZ (ΔUAS)	pGY79	1.4 (0.4)	2.1 (0.3)	1.5
CYC1-lacZ (4x P)	pCLM771	78.4 (11.8)	210.0 (54.2)	2.6
CYC1-lacZ (4x mutP)	pCLM772	2.8 (0.8)	10.4 (1.6)	3.7

^aβ-galactosidase activities are reported in Miller units (Miller, 1972) as the average of at least four trials. SD, standard deviation.

^bStrains: *SLN1 skn7*Δ, JF1738; *sln1** *skn7*Δ, JF1739. *SKN7* plasmid, pSL232.

^cpGY79 is a 2μ vector carrying a *CYC1-lacZ* fusion gene that includes *CYC1* sequences from +4 to -250 relative to the *CYC1* ATG. Plasmids pCLM771 and pCLM772 are derivatives of pGY79 into which wild-type (pCLM771) or mutant (pCLM772) P site oligonucleotides have been cloned.

the *ypd1 ssk1* double mutant showed any defect in oxidative-stress activation relative to the wild-type strain (Table V). These data support the conclusion that Skn7p is a multifunctional protein with distinct roles in oxidative-stress sensing and in *SLN1* signaling.

SKN7*-dependent regulation of *TRX2* by *Sln1p

Earlier studies suggesting a role for the Mcm1p-binding site in the *P-lacZ* vector in *sln1** activation were based on a comparison between the *P-lacZ* reporter, pGY48 (Yu et al., 1995) which is a derivative of pLG670Z (Guarente and Ptashne, 1981) and reporters harboring alternate UAS elements (Yu et al., 1995). For example, in contrast to the 5-fold effect of *sln1** mutations on the *P-lacZ* reporter, *sln1** mutations had a negligible effect on a reporter containing the natural *CYC1* UAS. To test further the role of the Mcm1-binding site in *sln1** activation, a pair of reporters was constructed consisting of four perfect Mcm1p-binding sites or four mutant binding sites cloned in tandem into the unique *XhoI* site of pGY79 (Materials and methods). The binding-site mutation TTT-CCTAATTAGGAAA→TTTCCTAATTAATAAA is predicted to reduce DNA binding by Mcm1p by ~90% relative to the wild-type site (Acton et al., 1997). As expected, basal activity of pGY79 was increased significantly by the presence of the four Mcm1-binding sites (Table VI) but not by the presence of four mutated Mcm1-binding sites. Furthermore, activity of the 4x *P-lacZ* reporter was increased by the *sln1** mutation. Unexpectedly, however, the 4x mutP-*lacZ* reporter was also stimulated by the *sln1** mutation to an extent indistinguishable

from the 4x *P-lacZ* reporter (Table VI). These results introduce the possibility that the site of activation by *sln1** may be distinct from the binding site for Mcm1p. However, an alternative explanation, that *sln1** activation of the 4x mutP-*lacZ* reporter might be due to residual binding of Mcm1 to the mutant P site, can not be ruled out.

Although the artificial reporter system allowed us to detect the effects of *sln1** mutations and to analyze the *SLN1*–*SKN7* pathway, the possibility that the Mcm1 site may not be critical for *sln1** activation led us to search for bona fide genes whose expression is increased in *sln1** mutants. Since our analysis of the *P-lacZ* reporter implied the presence of both D-dependent (osmotic) and D-independent (oxidative) regulatory elements, we tested whether known *SKN7*-dependent oxidative-stress responsive genes might also exhibit D-dependent regulation. *TRX2* appeared to be a good candidate, since a role for *SKN7* in the oxidative-stress induction of the *TRX2* gene has already been demonstrated (Morgan et al., 1997) and electrophoretic mobility-shift analysis revealed the presence of the Skn7 protein in a specific complex that forms at position -140 to -160 relative to the ATG in the *TRX2* promoter (Morgan et al., 1997). We, therefore, tested whether *TRX2* was responsive to *SLN1* by measuring the activity of a *TRX2-lacZ* reporter in wild-type and *sln1** strains. The *sln1** mutation increased *TRX2-lacZ* reporter activity 2.5-fold, suggesting that *SLN1* does in fact regulate an established oxidative-stress response gene (Table VII). In addition, *SKN7* is required for *sln1** activation of *TRX2*. The 2.5-fold increase in *TRX2-lacZ* activity seen in *sln1** mutants is eliminated in strains lacking *SKN7*. Likewise,

Table VII. Role of *SKN7* in basal and *sln1** activated expression of *TRX2*

Strain	Relevant genotype	β -galactosidase activity (SD) ^a	
		<i>SKN7</i> plasmid	<i>TRX2-lacZ</i> ^b
JF1787	<i>SLN1 skn7-Δ</i>	<i>SKN7</i>	163 (29.0)
JF1788	<i>SLN1 skn7-Δ</i>	<i>skn7 D427N</i>	118 (20.4)
JF1790	<i>SLN1 skn7-Δ</i>	<i>skn7-Δ</i>	13 (1.7)
JF1795	<i>sln1* skn7-Δ</i>	<i>SKN7</i>	394 (90.6)
JF1796	<i>sln1* skn7-Δ</i>	<i>skn7 D427N</i>	118 (30.0)
JF1798	<i>sln1* skn7-Δ</i>	<i>skn7-Δ</i>	15 (2.8)

^a β -galactosidase activities are represented in Miller units (Miller, 1972) and calculated as the average of at least four trials. SD, standard deviation.

^bThe *TRX2-lacZ* reporter plasmid is pJF1078. pJF1078 consists of a 2 μ vector containing 400 bp of sequence upstream of the *TRX2* ATG.

substitution of the Skn7 D427N mutant protein for wild-type Skn7p also eliminated the *sln1** effect (Table VII). Interestingly, deletion of *SKN7* caused a 10-fold reduction in *TRX2* expression in a *SLN1* strain, thus suggesting an additional role (discussed below) for *SKN7* in *TRX2* expression.

Discussion

A *SLN1*–*YPD1*–*SKN7* phosphorelay

The activity of the *P-lacZ* reporter gene was used as a tool to identify intermediates in a new *SLN1*-signaling pathway. We found previously that a certain category of *SLN1* mutations (*sln1**) increased the activity of the *P-lacZ* reporter (Yu *et al.*, 1995). In this study we sought additional genes whose overexpression would cause an increase in the activity of this reporter. This screen led to the re-identification of the *SKN7* gene which encodes a protein containing a domain related to the receiver domain of bacterial two-component proteins (Brown *et al.*, 1993). Our genetic and biochemical analysis supports the idea that both Skn7 and the Ypd1 phosphorelay proteins operate downstream of Sln1p in a signaling pathway leading to activation of the *P-lacZ* reporter and genes such as *TRX2*. Our studies also demonstrate that the conserved aspartate in the receiver domain of Skn7p is essential for this signaling pathway. These genetic data are supported by *in vitro* experiments demonstrating Ypd1p-dependent phosphotransfer from Sln1p to Skn7p, and are consistent with a model in which Sln1p phosphorylation status regulates the activities of two distinct response regulators, Ssk1p and Skn7p. These studies provide direct experimental evidence supporting the model suggested previously by Morgan *et al.* (1997) to explain the apparently distinct roles of Skn7p in D-dependent processes (regulation of cell wall and *G₁* cyclin gene expression) and the D-independent oxidative-stress response, and by Ketala *et al.* (1998) to explain genetic data showing that Skn7p functions downstream of Sln1p and exhibits genetic interactions with the HOG pathway.

Examples of pathways that bifurcate at the level of the response regulator protein are relatively rare. One organizational counterpart is represented by the bacterial chemotaxis system in which the kinase, CheA transduces signals to two response regulators, CheY and CheB.

Phosphorylated CheY interacts with the flagellar switch to cause tumbling behavior while CheB-P is a methyl-esterase that adjusts the signaling state of the receptor (Amsler and Matsumura, 1995). In contrast to the chemotaxis system in which both response regulators directly affect protein activities, the pair of *Escherichia coli* response regulators, NarL and NarP, function by altering patterns of gene expression (Stewart and Rabin, 1995). In the Nar system for the control of nitrate and nitrite metabolism under anaerobic conditions, the circuitry is further complicated by the presence of two sensor kinases. The physiological rationale for a system with two sensor kinases is possibly related to the differential phosphotransfer (kinase and phospho-response regulator phosphatase) activities inherent in the two sensor proteins. Activation of the dual Nar response regulators, each of which also contains a DNA-binding domain, results in more complex patterns of nitrate- and nitrite-regulated gene expression than would be possible in a system with a single response regulator.

On the one hand, in the *SLN1* signaling system, Ssk1p more closely resembles the CheY/B type of response regulator in that its phosphorylation state controls its interaction with and hence the activity of the MEK kinase, Ssk2p (Posas and Saito, 1998). Skn7p, on the other hand, possesses a (HSF-like) domain which may mediate DNA interaction and the capability to activate transcription (Brown *et al.*, 1994; Morgan *et al.*, 1995). Whether these Skn7p activities are relevant to *SLN1*–*YPD1*–*SKN7* signaling is currently under investigation. We postulate that the rationale for the complex organization of *SLN1* signaling pathways is to coordinate metabolic and cell-cycle adjustments to changes in environmental osmolarity.

The conserved aspartate of the Skn7p receiver is required for *SLN1* signaling but not oxidative-stress activation of the *P-lacZ* reporter

The Skn7 protein has several recognizable domains in addition to the receiver domain. As first noted by Brown *et al.* (1993), its N-terminus (aa 87–150) is characterized by its similarity to the DNA-binding domain of members of the eukaryotic heat-shock transcription factor (HSF). Adjacent to the potential HSF DNA-binding domain, Morgan *et al.* (1997) noted a coiled-coil domain also found in HSF, probably involved in protein–protein interactions and required for many aspects of Skn7 function (Alberts *et al.*, 1998). The C-terminus of the protein (497–622 end) is glutamine rich. Whether these domains function together with or independent of the receiver is unclear. Although the presence of a receiver domain suggests that the activity of the Skn7 protein may be regulated by aspartyl phosphorylation, mutation of the critical aspartate to asparagine (D427N) abolishes some but not all Skn7p functions. For example, the D427N mutation eliminates suppression of both the *kre9-Δ* cell-wall defect and the *swi6-Δ* cell-cycle gene expression defect (Brown *et al.*, 1994; Morgan *et al.*, 1995), but does not confer sensitivity to oxidative stress (Morgan *et al.*, 1997). These observations indicate that the various functions of Skn7p in the cell may involve distinct mechanisms.

Our studies on the effects of the *skn7* D427N mutation on *P-lacZ* expression are consistent with the conclusion that Skn7p has distinct roles to play in the cell. As

Fig. 2. Model for the coordinated regulation of osmotic response and oxidative response genes by the SLN1 and SKN7 pathways. The Sln1p equilibrium is shifted to favor the phosphorylated or unphosphorylated state depending on the osmotic environment. Hyperosmotic stress diminishes Sln1p phosphorylation (Posas *et al.*, 1996), and hypo-osmotic conditions appear to increase Sln1p phosphorylation (Tao *et al.*, 1998), as do *sln1** mutations (Fassler *et al.*, 1997). The phosphorylation status of Sln1p dictates the phosphorylation state of the downstream receivers, Ssk1p and Skn7p. Accumulation of unphosphorylated Ssk1p is required for activation of the Hog1 MAP kinase pathway and upregulation of osmotic stress genes, whereas accumulation of D-phospho-Skn7 is required for upregulation of the P-lacZ reporter and *TRX2*. The Skn7-D-P-dependent *CLN1* and *CLN2* genes (Morgan *et al.*, 1995) and as yet unidentified cell-wall genes are predicted to be regulated similarly. Oxidative-stress shifts unphosphorylated Skn7p into a distinct modification state (mod) via an unknown mechanism. Accumulation of this pool of Skn7p contributes to the activation of a set of oxidative-stress genes.

expected from the previous observation that oxidative-stress hypersensitivity of *skn7* deletion mutants could be rescued by a *SKN7* allele in which the conserved aspartate 427 had been mutated to asparagine (Morgan *et al.*, 1997), P-lacZ expression continued to be oxidative-stress inducible in a *skn7* D427N mutant (Table V). In contrast, the receiver domain aspartate in Skn7p is required for *SLN1*- and *YPD1*-dependent signal transmission. Multiple copies of the gene increase expression of the downstream P-lacZ reporter in a D-dependent manner. Likewise, *sln1** activation is eliminated in strains containing a D427N mutant allele of the *SKN7* gene. These results support our hypothesis based on earlier data that phosphorylated Sln1p signals to target genes via a phosphotransfer mechanism (Fassler *et al.*, 1997).

We further show that mutation of the conserved receiver domain aspartate to glutamate enhanced D-dependent Skn7p activities, mimicking somewhat the effects of the *sln1** mutation. The hyperactivity of Skn7p D/E was first noticed by Brown *et al.* (1994). Based on the absence of *in vitro* phosphorylation of D/E mutant alleles of several bacterial response regulators (Bourret *et al.*, 1990; Green *et al.*, 1991; Pazour *et al.*, 1992), the hyperactivity of this allele was suggested to reflect constitutive phosphorylation-independent activation (Brown *et al.*, 1994). The observation that the *sln1** mutation which causes accumulation of phosphorylated Sln1p did not further stimulate the elevated P-lacZ activity in the *skn7* D427E mutant is consistent with this hypothesis.

The role of SKN7 in oxidative stress is independent of SLN1 and YPD1

Oxidative-stress induction of the P-lacZ reporter gene did not require *SLN1* or *YPD1*. The nature of the Sln1p-independent effect of oxidative stress on Skn7p is unknown. However, previous studies showing that Skn7p phosphorylation is not limited to the receiver domain aspartate (Brown *et al.*, 1994) are consistent with the possibility that the oxidative-stress signal may affect the (serine/threonine) phosphorylation status of Skn7p.

Regulation of TRX2 by Skn7p and by the SLN1-SKN7 pathway

The *TRX2* gene was considered a candidate for regulation by the SLN1-SKN7 pathway based on previous work establishing the presence of a Skn7p-containing complex associated with sequences ~150 bp upstream of the *TRX2* ATG (Morgan *et al.*, 1997). The 2.5-fold increase in *TRX2* expression due to *sln1** mutation confirms that *TRX2* is one target of the SLN1-SKN7 pathway. In composite, three distinct Skn7p functions have been revealed with respect to *TRX2* gene regulation. First, as shown by Morgan *et al.* (1997), Skn7p is involved in oxidative-stress responsiveness of *TRX2*. Secondly, we show here that Skn7p is involved in receiving the SLN1 regulatory signal; and thirdly, the 10-fold reduction in uninduced levels of *TRX2-lacZ* gene expression shown in Table VII suggests a possible role for Skn7p in basal transcription of *TRX2*. We are currently investigating which of these

Table VIII. Yeast strains used in this work^a

Strain name	Genotype and related strains	Derivation and comments
JF1331	<i>MATa his4-917 lys2-1288 leu2 trp1Δ1 ura3-52</i> ; pGY48	
JF1359	<i>MATa sln1-21 his4-917 lys2-1288 leu2 trp1Δ1 ura3-52</i> ; pGY48	
JF1432	<i>MATa ssk1Δ::LEU2 his4-917 lys2-1288 leu2 trp1Δ1 ura3-52</i> ; pGY48	<i>ssk1Δ::LEU2</i> derivative of JF1331; one-step transplacement
JF1433	<i>MATa ssk1Δ::LEU2 sln1-21 his4-917 lys2-1288 leu2 trp1Δ1 ura3-52</i> ; pGY48	<i>ssk1Δ::LEU2</i> derivative of JF1359; one-step transplacement
JF1493	<i>MATa his4-917 lys2-1288 leu2 trp1Δ1 ura3-52</i> ; <i>URA3::pGY109</i>	derivative of JF1331; starting strain for high-copy screen
JF1735	<i>MATa sln1Δ::TRP1 ssk1Δ::LEU2 his4-917 lys2-1288 leu2 trp1Δ1 ura3-52</i> ; pGY48	
JF1738	<i>MATa skn7Δ::TRP1 his4-917 lys2-1288 leu2 trp1Δ1 ura3-52</i> ; pGY48	derivative of JF1331
	Related strains JF1787, JF1738 carrying pSL232; JF1788, JF1738 carrying pCLM699; JF1789, JF1738 carrying pCLM700; JF1790, JF1738 carrying pRS315;	
JF1739	<i>MATa skn7Δ::TRP1 sln1-21 his4-917 lys2-1288 leu2 trp1Δ1 ura3-52</i> ; pGY48	derivative of JF1359
	Related strains JF1795, JF1739 carrying pSL232; JF1796, JF1739 carrying pCLM699; JF1797, JF1739 carrying pCLM700; JF1798, JF1739 carrying pRS315;	
RJY1209	<i>MATa ypd1Δ::TRP1 ssk1Δ::LEU2 his4-917 lys2-1288 leu2 trp1Δ1 ura3-52</i> ; pGY48	derivative of JF1432
RJY1212	<i>MATa ypd1Δ::TRP1 ssk1Δ::LEU2 sln1-21 his4-917 lys2-1288 leu2 trp1Δ1 ura3-52</i> ; pGY48	derivative of JF1433

^aAll strains were from our collection or constructed for this study.

Skn7p functions is mediated by the Skn7p-binding site identified by Morgan *et al.* (1997).

Since the *skn7* D427N allele elevates basal *TRX2* activity back to near wild-type levels (Table VII), the role of Skn7p in basal *TRX2* transcription, like the role of Skn7 in oxidative-stress induction of *TRX2*, is D independent. Thus, only Sln1-mediated stimulation of *TRX2* expression involves aspartate 427 of Skn7p.

Curiously, the reduction in basal activity of *TRX2* we observe in *skn7*-Δ mutants was not detected previously at the RNA level or in reporter assays (Morgan *et al.*, 1997). The discrepancy between the reporter data presented here and previous reporter and RNA analysis may be due to strain background differences and is currently under investigation.

At this time, it is not clear whether the D-phosphorylated form of Skn7p interacts directly with DNA or transmits a signal to the nucleus via additional intermediates. The genes identified in our high-copy activation screen (Table I) are obvious candidate intermediates, and it is interesting to note that at least one of them, *RCK2/CLK1*, is known to have serine/threonine kinase activity (Melcher and Thorner, 1996).

Is the Mcm1p transcription factor downstream of the SLN1-SKN7 signal-transduction pathway?

Our initial studies on *sln1** mutants employed an *MCMI*-dependent reporter gene whose activity was responsive to activated alleles of *SLN1* relative to reporters harboring non-*MCMI*-dependent UAS elements. Our early model held that the increased pool of phosphorylated Sln1p in a *sln1** mutant strain might cause a change in Mcm1p activity, thereby changing the pattern of *MCMI*-regulated genes in response to osmotic stress (Yu *et al.*, 1995; Fassler *et al.*, 1997). Our current data showing that

mutation of the Mcm1p binding does not impair the *sln1** response call into question the previous conclusion that Mcm1p might be a target of the SLN1-SKN7 pathway. An alternative explanation for our results, which has not been ruled out, is that the mutant Mcm1p-binding site may retain sufficient Mcm1-binding activity to allow the stimulatory effect of *sln1**. It should be noted that expression of the Mcm1-regulated genes, *Mfa1* and *PMA1* was reduced significantly in *sln1*-Δ mutants (Yu *et al.*, 1995). However, no effect of the *sln1**-activating mutation could be found on expression of these genes (G.Yu and J.Fassler, unpublished data). Finally, since we find no recognizable Mcm1p-binding site in the promoter of *TRX2*, there appear to be additional transcription factor targets of the SLN1-SKN7 pathway. Further analysis of genes such as *TRX2*, which respond to *SLN1* via phosphorylation of the Skn7p response regulator, will ultimately be required to reveal the nature of the *trans*-acting factors and *cis*-acting elements required for the *sln1** response.

What is the role of SLN1-YPD1-SKN7 signaling in osmotic response?

Taken together, our data are consistent with the model depicted in Figure 2. *SLN1* coordinately regulates expression of the osmotic-response genes and a second set of genes by phosphorylation of the two response regulators, Ssk1p and Skn7p. Phosphorylation of Ssk1p maintains the HOG pathway in its inactive state, while phosphorylation of Skn7p promotes signaling to the P-*lacZ* reporter and genes such as *TRX2*. Since expression of the P-*lacZ* reporter and the *TRX2* gene were responsive to both D-dependent and D-independent Skn7 regulation, we speculate that there may exist a family of target genes responsive to both the osmotic and the oxidative-stress pathways. Earlier studies (Brown *et al.*, 1993; Morgan *et al.*, 1995)

in which a high-copy *SKN7* plasmid was shown to suppress the effects of a mutation in the *KRE9* gene, involved in cell-wall biosynthesis, and separately to bypass the requirement for the known G_1 transcription factors, SBF and MBF in a D-dependent manner indicate that cell-wall- and cell-cycle-regulated genes are among the targets of the SLN1–SKN7 pathway. Whether the Skn7-regulated cell-cycle genes, *CLN1* and *CLN2*, and unknown cell-wall genes are also responsive to oxidative stress has not yet been examined.

In the SLN1 system the two response regulators have distinct roles to play. Under conditions that cause accumulation of phospho-Sln1p, phosphorylated Ssk1p keeps the HOG1 MAP kinase pathway inactive, while Skn7p-D-P activates a set of genes including *TRX2* and the *P-lacZ* reporter. Conditions that cause the accumulation of dephospho-Sln1p, however, activate the HOG1 MAP kinase pathway to allow survival under osmotic-stress conditions. The reciprocal regulation of the two SLN1 branches in response to osmotic stress would indicate that the genes downstream of the SLN1–SKN7 branch also play a role in the osmotic-stress response. We have shown previously that the SLN1–SKN7 branch is activated by a hypo-osmotic stimulus (Tao *et al.*, 1998). Hence, the bifurcated SLN1 pathway allows responsiveness to different aspects of osmotic stress.

The identification of Skn7 at the junction of the oxidative and osmotic-stress pathways implies that this molecule has an important and general role to play in further integrating cellular stress responses. The multi-functional nature of the Skn7 protein makes it uniquely suited for the integration of multiple signals and their relay to the nucleus. Future structure–function studies on the Skn7 protein will reveal the domains and activities specifically required for each pathway.

Materials and methods

Strains, media and yeast techniques

The yeast strains used in this study are listed in Table VIII. *skn7* disruption strains, JF1738 and JF1739 were generated by transformation of strains JF1331 and JF1359 with the *SacI* fragment from a *skn7* disruption plasmid from H. Bussey (Brown *et al.*, 1993) and selection on SC-Trip plates containing 1.5 M sorbitol. Using this procedure, the fraction of transformants with true disruptions of the *SKN7* gene was low. Transformants were therefore screened for hypersensitivity to hydrogen peroxide prior to Southern analysis to enrich for *skn7* disruptions. *SKN7* disruption was confirmed by Southern hybridization analysis of *Bgl*II- and *Xba*I-digested DNA using a 0.9-kb probe generated from the PCR fragment amplified using primers SKN7-911F 5'-GATGACG-AATTGTGTCAGTGG-3' and SKN7+6R 5'-GCTCATATGGGATAT-CAAAAGTAAGC-3'.

YPD1 disruption was achieved by digestion of plasmid pCLM656 with *Spe*I and *Sal*I prior to transformation of *ssk1*-Δ yeast strains. *YPD1* disruption was confirmed by the reduced size of the 1.7-kb *Eco*RI band in *Eco*RI-digested DNA hybridized to a radiolabeled 1.0-kb *Spe*I–*Eco*RI *YPD1* fragment.

Solid and liquid media were prepared as described by Sherman *et al.* (1986) and included synthetic complete medium (SC) lacking one or more specified amino acids (e.g. SC-leucine) and rich medium (YPD). Plates for the detection of β-galactosidase activity contained various amounts of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal)/ml and were prepared as described by Larson *et al.* (1983). 5-FOA plates used in recombinational loss of the *hisG::URA3::hisG* allele were prepared according to Boeke *et al.* (1987). The growth temperature for yeast cultures was 30°C. Responsiveness to oxidative stress was tested using *t*-butyl hydrogen peroxide (Sigma) at a final concentration of 0.5 mM. Early log-phase cultures were exposed to the reagent for 1 h.

Yeast transformation was performed by a modified LiOAc method (Ito *et al.*, 1983). Plasmids were recovered out of yeast by the 10-min glass bead method (Hoffman and Winston, 1987).

Plasmids

The *SKN7* CEN plasmid, pSL232, was constructed by cloning a 3.5-kb *Sal*I–*Hind*III fragment from pSL49-1 (genomic library plasmid; YEpl3) (using the *Sal*I and *Hind*III sites of YEpl3) into the *Sal*I and *Hind*III sites of the pRS315 (Sikorski and Hieter, 1989) polylinker. The D427N derivative was made by substituting the *Nsi*I–*Msc*I fragment from the *SKN7* CEN plasmid, YCpASN (Morgan *et al.*, 1995). The D427E mutation was introduced by *in vitro* mutagenesis using oligos OLI-352 5'-AAGTATAGGTATGATTGGTTTGTATGGAAATTGTTATGCCA-AACC-3' and OLI-353 5'-GGTTTGGCATAACAATTTCATCAAA-ACCAATCATACCTATACTT-3'. These mutant derivatives were substituted for the wild-type by swapping the *Nsi*I–*Msc*I fragment in the CEN *SKN7* vector to generate plasmids pCLM699 (D427N) and pCLM700 (D/E). Both alleles were tested for function by examining the oxidative-stress phenotype of strains containing the mutant *skn7* derivatives as their sole source of Skn7 protein. Since D427 is not required for oxidative stress, both mutant derivatives were expected (and found) to be resistant to media containing *t*-bH₂O₂.

The GST–Skn7 expression plasmid, pAA841, was constructed by cloning PCR amplified *SKN7* [primers: OLI-341 (5'-TTGTTTCGAATTCTTAGCTTTTCCACCATAAATAGCAACG-3' containing an *Eco*RI site and OLI-340 (5'-GATGTGCTCGAGTGATAGCTGGTTTCTTG-AAGTGTAG-3') containing an *Xho*I site into *Eco*RI and *Xho*I digested pGEX-KG (Guan and Dixon, 1991)].

The *YPD1* gene was PCR amplified from ATCC clone 71003 using primer OLI-253 (5'-GAACATTTAACTAGTGTCATTCAG-3'), and OLI-255 (5'-GAAGGATTCTGTGCGACTTTGTTGGTAC-3') which contain novel *Spe*I and *Sal*I sites respectively. The *Spe*I, *Sal*I double-digested PCR product was cloned into Litmus 38 (NEB) to create pCLM636. To construct the GST *YPD* fusion, pCLM655, *YPD1* was amplified from the same template [primer: OLI-254 (5'-TCGATCGAGG-ATCCATGTCTACTATTCCTCAGAAATC-3') containing a *Bam*HI site and primer OLI-255 described above]. The PCR product was digested with *Bam*HI and *Sal*I and cloned into pGEX-KG (Guan and Dixon, 1991) to generate plasmid pCLM655. Plasmid pCLM659 was generated by linearizing pCLM636 with *Sna*BI, converting the ends to *Sal*I using linkers, digesting with *Sal*I to generate a 1.8-kb fragment and cloning into the *Sal*I site of pCLM654. pCLM654 is a modified YCp50 plasmid in which the *URA3* gene was removed by *Sma*I–*Sal*I digest, *LYS2* added as an *Eco*RI–*Hind*III fragment and the *Hind*III site converted to a *Sal*I site using *Sal*I linkers.

The *YPD1Δ::TRP1* disruption plasmid, pCLM656, was constructed in several steps. pCLM636 was digested with *Age*I, filled in with Klenow, ligated in the presence of phosphorylated *Eco*RI linkers, digested with *Eco*RI and religated to create a 400-bp deletion. *TRP1* was amplified from pRS314 (Sikorski and Hieter, 1989) using primers complementary to *TRP1* sequences and flanked by *Eco*RI sites. The PCR product was digested with *Eco*RI and ligated into the *Eco*RI site of the modified pCLM636 plasmid.

The GST–Sln1p receiver domain plasmid (pAA689) was constructed by cloning the PCR-amplified receiver domain [primers SLN1-3200F (5'-ACATCAAGTAGAAGAATTCCACAGCAAGACG-3') containing an *Eco*RI site and OLI73573 (5'-CGCGCAAGCTTTTGATTCTC-3') containing a *Hind*III site] in frame into *Eco*RI–*Hind*III-cut digested pGEX-KG (Guan and Dixon, 1991). The GST–Sln1p kinase domain plasmid (pHL581) was constructed by cloning the PCR-amplified kinase domain [primers OLI-195 (5'-ATGACAGACGCATGAATTCACAT-TATGCTCTCTAG-3') containing an *Eco*RI site and OLI-176 (5'-CTTTCTACTCTCGAGGATTAAATTCGTC-3') containing a *Xho*I site into *Eco*RI–*Xho*I-digested pGEX-KG (Guan and Dixon, 1991)].

The *MCM1*-dependent reporter, *P-lacZ* is carried on the previously described 2μ plasmid, pGY48 (Yu *et al.*, 1995). pGY48 consists of a 16-bp palindromic *Mcm1*-binding site inserted into the *Xho*I site of the *CYC1-lacZ* vector, pLG670Z (Guarente and Ptashne, 1981). For integration, plasmid pGY48 was converted to integrating plasmid, pGY109 by deletion of a 2.2-kb fragment containing the 2μ replication origin by partial digestion of the plasmid with *Eco*RI. The resulting integrating plasmid was linearized in the *URA3* gene with *Apa*I prior to transformation.

Reporters pCLM771 and pCLM772 were constructed in the pGY79 vector which is a derivative of pLGA312 (Guarente and Mason, 1983) from which sequences between the *Sma*I site at position –312 and the *Xho*I site at position –178 relative to the initiation site of *CYC1* have

been deleted. Because of the details of the construction, an *XhoI* site remains at the deletion junction. This vector therefore retains *CYC1* sequences to -178 relative to the initiation site (-250 relative to the ATG), but has no *CYC1* sequences upstream of the *XhoI* site. Phosphorylated oligonucleotides, P-pal (5'-TCGAGTTTCCTAATTA-GGAAAC-3'), or mutP1 (5'-TCGAGTTTCCTAATTAATAAAC-3') and mutP2 (5'-TCGAGTTTATTAATTAGGAAAC-3') all of which are flanked by *XhoI* sticky ends were allowed to anneal and then ligated separately into the *XhoI* site of pGY79. Transformants with four palindromic or four mutant oligonucleotide inserts were found by sequencing and designated pCLM771 (4x P-*lacZ*) and pCLM772 (4x mutP-*lacZ*).

The *TRX2-lacZ* (pJF1078) plasmid was constructed by cloning the PCR-amplified *TRX2* promoter fragment (primers: TRX2 -393F, 5'-TCACCGGGGCGAGGTCACCATTCGAAGCATTCG-3' containing an added *SmaI* site, and TRX2+9R, 5'-GTCAGGATCCAGTGACCAT-TATTGATGTGTTA-3' containing an added *BamHI* site) into *SmaI*- and *BamHI*-digested *lacZ* vector, pLG670Z (Guarente and Ptashne, 1981). The resulting plasmid lacks all *CYC1* regulatory sequences.

***β*-galactosidase assays**

Yeast protein extracts were prepared by glass-bead lysis from cultures grown at 30°C and harvested at a density of 10⁷ cells/ml. Extracts were cleared by centrifugation in all cases. Activities were calculated in Miller units (Miller, 1972) and are the averages of at least three different transformants except where otherwise noted.

Dideoxy sequencing analysis

Sequencing reactions were performed using the Sequenase kit (version 2.0; United States Biochemical Corporation) and [³²P]dATP (Amersham). Primers for sequencing the high-copy clone inserts consisted of oligonucleotides complementary to sequences on either side of the *BamHI*-cloning site in YEp13 (and pBR322) (YEp13B-F, 5'-GCTACTTGG-AGCCACTATC-3'; and YEp13B-R, 5'-GTGATGTCGGCGATATAG-3'). Reaction products were resolved on 6% acrylamide (19:1 acrylamide/bisacrylamide) sequencing gels containing 7 M urea. Additional sequencing was performed by University of Iowa DNA Core Facility.

Expression and purification of Sln1, Ypd1 and Skn7 fusion proteins

E.coli cultures containing GST-Sln1 kinase, GST-Sln1-associated receiver, GST-YPD1 or GST-SKN7 plasmids were grown to an OD₆₀₀ of 0.6 at 37°C at which time 0.2 mM IPTG was added and the cultures incubated for an additional 2 h. Cell pellets were resuspended in 2 ml buffer B (Posas *et al.*, 1996) per liter of culture. Extracts were prepared by lysis with a French press and removal of cell debris. For purification, aliquots corresponding to 1 l of culture were thawed and incubated with 1.0 ml washed 50% slurry of glutathione beads for 1 h at 4°C. Beads were removed by centrifugation and washed four times in an equal volume of buffer B, and four times in an equal volume of buffer C (Posas *et al.*, 1996) modified to contain 5 mM MgCl₂ and 0.12 mM β-mercaptoethanol instead of dithiothreitol. Beads were resuspended as a 50% slurry in buffer C and stored at -20°C. Skn7p and Ypd1 proteins were eluted as GST fusion proteins by incubation in elution buffer (10 mM glutathione in buffer C) for 1 h at 4°C. The supernatant was collected by gravity flow from a chromatography column. Sln1p-associated receiver was released from the glutathione beads by thrombin cleavage. Beads were washed twice in thrombin cleavage buffer (50 mM Tris, 50 mM NaCl, 2.5 mM CaCl₂) and incubated with biotinylated thrombin for 1 h at 25°C. Thrombin was removed according to the protocol in the Thrombin Cleavage Capture Kit (Novagen). The supernatant was collected by gravity flow from a chromatography column.

Phosphotransfer assays

Phosphotransfer assays were performed essentially as described previously (Posas *et al.*, 1996) with the following modifications. GST-tagged Sln1p kinase domain (1 μg) bound to glutathione-agarose beads was incubated with [γ-³²P]ATP (30 μM, 25 Ci/mmol) for 10 min at 25°C, at which time 1 ml of buffer C (50 mM Tris pH 7.5, 50 mM KCl, 5 mM MgCl₂, 0.1% β-mercaptoethanol) was added, the beads washed four times and resuspended in 1.0 ml Buffer C. For each phosphotransfer reaction, an aliquot of 100 μl of bead suspension was centrifuged to remove the supernatant. Purified GST-Ypd1p (0.5 μg) and GST-Skn7p (0.25 μg) in buffer C and purified Sln1p-associated receiver (0.5 μg) in thrombin-cleavage buffer were added as indicated and the final volume adjusted to 45 μl. After 15 min, reactions were quenched by the addition

of 15 μl loading buffer. Protein phosphorylation was analyzed after SDS-PAGE (10%), transfer to nitrocellulose and autoradiography.

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