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Proper Protein Glycosylation Promotes Mitogen-Activated Protein Kinase Signal Fidelity

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Supporting Information

ABSTRACT: The ability of cells to sense and respond appropriately to changing environmental conditions is often mediated by signal transduction pathways that employ mitogen-activated protein kinases (MAPKs). In the yeast *Saccharomyces cerevisiae*, the high-osmolarity glycerol (HOG) and filamentous growth (FG) pathways are activated following hyperosmotic stress and nutrient deprivation, respectively. Whereas the HOG pathway requires the MAPK Hog1, the FG pathway employs the MAPK Kss1. We conducted a comprehensive screen of nearly 5000 gene deletion strains for mutants that exhibit inappropriate cross-talk between the HOG and FG pathways. We identified two novel mutants, *mnn10*Δ and *mnn11*Δ, that allow activation of Kss1 under conditions that normally stimulate Hog1. *MNN10* and *MNN11* encode mannosyltransferases that are part of the N-glycosylation machinery within the Golgi apparatus; deletion of either gene results in N-glycosylated proteins that have shorter mannan chains. Deletion of the cell surface mucin Msb2 suppressed the *mnn11*Δ phenotype, while mutation of a single glycosylation site within Msb2 was sufficient to confer inappropriate activation of Kss1 by salt stress. These findings reveal new components of the N-glycosylation machinery needed to ensure MAPK signaling fidelity.

The ability of cells to sense and respond to changes in their environment is fundamental to cell survival. The mitogen-activated protein kinases (MAPKs) are commonly employed to transduce extracellular signals and to evoke specific intracellular responses.[1] MAPKs are part of an evolutionarily conserved three-tiered signaling cascade consisting of the MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). In mammalian cells, MAPKs mediate responses to a variety of stimuli such as hormones, stresses, and cytokines, which promote cell proliferation, differentiation, and inflammation. Clearly, the signals that initiate these events must be transmitted in a tightly regulated manner. The complexities of these pathways, however, have hindered our understanding of MAPK regulation.

MAPK pathways are also present in the unicellular eukaryote *Saccharomyces cerevisiae* (hereafter, yeast). Because of its relative simplicity and the ease of genetic manipulation, yeast has emerged as an excellent model system for investigating the MAPK pathway and points of regulation. Indeed, studies in yeast have revealed key pathway components and improved our understanding of how these components are regulated.[2]

Among the MAPK pathways in yeast, the most well-studied are the pheromone response pathway, the high-osmolarity glycerol (HOG) pathway, and the filamentous growth (FG) pathway (detailed in Figure 1). Each of these pathways is activated by a different stimulus and employs a distinct MAPK. In the HOG pathway, high osmotic stress activates the three-component kinase cascade: Ste11 (or Ssk2/Ssk22), Pbs2, and the MAPK Hog1. Activated Hog1 promotes events leading to stress adaptation, including cell cycle arrest and an increased

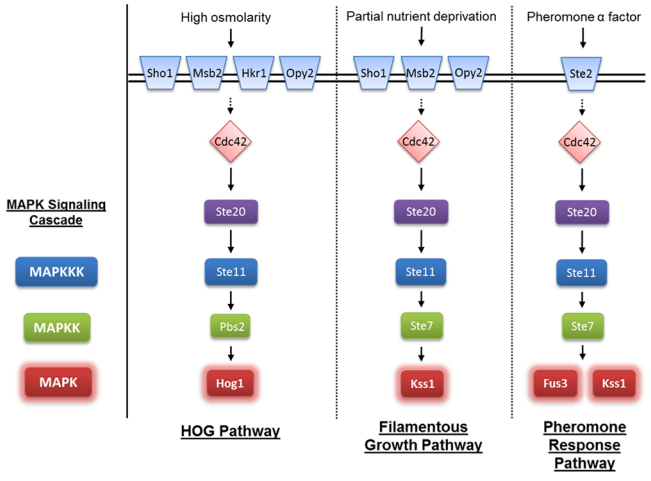

Figure 1. Components of the HOG, FG, and mating pheromone pathways. Some components have been omitted for the sake of brevity.

level of glycerol production to restore osmotic balance.[2−5] The filamentous growth pathway responds to nutrient deprivation by activating Ste11, Ste7, and the MAPK Kss1, which promotes enhanced cell−cell adhesion, enhanced cell−substratum adhesion, and an enhanced ability of cells to penetrate the

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substratum. These phenotypes may comprise a foraging response to allow nonmotile cells to grow into their surroundings in a search for additional nutrients.[2,6,7] In haploid yeast cells, this response is termed "invasive growth",[8] while in diploids, it is termed "pseudohyphal growth".[9,10] Finally, in the mating response pathway, a pheromone signal leads to the activation of Ste11, Ste7, and the MAPK Fus3, which initiates a series of changes that include cell cycle arrest, cytoskeletal rearrangements, and new gene transcription that lead to cell fusion and mating.[2,11−13]

The HOG, FG, and mating pathways share several components (Figure 1), yet they exhibit remarkable signal fidelity when stimulated individually. The prevalence of shared components raises the question of how pathway fidelity is regulated and maintained so that a signal transmitted though one pathway does not cross-activate another pathway. Prior work has revealed dynamic mechanisms that help these pathways function in a mutually exclusive manner. For instance, when exposed to a hyperosmotic environment, cells lacking Pbs2 or Hog1 exhibit sustained activation of Kss1 and undergo filamentous growth.[14−17] This finding suggests that activated Hog1 normally phosphorylates proteins that serve to limit activation of the competing FG pathway and does so in a dynamically regulated (stimulus-dependent) manner. Hog1 was later shown to repress Kss1 in part through phosphorylation of the MAPKKK adaptor Ste50.[18,19]

In addition to dynamic feedback mechanisms, pathway fidelity can also be maintained by static mechanisms involving scaffold proteins. The Ste11 adaptor protein Ste50 behaves as a dynamic integrator of multiple signals.[18−20] The kinase scaffold protein Ste5 was originally thought to serve as a signal insulator,[2,21] although current evidence indicates that it is also a dynamic regulator of Fus3 activity.[22,23] To identify new components that regulate cross-pathway signaling, we screened for mutants that display inappropriate MAPK responses in a high-salt environment. By this approach, we identified gene deletion mutants that exhibit hyperactivation of Kss1 in response to osmotic stress stimulation. These genes, *MNN10* and *MNN11*, each encode a subunit of the Golgi mannosyltransferase complex needed for proper N-glycosylation of proteins.[24] Deletion of a known glycoprotein (Msb2), potential Msb2 binding partners at the plasma membrane (Sho1 and Opy2), or downstream effectors of Msb2 (Ste20, Ste50, Ste11, and Ste7) was sufficient to suppress hyperactivation of Kss1. Finally, mutation of a single putative N-glycosylation site within a single shared component, Msb2, led to inappropriate activation of Kss1, in the manner of *mnn10*Δ and *mnn11*Δ. We propose that N-glycosylation of Msb2 is needed for insulation of pathway signaling components and to promote MAPK signaling fidelity.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions. Standard procedures for the growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used throughout. Yeast *S. cerevisiae* strain BY4741 (*MAT***a** *leu2 met15*Δ *his3-1 ura3*Δ) was used in this study. Strains from the Yeast Knockout (YKO) gene deletion library[25] were used for the initial genetic screen (Table S1 of the Supporting Information). Aside from Table S1, all of our analysis was done using mutant strains that had been reconstructed by polymerase chain reaction (PCR) amplification of YKO genomic DNA and homologous recombination in BY4741. All double mutants were constructed by PCR amplification of *LEU2*, *URA3*, or *HIS3* cassettes and homologous recombination in *mnn11*Δ. The *mnn10*Δ*mnn11*Δ double mutant is reported to be viable and to exhibit the same phenotype as the individual mutations.[26] However, we have not been able to sporulate the heterozygous diploid in our strain background, so we were not able to characterize the double mutant or to verify the earlier findings.

Cells were routinely grown at 30 °C in synthetic complete dextrose (SCD) liquid medium (yeast nitrogen base without amino acids, ammonium sulfate, adenine, amino acids, and dextrose) or in selective medium where needed for plasmid maintenance.

Plasmid Construction. pRS313-MNN11, pRS316-SNF3, pRS315-SHO1, pRS313-RGT2, and pRS316-STE50 were constructed by PCR amplification from BY4741 genomic DNA, restriction digestion, and ligation into the indicated pRS series vectors (ATCC). pRS316-MSB2 was constructed by PCR amplification of *MSB2* from BY4741 genomic DNA and gap repair with pRS316 in yeast. Human influenza hemagglutinin (HA) epitope tags were introduced by site-directed mutagenesis to construct pRS316-SNF3-HA, pRS313-RGT2-HA, and pRS316-MSB2-HA. The FLAG epitope tag was introduced by site-directed mutagenesis to construct pRS315-SHO1-FLAG. pRS316-ste50^{5A} was constructed as described previously.[18,19] All N-glycosylation-site mutations were constructed with the QuikChange site-directed mutagenesis kit (Stratagene), according to the manufacturer's directions. Mutagenic oligonucleotides [plus the complementary strand (not shown)] were as follows: *msb2*^{N30A}, 5′-CCT TCG ACT TTA TAT TCG GCG CTG GAA CGC AAC AAG CTC AGA GCC-3′; *snf3*^{N383A}, 5′-CTA CGG TGT CAA TTT CTT CGC TAA GAC AGG AGT CAG TAA TAG-3′; *rgt2*^{N136A}, 5′-GTT AAA ACC TAC ATT GCT CCG GCC CAT TCA TAT TTC ACC ACT AGC-3′; *rgt2*^{N385A}, 5′-CTA TGG AGT TAA TTT TTT CGC CAA CAC AGG GGT GGA CAA C-3′; *sho1*^{N59A}, 5′-CCA TCT CAT CTG CAT CCA CCG CTG AAT CCT TCC CAC GTT TTA CTT GG-3′.

Genetic Screen of the YKO Gene Deletion Library. Cells from the YKO gene deletion library were inoculated into a 96-well plate containing 100 μL of SCD liquid medium in each well. After incubation overnight, 100 μL of SCD liquid medium was added to each culture, and the cells were grown for an additional 24 h. Each mutant strain was spotted in duplicate onto agar plates made from SCD medium (2% agar) with either no salt, 0.5 M KCl, or 1.5 M KCl. Spots (5 μL) were made using a multichannel pipet.

After the plates had been incubated for 24 h at 30 °C, the colonies on each plate were scored for diminished levels of growth under high-salt conditions. After the plates had been incubated for a minimum of 2 days, they were rinsed with deionized water, and each colony was scored for penetration into the agar.

Cell Extract Preparation and Immunoblot Analysis. Cells were grown overnight to saturation in SCD medium and reinoculated into SCD and grown to an A_{600} of ∼1.0. Cells were then treated with 0.5 M KCl for 5 min (unless otherwise indicated), 1 M sorbitol for 5 min, or 3 μM α factor pheromone for 30 min or left untreated at 30 °C. Cells were harvested by addition of 100% trichloroacetic acid, centrifugation at 3000*g* for 2 min, being washed with 500 μL of 10 mM NaN_3, and being stored at −80 °C. Protein extracts were prepared by glass bead lysis in trichloroacetic acid as described previously.[27] The

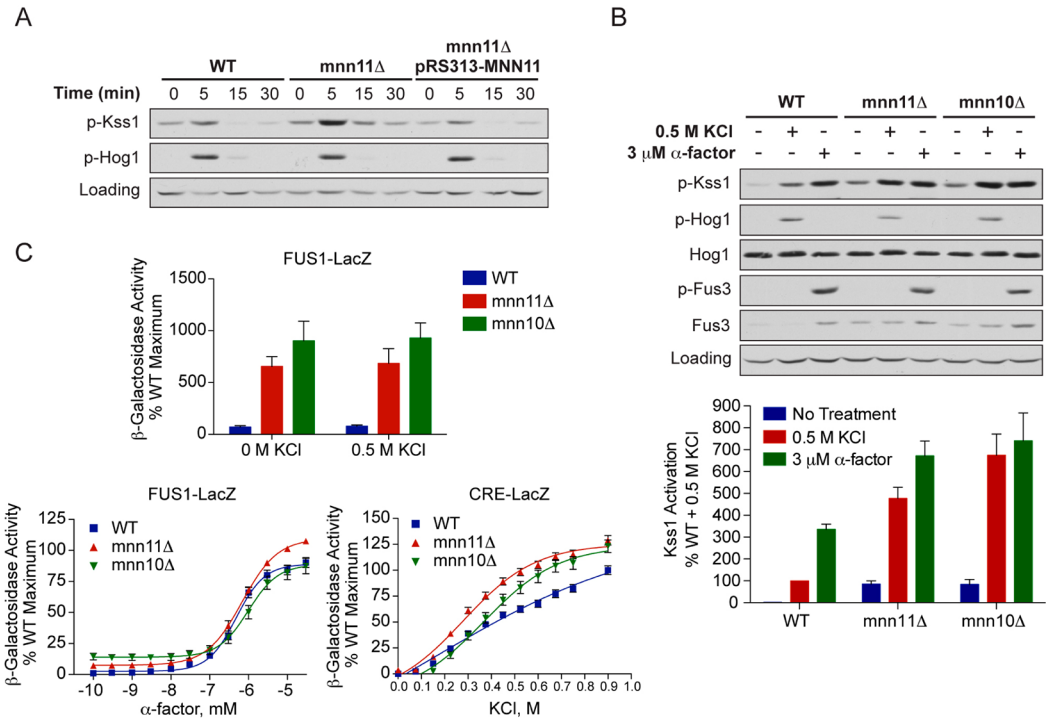

Figure 2. Deletion of *MNN11* results in hyperactivation of Kss1 and the filamentous growth pathway. (A) Activation of Kss1 and Hog1. Wild-type (WT), *mnn11*Δ, and pRS313-MNN11-transformed *mnn11*Δ cells were stimulated with 0.5 M KCl for the indicated times. Cell lysates were resolved by 10% SDS−PAGE. Phospho-Kss1 (p-Kss1) and phospho-Hog1 (p-Hog1) were detected by immunoblotting with phospho-p44/42 and phospho-p38 antibodies, which recognize the dually phosphorylated and activated forms of Kss1 and Hog1, respectively. G6PDH served as a loading control. (B) Activation of Kss1, Hog1, and Fus3. WT, *mnn10*Δ, and *mnn11*Δ cells were stimulated with 0.5 M KCl for 5 min or with 3 μM α-factor pheromone for 30 min. Cell lysates were resolved by 10% SDS−PAGE. Specific antibodies were used to detect the activated form of Hog1, Kss1, and Fus3 (p-Fus3). The total abundance of Hog1 and Fus3 was determined with Hog1 and Fus3 antibodies. G6PDH served as a loading control. All primary antibodies were recognized by chemiluminescent detection and quantified by scanning densitometry (ImageJ). The bottom panel shows averaged scanning densitometry data for three individual experiments. Error bars represent the standard error of the mean. (C) Transcriptional activation (β-galactosidase activity) measured spectrofluorometrically in WT, *mnn10*Δ, and *mnn11*Δ cells transformed with a plasmid containing either a pheromone-inducible reporter (FUS1-lacZ) or a salt-inducible reporter (CRE-lacZ). Transcription was induced by the addition of the indicated concentrations of KCl or α-factor pheromone. Data are means ± the standard error of four individual colonies measured in triplicate and presented as a percentage of the wild-type maximum.

protein concentration was determined by the DC protein assay, according to the manufacturer's instructions (Bio-Rad Laboratories). Protein extracts were resolved by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and immunoblotted with phospho-p44/42 MAPK antibodies (9101L, Cell Signaling Technology) at a 1:500 dilution, phospho-p38 MAPK antibodies (4631L, Cell Signaling Technology) at a 1:500 dilution, Fus3 antibodies (sc-6773, Santa Cruz Biotechnology) at a 1:500 dilution, and Hog1 antibodies (sc-6815, Santa Cruz Biotechnology), Kss1 antibodies (sc-28547, Santa Cruz Biotechnology), and glucose-6-phosphate dehydrogenase (G6PDH) antibodies (A9521, Sigma-Aldrich) at 1:10000 dilutions. Immunoreactive species were visualized by chemiluminescent detection (PerkinElmer Life Sciences LAS) of horseradish peroxidase-conjugated antibodies (170-5047 and 170-5046, Bio-Rad). Image densitometry was conducted consistently throughout using NIH ImageJ.[28] Statistical analysis was done using GraphPad Prism 4 for at least three independent experiments.

Transcription Reporter Assay. Pheromone-dependent FUS1-lacZ transcription reporter assays were conducted as described previously.[29] Cells transformed with pRS423-FUS1-lacZ[29] were grown to an A_{600} of ∼1.0, then dispensed at 90 μL per well into a 96-well plate, and mixed with 10 μL of α-factor pheromone at the indicated concentrations for 90 min at 30 °C. The reaction was started by adding 20 μL of the FDG solution [130 mM PIPES (pH 7.2), 0.25% Triton X-100, and 0.5 mM fluorescein di-β-galactopyranoside (M0250, Marker Gene Technologies)] for 1 h at 37 °C. The reaction was stopped by adding 20 μL of 1 M sodium bicarbonate. Fluorescence was quantified using a fluorescence plate reader with excitation at 485 μm and emission at 580 μm (SpectraMax M5, Molecular Devices).

Salt-dependent FUS1-lacZ transcription reporter assays were conducted as described above, except cell cultures at an A_{600} of ∼1.0 were dispensed at 65 μL per well and mixed with 35 μL of KCl at the indicated concentrations for 90 min at 30 °C. Each well was then mixed with 20 μL of the FDG solution for 3 h at 37 °C. Salt-dependent CRE-lacZ transcription reporter assays were conducted in the same manner, except using the pRS423-CRE-lacZ reporter plasmid[30] and treating with FDG for 6 h. Statistical analysis was done using GraphPad Prism 4 for at least three independent experiments.

RESULTS

A Genomewide Screen Reveals a New Regulator of Osmostress Signaling. Under normal circumstances, the HOG pathway is strongly activated by osmotic stress. Under the same conditions, however, certain yeast mutants incorrectly cross-activate Kss1 and the filamentous growth pathway. Most

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notably, mutants of the MAPK Hog1, or its upstream activator, Pbs2, grow invasively and penetrate their substratum when exposed to an osmotic stimulus. When grown on agar plates with salt, these cells cannot be easily rinsed off the plate.[15]

To identify additional regulators of cross-pathway activation, we used the invasive growth assay to screen the complete YKO yeast gene deletion strain collection.[25] The screen did not reveal any mutants that invaded the agar to the same degree as *hog1*Δ or *pbs2*Δ. Nevertheless, some mutants penetrated the agar in a weak but detectable manner. When they were retested, all but one of the mutants grew invasively in the absence of salt, and those mutants were not pursued (Table S1 of the Supporting Information). One remaining mutant, *mnn11*Δ, was invasive in the presence but not in the absence of salt and was characterized in detail.

Mnn11 Impedes the Activation of Kss1 by Osmotic Stress. We began by reconstructing the *mnn11*Δ strain and in this case observed no FG behavior, suggesting that the original YKO strain contained one or more enhancer mutations. Substratum penetration is often mediated by activation of the MAPK Kss1. To determine if the *mnn11*Δ mutation conferred an enhanced MAPK response, we monitored the activity of Kss1 directly by immunoblotting with an antibody that recognizes the dually phosphorylated, fully activated form of the kinase (phospho-Kss1). Kss1 activity was indeed elevated in *mnn11*Δ as compared with that of wild-type cells, and the level of activation was elevated further upon stimulation with 0.5 M KCl. Elevated MAPK activity was also evident in the reconstructed *mnn11*Δ strain, treated with either 0.5 M KCl or 1 M sorbitol (Figure 2A and Figure S1 of the Supporting Information). Introduction of *MNN11* on a single-copy plasmid restored normal Kss1 activity [pRS313-MNN11 (Figure 2A)].

By immunoblotting with an antibody that recognizes Kss1, we determined that *mnn11*Δ cells express ∼2-fold more Kss1 than wild-type cells (Figure S2 of the Supporting Information). This difference in expression is likely due to the 2-fold transcriptional induction of *KSS1* when the FG pathway is activated, for example, using an activated allele of Msb2.[31] Stronger Kss1 expression, however, cannot account for the 5-fold increase in the level of Kss1 phosphorylation in the *mnn11*Δ strain. Taken together, these results show that Mnn11 regulates the filamentous growth pathway by limiting the activity of Kss1 under osmotic stress conditions.

Mnn11 Selectively Regulates Kss1. The findings described above reveal that *MNN11* is needed to prevent inappropriate activation of Kss1 by osmotic stress. To determine if Mnn11 regulates the functions of any other MAPKs, we examined the activity of Hog1, Fus3, and Kss1 following stimulation with either salt (KCl) or the pheromone α-factor. For these experiments, we also tested an *mnn10*Δ mutant strain. *MNN10*, like *MNN11*, encodes an α-1,6-mannosyltransferase present in the lumen of the Golgi.[24] Moreover, Mnn10 and Mnn11 are part of the same macromolecular complex, and both proteins are required for the extension of mannan chains on N-glycosylated proteins.[24]

Once again, we monitored activation of the MAPKs by immunoblotting with antibodies that recognize the phosphorylated, activated forms of each kinase. As shown in Figure 2B, the *mnn10*Δ and *mnn11*Δ mutants exhibited elevated phospho-Kss1 levels, in the absence and presence of a salt stimulus. In the stimulated cells, the level of phospho-Kss1 was 5- and 7-fold higher in *mnn11*Δ and *mnn10*Δ, respectively, as compared with that in wild-type cells. This increase in the level of phospho-Kss1 was well above the 2-fold stronger expression of total Kss1, noted above (Figure S2 of the Supporting Information). In contrast, the mutants had no effect on the activation of the osmosensitive MAPK Hog1. Activation of the mating-specific MAPK Fus3 was likewise unaffected, although there was a modest elevation in the abundance of Fus3. Thus, Mnn10 and Mnn11 serve to limit salt activation of Kss1 but not that of other MAPKs.

We then examined whether Mnn10 or Mnn11 alters the response to α-factor pheromone (Figure 2B), which normally activates Fus3 and Kss1 but not Hog1. When stimulated with α-factor, Fus3 activation in the mutants was comparable to that of the wild type. Here, the level of Kss1 activation increased ∼2-fold in *mnn10*Δ and *mnn11*Δ cells. This difference mirrors the 2-fold higher level of expression of Kss1 (Figure S2 of the Supporting Information). Under no circumstance was Hog1 activated by pheromone. Hence, these data reveal that Mnn10 and Mnn11 limit the activation of Kss1 under salt stress conditions. These effects are highly selective, because the same mutants did not affect activation of Kss1 by pheromone. Moreover, these mutants did not affect activation of either Fus3 or Hog1 in cells treated with either salt or pheromone.

Our findings indicate that Mnn10 and Mnn11 specifically limit activation of Kss1 by salt. One of the functions of MAPKs is to activate downstream transcription factors that induce the expression of a specific set of genes. To corroborate the MAPK activity data, we monitored gene induction downstream of Kss1. To this end, we used a reporter comprised of the β-galactosidase gene fused to the *FUS1* promoter (FUS1-lacZ). *FUS1* is strongly induced upon activation of either Fus3 or Kss1.[12] Fus3 is not activated by osmotic stress, however (Figure 2B), so under these conditions, FUS1-lacZ induction is mediated solely by Kss1. As shown in Figure 2C, the basal level of FUS1-lacZ expression was significantly higher in both *mnn10*Δ and *mnn11*Δ mutants than in wild-type cells. This increase is consistent with the elevated Kss1 activity. However, in no case did we detect a further increase following treatment with 0.5 M KCl. The absence of any stimulus-dependent change in transcription reporter activity contrasts with the marked increase in the level of activation of Kss1 in the mutant strains. As shown in Figure 2A, Kss1 activation was quite transient, however, and may not have been sufficient to fully activate the downstream transcription factors. Just as transient activation of Kss1 is not sufficient to initiate the mating response in wild-type yeast,[32] transient activation of Kss1 did not initiate mating responses in *mnn10*Δ or *mnn11*Δ cells. A second possibility is that, under these specific circumstances, Kss1 activates an alternative transcription program.

We then monitored FUS1-lacZ as a reporter for the pheromone response pathway mediated by Fus3 (Figure 2C). Once again, the *mnn10*Δ and *mnn11*Δ strains exhibited elevated basal activity, but no further induction with α-factor pheromone, as compared with wild-type cells. Given that deletion of Mnn11 and -10 does not influence the activation status of Fus3 (Figure 2B), we would not expect these mutants to significantly alter the FUS1-lacZ response to pheromone. The pheromone response pathway is mediated primarily by the MAPK Fus3 and signals through Kss1 only when Fus3 is absent.[2] When Fus3 is present, Kss1 activation is repressed.[33] Finally, to examine transcriptional induction downstream of Hog1, we used a reporter comprised of the β-galactosidase gene fused to the CRE (cAMP-responsive element) promoter motif

(CRE-lacZ).[30] As shown in Figure 2C, *mnn10*Δ and *mnn11*Δ mutants exhibited full induction of CRE-lacZ, although maximal induction occurred at slightly (<2-fold) lower concentrations of salt than in wild-type cells. Taken together, the transcription reporter data support the conclusion that Mnn10 and Mnn11 restrict Kss1 activity under basal (unstimulated) conditions. Other unidentified mechanisms appear to limit transcriptional induction by Kss1 under salt-stimulated conditions. These mechanisms may account as well for the absence of a reproducible invasive growth phenotype noted for the *mnn11*Δ mutant.

Genetic Analysis Reveals Potential Targets of Mnn11. *MNN11* was shown previously to be required for proper N-glycosylation of proteins. Given that the absence of *MNN11* allows inappropriate activation of Kss1, we reasoned that deletion of a glycosylated target protein might suppress the observed *mnn11*Δ phenotype. To this end, we deleted a series of candidate regulators in conjunction with the *mnn11*Δ mutation. We then monitored the activation state of Kss1, in the absence or presence of 0.5 M KCl and in the absence or presence of *MNN11*.

We began our analysis with components of the FG pathway that are shared with the salt stress pathway, any of which could limit inappropriate activation of Kss1 by salt; these include Ste20 (MAPKKKK), Ste11 (MAPKKK), Ste50 (Ste11 adaptor), and Ste7 (MAPKK).[2] We also tested the pheromone receptor (Ste2) and the G protein β subunit (Ste4) because they, too, have the potential to activate Kss1. Of all these pathway components, only Ste2 is known to undergo N-glycosylation.[34]

As shown in Figure 3, Kss1 was no longer activated by salt in the absence of Ste20, Ste50, Ste11, or Ste7. The *mnn11*Δ*ste20*Δ mutant did exhibit a slight elevation in the basal level of Kss1 activation, presumably because of the presence of kinases related to Ste20 (Cla4 and Skm1). We were not able to test this hypothesis because the *ste20*Δ*cla4*Δ double mutant is not viable.[35] Nevertheless, the *mnn11*Δ*ste20*Δ double mutant remained unresponsive to salt. In contrast, Kss1 was still hyperactivated by salt in the *ste2*Δ*mnn11*Δ and *ste4*Δ*mnn11*Δ double-mutant strains, even though deletion of *STE4* reduces the basal level of signaling and expression of several pheromone pathway components, both upstream and including Kss1.[2] These data indicate that components of the FG pathway are needed for Kss1 hyperactivation (Ste20, Ste50, Ste11, and Ste7), whereas components unique to the pheromone response pathway (Ste2 and Ste4) are not. Taken together, these data suggest that Mnn11 targets a membrane protein other than Ste2, but one that is upstream of protein kinases Ste20, Ste11, Ste7, and Kss1.

We next considered candidate targets of Mnn11 in the FG pathway. To this end, we tested a group of cell surface proteins known to act upstream of Kss1;[36] these include Sho1, Msb2, Opy2, and Hkr1. Sho1 is a transmembrane protein that can form a hetero-oligomeric complex with the signaling mucin Msb2.[2,31,37] Both proteins are likely to be N-glycosylated and function at the head of both the FG and HOG pathways.[2,31,36,38,39] Opy2 is an integral membrane protein that interacts with and recruits Ste50 to the plasma membrane; Opy2 is likewise necessary for both the FG and HOG responses.[2,20,40] While it does not have any luminal N-glycosylation sites (N-X-S/T)[41,42] and therefore cannot be targeted directly by Mnn11, Opy2 could associate with another glycosylated binding partner such as Msb2. Finally, Hkr1 is another signaling mucin that functions as an osmosensor in complex with Sho1.[36]

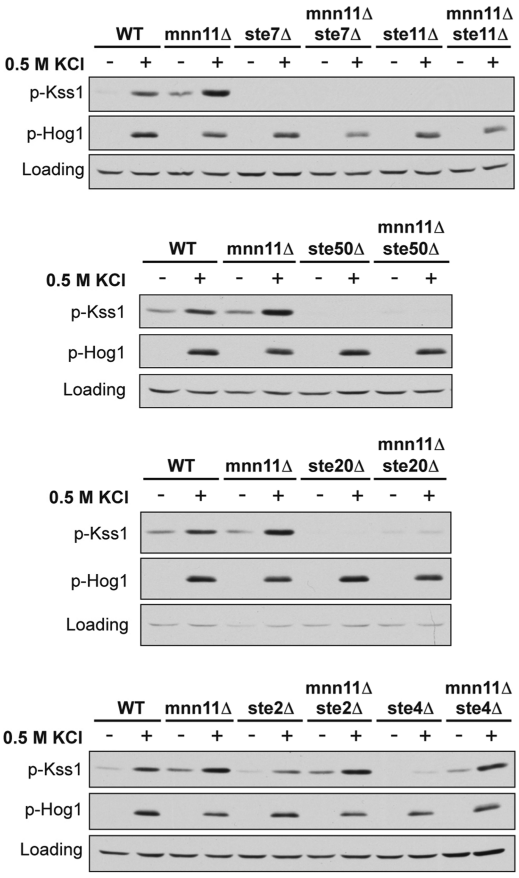

Figure 3. Mnn11 limits Kss1 activation through the filamentous growth pathway and not the pheromone response pathway. Activation of Kss1 and Hog1. *mnn11*Δ and the indicated double-gene deletion mutant cells were stimulated with 0.5 M KCl for 5 min. Cell lysates were resolved by 10% SDS−PAGE and immunoblotting. Specific antibodies were used to detect the dually phosphorylated, fully activated forms of Kss1 (p-Kss1) and Hog1 (p-Hog1). G6PDH served as a loading control. Data are representative of three individual experiments.

Once again, we constructed double-mutant strains lacking Mnn11 and each of the candidate target proteins. We then exposed these mutant strains to hyperosmotic stress and immunoblotted for activated Kss1. As shown in Figure 4, Kss1 was hyperactivated by salt in the *hkr1*Δ*mnn11*Δ strain. The level of Kss1 activation was also slightly elevated in cells lacking only Hkr1, consistent with its role as a negative regulator of the FG response.[39] In contrast, Kss1 was no longer activated by salt in the *msb2*Δ*mnn11*Δ, *sho1*Δ*mnn11*Δ, or *opy2*Δ*mnn11*Δ strain. These data suggest that Mnn11 targets Msb2, Sho1, or Opy2. However, while these proteins are components of the FG pathway, only Msb2 and Sho1 have consensus N-glycosylation sites. Thus, the effects of Mnn11 on Opy2 are likely to be indirect.

We then considered proteins that act in conjunction with Opy2, which may in turn be regulated by Mnn11. It was proposed recently that Opy2 is phosphorylated and regulated by the casein kinases Yck1 and Yck2.[20] These kinases appear to play a role in pathway fidelity, because unphosphorylated Opy2 activates Kss1 in preference to Hog1, while phosphorylated Opy2 activates Hog1 in preference to Kss1.[20] Yck1 and Yck2, in

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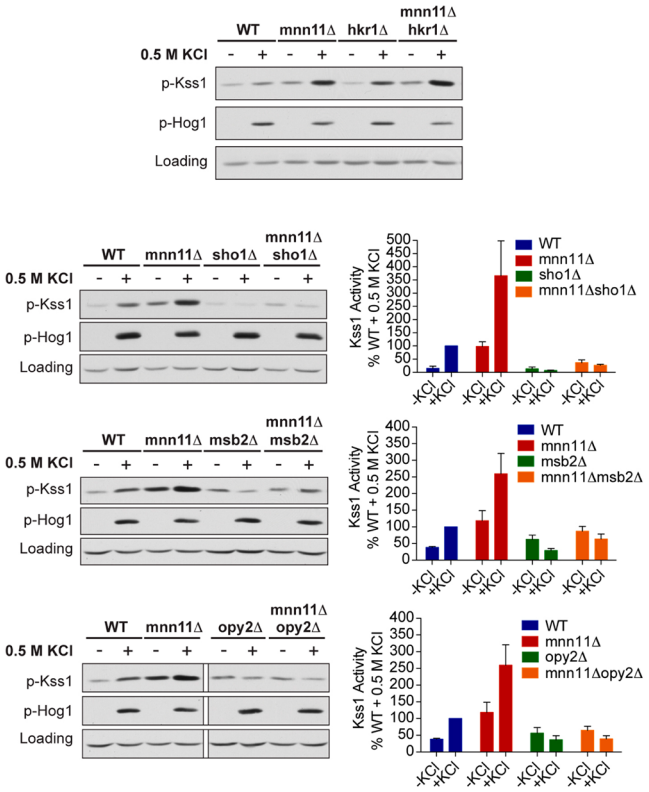

Figure 4. Mnn11 may target the glycoproteins Msb2 or Sho1, or a glycoprotein associated with Opy2. Activation of Kss1 and Hog1. *mnn11*Δ and the indicated double-mutant cells were stimulated with 0.5 M KCl for 5 min. Cell lysates were resolved by 10% SDS−PAGE and immunoblotting. Specific antibodies were used to detect the dually phosphorylated, fully activated forms of Kss1 (p-Kss1) and Hog1 (p-Hog1). G6PDH served as a loading control. The panels on the right show quantitation for those conditions that diminished Kss1 activity. The bottom panel is a composite image from a single exposure of a single gel. Error bars represent the standard error of the mean.

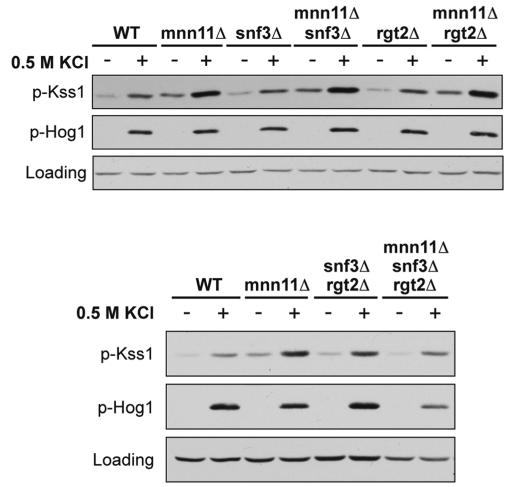

Figure 5. Both Snf3 and Rgt2 are required to limit the activation of Kss1 by osmotic stress. Activation of Kss1 and Hog1. *mnn11*Δ and the indicated double- or triple-mutant cells were stimulated with 0.5 M KCl for 5 min. Cell lysates were resolved by 10% SDS−PAGE and immunoblotting. Specific antibodies were used to detect the dually phosphorylated, fully activated forms of Kss1 (p-Kss1) and Hog1 (p-Hog1). G6PDH served as a loading control. Data presented are representative of three individual experiments.

turn, are thought to be regulated by Snf3 and Rgt2. These proteins are 12-pass transmembrane glucose sensors responsible for inducing the expression of hexose transporters.[20,43,44] Thus, there is an established functional relationship among the two glucose sensors, Opy2, and pathway fidelity. Indeed, the deletion of both Snf3 and Rgt2 together, but not deletion of either alone, was able to fully suppress Kss1 hyperactivation in the *mnn11*Δ background (Figure 5). In fact, we observed a stronger salt-dependent activation of Kss1 in the *snf3*Δ*rgt2*Δ strain than in the *mnn11*Δ*snf3*Δ*rgt2*Δ strain. This observation lends further support to the idea that the glucose sensors Snf3 and Rgt2 might potentially be in the same pathway with Mnn11, given the diminution of Kss1 activity when Mnn11 is absent.

Analysis of Glycosylation-Site Mutants. The data presented above reveal several proteins required to manifest the *mnn11*Δ phenotype. Among these are plasma membrane proteins that contain N-glycosylation sites. These include the glucose sensors Snf3 and Rgt2, as well as the putative osmosensors Msb2 and Sho1. Having shown that these proteins are needed to confer Kss1 hyperactivation in salt-treated *mnn11*Δ cells, we sought to identify specific sites of glycosylation that may be targeted by Mnn11. For each of the mutant strains, we introduced the corresponding gene, expressed from a single-copy plasmid, in which all potential N-glycosylation sites had been replaced with alanine: Asn383 in Snf3 ($snf3^{N383A}$), Asn136 and Asn385 in Rgt2 ($rgt2^{N136A/N385A}$), Asn59 in Sho1 ($sho1^{N59A}$), and Asn30 in Msb2 ($msb2^{N30A}$). Notably, aside from Asn30, Msb2 has six additional potential N-glycosylation sites, which have all been previously mutated (Msb2−6N/A) without an effect on basal FUS1-lacZ expression.[40] We then exposed these cells to hyperosmotic stress and immunoblotted for activated Kss1. Of these mutants, only $msb2^{N30A}$ was able to restore Kss1 hyperactivation to the corresponding deletion strain (*msb2*Δ) when either expressed from a plasmid (Figure 6A) or integrated into the genome at the *MSB2* locus (Figure 6B). Although we repeatedly and consistently observed Kss1 hyperactivation following construction of the $msb2^{N30A}$ mutant strains, the hyperactivation phenotype was less consistent as we propagated the strains, possibly as a consequence of genetic reversion or the acquisition of suppressor mutations. Given this instability, we did not attempt to construct any double-mutant strains containing $msb2^{N30A}$. In contrast, Kss1 responded normally in cells expressing $snf3^{N383A}$ and $rgt2^{N136A/N385A}$ (*snf3*Δ*rgt2*Δ strain) or $sho1^{N59A}$ (*sho1*Δ) (Figure 6A). Thus, Msb2, Sho1, and either Snf3 or Rgt2 can limit inappropriate activation of Kss1 by salt. Of these, only Msb2 requires an intact putative N-glycosylation site to function properly as a pathway insulator.

DISCUSSION

MAPK pathways often share common signaling components yet exhibit remarkably little cross-pathway activation. In yeast, salt stress leads to activation of the MAPK Hog1 but not Fus3, while mating pheromone activates Fus3 but not Hog1. Transient activation of Kss1 has been observed in response to pheromone[32] and osmotic stress.[18,45] However, neither stimulus is sufficient to invoke the filamentous growth response; this restriction is due in part to Fus3 phosphorylation of a transcription factor in the invasive pathway, Tec1. Tec1 is then desumoylated, ubiquitinated, and subsequently degraded.[46−50] Another mechanism entails Hog1 phosphorylation

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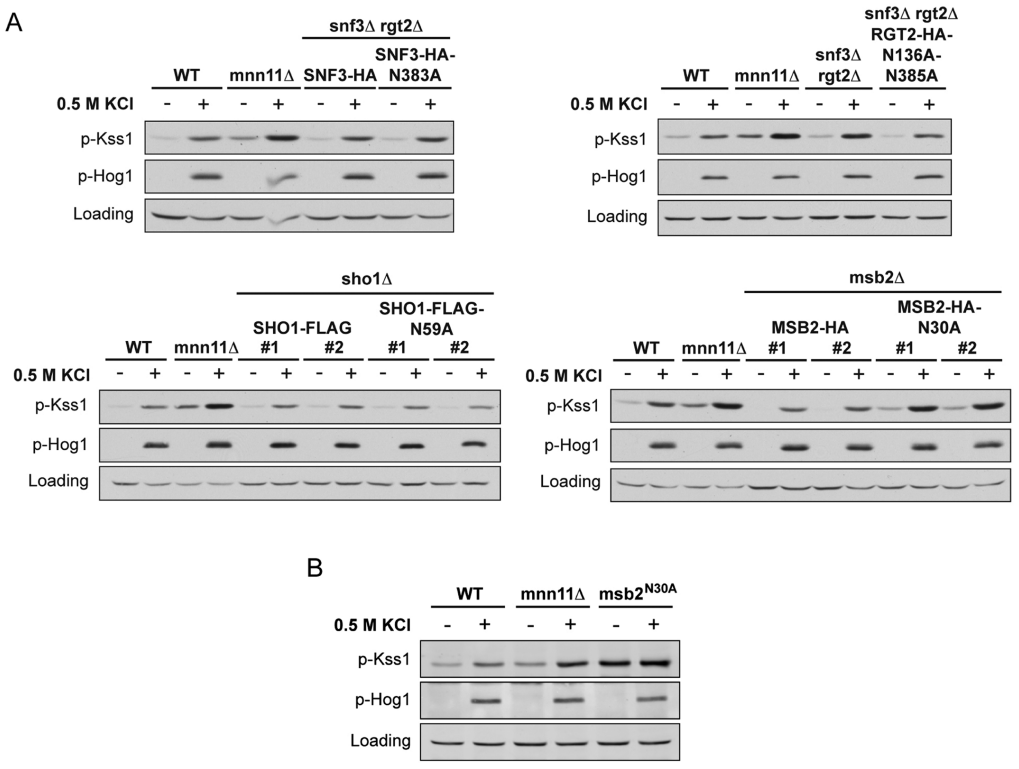

Figure 6. Msb2 Asn30 is a putative N-glycosylation site required to limit the activation of Kss1 by osmotic stress. (A) Activation of Kss1 and Hog1. *mnn11*Δ, *snf3*Δ*rgt2*Δ, *sho1*Δ, and *msb2*Δ mutant strains, transformed with pRS316-SNF3-HA, pRS313-RGT2-HA, pRS315-SHO1-FLAG, and pRS316-MSB2-HA, respectively, or the indicated glycosylation-site mutants were stimulated with 0.5 M KCl for 5 min. Cell lysates were resolved by 10% SDS−PAGE and immunoblotting. Specific antibodies were used to detect the dually phosphorylated, fully activated forms of Kss1 (p-Kss1) and Hog1 (p-Hog1). G6PDH served as a loading control. For the relevant uncomplemented deletion strains, refer to Figures 4 and 5. (B) Activation of Kss1 and Hog1 in the wild-type, the *mnn11*Δ mutant, or the integrated *msb2*^{N30A} mutant, stimulated with 0.5 M KCl for 5 min.[69]

of Ste50, one of several components shared by all three MAPK pathways. However, the role of Ste50 appears to be limited to cross talk between the FG pathway and the Sho1 branch of the HOG pathway, because Ste50 phosphorylation-site mutants sustain Kss1 signaling in *ssk1*Δ cells[18] more so than in wild-type cells, where both branches of the HOG pathway are functioning[20] (see Figure S3 of the Supporting Information). Nevertheless, the realization that Kss1 is activated by pheromone or osmotic stress, as well as by nutrient deprivation, challenged a long-held notion that cross activation would occur only upon loss of Fus3 or Hog1 function. On this basis, we initiated a genome-scale search for additional mutants that disrupt pathway fidelity. Our approach was modeled after previous efforts to analyze pathway specificity using comprehensive genomic,[31,45,51,52] proteomic,[53] and expression profiling strategies.[12,31,54,55] This screen led to the identification of *mnn11*Δ from the YKO collection, which could penetrate its substratum in response to hyperosmotic stress. Although we could not recapitulate the original invasive growth phenotype in a reconstructed *mnn11*Δ strain, we observed an increase in the level of Kss1 phosphorylation, indicative of activation. On the basis of these findings, we postulate the existence of additional, as yet unidentified, genes that limit invasive growth in response to Kss1. For example, this could explain why we did not identify *MNN10* in our original screen.

Hence, our effort revealed two new mutants that exhibit inappropriate activation of Kss1; when *MNN10* or *MNN11* is absent, Kss1 is hyperphosphorylated in response to salt stress. In comparison to that of the wild type, the abundance of Kss1 is elevated by 2-fold in the deletion strain (Figure S2 of the Supporting Information). The level of phospho-Kss1 is likewise elevated but increases an additional 2.5-fold under salt-stimulated conditions (Figure 2B). Notably, this salt-dependent increase in the level of phospho-Kss1 is evident only in the mutant strains. We conclude that Mnn11 and -10 limit the overall expression of Kss1 under basal conditions and also limit the activation of Kss1 under salt stress conditions. The same gene mutations do not alter the response of Kss1 to pheromone, and they do not alter the response of Fus3 or Hog1 to either pheromone or salt.

MNN10 and *MNN11* encode α-1,6-mannosyltransferases associated with the Golgi apparatus. These enzymes are responsible for extending the mannan chains on all N-glycosylated proteins destined for the cell surface.[24] Deletion of either *MNN10* or *MNN11* leads to a reduction in the length of mannan chains on N-glycosylated proteins.[24,56] On the basis of the known function of Mnn10 and Mnn11, we postulated that one or more N-glycosylated proteins serve to limit the activity of Kss1 under hyperosmotic conditions and that the substrate must be glycosylated to function properly. We subsequently established that deletion of a known glycoprotein (Msb2) is sufficient to suppress hyperactivation of Kss1 in the *mnn11*Δ mutant. Finally, we asked whether loss of a particular N-glycosylation site would lead to hyperactivation of Kss1, even in the absence of Mnn11. N-glycosylation-site mutations in Sho1, Snf3, or Rgt2 had no effect on Kss1. In contrast, mutation of a single candidate N-glycosylation site (Asn30) in Msb2 led to inappropriate activation of Kss1, in the manner of *mnn11*Δ.

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Previous studies have indicated that protein glycosylation is important for proper MAPK signaling, particularly within the FG pathway.[40,57−59] One study reported a high basal level of activation of Kss1 in cells bearing partial loss-of-function alleles of genes needed for O-linked and N-linked glycosylation.[57] Another study reported FUS1-lacZ induction when a specific type of O-glycosylation was absent and when the cells were treated with tunicamycin, a pharmacological inhibitor of protein N-glycosylation. That analysis pointed to Msb2 as the most likely target for O-glycosylation, and that some combination of O- and N- glycosylation was needed to maintain signal fidelity.[40] Our study advances earlier findings by identifying a specific mannosyltransferase activity as well as a single N-glycosylation site that is evidently needed for Msb2 to function as a pathway insulator.

While it is well established that Msb2 functions at the head of the FG MAPK pathway,[31,36] a long-standing question is how Msb2 initiates a signaling cascade leading to Kss1. The *MSB2* gene was originally identified as a high-copy number suppressor of temperature sensitive alleles of *cdc42* and *cdc24*.[60] Cdc42 is a Rho family GTPase, while Cdc24 is an exchange factor that activates Cdc42. Whereas Msb2 associates with Cdc42,[31] Sho1 binds Cdc24.[37] Thus, assembly of Msb2 with Sho1 could bring Cdc42 into the proximity of its activator.[36] Once activated, Cdc42 stimulates a protein kinase cascade beginning with Ste20[61,62] and culminating with Kss1.[31,36] It is also well established that diminished nutrient availability leads to filamentous growth in yeast.[6−8,10,63] Indeed, our data implicate a new role for the glucose sensors Snf3 and Rgt2 in the filamentous growth pathway, as these proteins are needed to confer Kss1 hyperactivation in salt-treated cells. However, it is not established how the pathway can detect changes in nutrient levels, levels that must fall somewhere between what is needed to support vegetative growth and what triggers entry into the stationary phase.[6] One possibility is that a reduction in glucose availability results in a diminished level of glycosylation of key signaling proteins.[64] In this regard, Msb2 is an attractive target of regulation. Perturbations to the Msb2 extracellular domain were shown previously to trigger Kss1 activation.[31,37,40] Our own data reveal a critical role for a candidate N-glycosylation site in Msb2, Asn30, as well as for two mannosyltransferases, Mnn10 and Mnn11, in dictating the activity of Kss1.

While much has been learned, important questions remain. How does protein glycosylation in general, and Mnn10 and -11 in particular, regulate Msb2? Synthesis of N-glycans begins in the endoplasmic reticulum (ER), where a core oligosaccharide structure is assembled on nascent proteins during their translocation into the ER lumen.[65] These N-glycosylated proteins are then delivered to the Golgi apparatus, where the oligosaccharide core structure is elaborated with mannose in one of two ways. Proteins located on endomembranes of the internal organelles have a small N-linked glycan structure with only a few mannoses added onto the core oligosaccharide. Proteins that are incorporated into the cell wall and/or plasma membrane, however, have a large mannan structure that consists of a backbone of ∼50 mannose residues with short side branches.[56] Mnn10 and Mnn11 are part of the M-pol II complex in the Golgi. M-pol II is dedicated to extending the mannan chain on all N-glycosylated proteins, and within this complex, both Mnn10 and Mnn11 are thought to be responsible for cellular α-1,6-mannosyltransferase activity.[24] When either of these enzymes is absent, the consequence is a shorter mannan chain on N-glycosylated proteins.[24,66]

How might nutrient limitation affect Msb2 glycosylation? Both Mnn10 and Mnn11 use mannose as a substrate. Mannose is derived from glucose 6-phosphate and glucose. As glucose becomes limiting, the extent of mannose synthesis will likely be diminished, and this could prevent Mnn10 and Mnn11 from properly modifying Msb2. Therefore, if underglycosylated Msb2 represents the activated form of the protein, the absence of Mnn10 or Mnn11 could be mimicking the events that occur normally following nutrient deprivation. The consequence in either case is Kss1 activation. Given the similarity between Msb2 and the signaling mucin MUC1 in animals, and given that both proteins activate MAP kinases (reviewed in refs 67 and 68), the mechanisms reported in yeast could point to similar processes in humans.

Further studies are needed to determine whether Msb2 is modified by Mnn10 or Mnn11 directly and whether Msb2 has a large mannan structure characteristic of M-pol II substrates. Addressing this question rigorously would require the reconstitution of M-pol II components Mnn11, Mnn9, Anp1, Mnn10, and Hoc1,[24] as well as the ability to detect and quantify the properly modified and mismodified forms of Msb2. Indeed, our efforts to monitor Msb2 modifications by immunoblotting were impeded by the poor detection of our epitope-tagged Msb2, the induction of Msb2 following Kss1 activation, and the existence of proteolytically processed forms of the protein in vivo.[31,37]

In summary, we have identified novel mutations that exhibit hyperactivation of Kss1 in response to osmotic stress stimulation. Our findings indicate that Mnn10 and Mnn11 act specifically to limit activation of Kss1 by an inappropriate stimulus. We propose that N-glycosylation of Msb2 in particular is needed for insulation of pathway signaling components. Together, these findings reveal new proteins and processes needed for proper MAPK signaling in cells.

■ ASSOCIATED CONTENT

Supporting Information

One table and four figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

CRE, cyclic adenosine monophosphate-responsive element; FG, filamentous growth; G6PDH, glucose-6-phosphate dehydrogenase; HOG, high-osmolarity glycerol; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MAPKKKK, mitogen-activated protein kinase kinase kinase kinase; SCD, synthetic complete dextrose; YKO, yeast knockout.

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