Glycosylation defects activate filamentous growth Kss1 MAPK and inhibit osmoregulatory Hog1 MAPK

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The yeast filamentous growth (FG) MAP kinase (MAPK) pathway is activated under poor nutritional conditions. We found that the FG-specific Kss1 MAPK is activated by a combination of an *O*-glycosylation defect caused by disruption of the gene encoding the protein *O*-mannosyl-transferase Pmt4, and an *N*-glycosylation defect induced by tunicamycin. The *O*-glycosylated membrane proteins Msb2 and Opy2 are both essential for activating the FG MAPK pathway, but only defective glycosylation of Msb2 activates the FG MAPK pathway. Although the osmoregulatory HOG (high osmolarity glycerol) MAPK pathway and the FG MAPK pathway share almost the entire upstream signalling machinery, osmostress activates only the HOG-specific Hog1 MAPK. Conversely, we now show that glycosylation defects activate only Kss1, while activated Kss1 and the Ptp2 tyrosine phosphatase inhibit Hog1. In the absence of Kss1 or Ptp2, however, glycosylation defects activate Hog1. When Hog1 is activated by glycosylation defects in *ptp2* mutant, Kss1 activation is suppressed by Hog1. Thus, the reciprocal inhibitory loop between Kss1 and Hog1 allows only one or the other of these MAPKs to be stably activated under various stress conditions.

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Introduction

Under optimal growth conditions, the budding yeast *Saccharomyces cerevisiae* grows as round-shaped, single cells. On nitrogen starvation or in the presence of poor carbon sources, however, diploid cells of certain strains (such as $\Sigma 1278$ h) initiate a fungus-like filamentous growth (FG)

(Gimeno *et al*, 1992). Haploid cells also undergo a related phenomenon whereby the elongated cells invade solid media (Roberts and Fink, 1994). These responses, which we will refer to collectively as FG, are considered as an adaptive mechanism that allows normally sessile yeast to forage for scarce nutrients or to evade harmful waste products. An FG response requires at least three distinct protein kinases: the cAMP-dependent protein kinase Tpk2, the 5'-AMP-dependent protein kinase Snf1, and the MAP kinase (MAPK) Kss1 (Chen and Thorner, 2007). This article focuses on the signalling pathway that activates Kss1, namely, the FG MAPK pathway, whose core is a three-kinase cascade composed of the Ste11 MAPKKK, the Ste7 MAPKK and the Kss1 MAPK (Roberts and Fink, 1994).

Another MAPK cascade, termed the high osmolarity glycerol (HOG) MAPK pathway, is activated by, and required for adaptation to, an increased external osmolarity (Brewster *et al*, 1993; Hohmann, 2002). The HOG MAPK pathway is composed of two functionally redundant upstream signalling branches that regulate the common Hog1 MAPK (Maeda *et al*, 1994, 1995). Of these branches, the SHO1 branch is closely related to the FG MAPK pathway. The SHO1 branch is initiated by an interplay between the membrane anchor protein Sho1 and the putative osmosensor membrane proteins Msb2 and Hkr1, leading to activation of Ste11, that is, the same MAPKKK that is involved in the FG MAPK pathway (Maeda *et al*, 1995; Tatebayashi *et al*, 2006, 2007). In the HOG pathway, however, activated Ste11 activates the Pbs2 MAPKK, which then activates the Hog1 MAPK (Posas and Saito, 1997) (see Figure 7A). Activated Hog1 governs a series of adaptive responses to high osmolarity, including temporarily arrest of the cell-cycle progression, readjustment of the transcription and translation patterns, and synthesis and intracellular retention of the compatible osmolyte glycerol (Bilsland-Marchesan *et al*, 2000; Teige *et al*, 2001; Hohmann, 2002; Escote *et al*, 2004; O'Rourke and Herskowitz, 2004).

Although both the FG and the HOG MAPK pathways involve Ste11, each controls completely different, and mutually incompatible responses. Naturally, osmostress normally activates only the Hog1 MAPK (Posas and Saito, 1997). However, osmostress can activate the FG MAPK pathway in mutant cells, such as *pbs2Δ* or *hog1Δ*, in which the Hog1 MAPK cannot be activated (O'Rourke and Herskowitz, 1998; Davenport *et al*, 1999). In other words, activated Hog1 inhibits undesirable cross-talk activation of the FG MAPK pathway by osmostress in normal cells. In the pathogenic *Candida albicans* yeast, its Hog1 homolog inhibits, or raises the activation threshold of, the filamentation-inducing Cek1 MAPK (the *Candida* homolog of Kss1) by various natural inducers, thereby affecting its virulence (Eisman *et al*, 2006). Thus, inhibition of Kss1 by Hog1 is a conserved feature of the yeast MAPK pathways.

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It is, however, unknown whether or how an inhibition of cross-talk in the reverse orientation occurs, that is, does activation of the FG pathway suppress the HOG pathway? Using a novel approach based on the use of glycosylation defective mutants, we show here that activation of the FG-specific Kss1 MAPK in fact suppresses a concomitant activation of the HOG-specific Hog1 MAPK. When activation of Kss1 is blocked by mutations, such as *ste7Δ* or *kss1Δ*, the Hog1 MAPK is activated by glycosylation defects. Kss1 inhibits Hog1 indirectly through the Ptp2 protein phosphatase. Thus, the FG MAPK pathway and the HOG MAPK pathway suppress each other so that only the relevant pathway is activated by a specific stimulus.

Results

A combination of O- and N-glycosylation defects induces *FUS1-lacZ* expression

To investigate the possible cross-regulation between the FG and the HOG pathways, a rapid and reliable method of activating the FG MAPK cascade is necessary. The commonly used method, namely the nutritional limitation, is inadequate, as it takes days to effect significant FG responses. A more promising approach is to use mutants that are defective in protein glycosylation. It has been shown that mutants of genes that are required for general glycosylation, such as *OCH1*, *PMI40* and *DPM1*, constitutively activate an FG-like response (Lee and Elion, 1999; Cullen *et al*, 2000). However, because mutants of these genes are severely defective in protein glycosylation, they are either very sick or nonviable. It was also reported that the inhibitor of N-glycosylation tunicamycin moderately activated the FG MAPK pathway in rich media after 16 h (Cullen *et al*, 2000). As we will describe in this article, however, tunicamycin alone could not appreciably activate the FG MAPK pathway, at least under our experimental conditions. We, therefore, examined the possibility that a combination of separate, and more limited, defects in O-glycosylation and N-glycosylation might induce the FG response more robustly.

We found that the commonly used reporters for the FG response, such as *FRE-lacZ* and *FG(TyA)-lacZ*, were unsuitable for our purposes because they had high baseline expression levels without stimulation and relatively moderate increases on stimulation (Mosch *et al*, 1996; Madhani and Fink, 1997; Davenport *et al*, 1999; Cullen *et al*, 2004). We, therefore, used the *FUS1-lacZ* reporter, which had much lower baseline expression levels and larger increases on stimulation (Cullen *et al*, 2000). Furthermore, all strains used in this study carried the *ssk2Δ ssk22Δ* double mutation unless otherwise specified, to eliminate any potential influence from the SLN1 branch of the HOG pathway.

To inhibit O-glycosylation, we disrupted the genes that encode a family of protein O-mannosyltransferases (Pmt) (Gentzsch and Tanner, 1996; Girrbach and Strahl, 2003). Disruption of any of the six *PMT* genes (*PMT1*–*PMT6*), individually, had no significant effect on expression of *FUS1-lacZ* (Figure 1). Inhibition of N-glycosylation by tunicamycin, in wild-type cells, also did not induce *FUS1-lacZ* expression. In clear contrast, tunicamycin treatment of *pmt4Δ* mutant cells strongly induced *FUS1-lacZ* expression (Figure 1). Tunicamycin treatment of other *pmt* single mutants did not induce *FUS1-lacZ* expression. Furthermore,

Figure 1 Induction of the *FUS1-lacZ* reporter gene by glycosylation defects. The indicated yeast mutant cells carrying the pFUS1-lacZ plasmid were grown exponentially and were treated with (+ tun) or without (–tun) tunicamycin (final conc. 25 μg/ml) for the indicated times. Activity of β-galactosidase in cell extracts was normalized using cell densities and expressed as Miller units (Miller, 1972). The strains used, and their full genotypes, are listed in Supplementary Tables S1 and S2, respectively.

disruption of one or two additional *PMT* genes in the *pmt4Δ* background (e.g. *pmt3/4/5Δ* or *pmt4/5/6Δ*) did not have any stronger effect than *pmt4Δ* alone (Supplementary Figure S1). Thus, *FUS1-lacZ* expression is induced when N-glycosylation and a specific type of O-glycosylation is inhibited.

Induction of *FUS1-lacZ* expression by glycosylation defects requires signalling elements of the FG pathway

We next examined whether induction of *FUS1-lacZ* expression by glycosylation defects is due to the activation of the FG pathway, because the *FUS1-lacZ* reporter can be induced also by the mating MAPK pathway. To determine which pathway is activated by the glycosylation defects, we disrupted, in the *pmt4Δ* background, genes that are specific for, or shared between, the mating and FG pathways. The results of an extensive survey are summarized in Figure 2A, which is a compilation of data from several different experiments. Although the mean value of the positive control strain (*pmt4Δ*) was somewhat variable from experiment to experiment (the range is between 34.3 and 61.9), only a typical control result is shown to save space. Thus, the exact values may not be directly comparable, but the following general conclusion is statistically valid.

From these data, two components in *FUS1-lacZ* expression are discernible: the basal component seen without tunicamycin treatment, and the tunicamycin-induced component. On the basis of the differential effect of the mutants on these two components, we could classify the mutants into four groups. The first group (*ste4Δ* and *ste5Δ*) abolished only the basal component without affecting the induced component. The second group, composed of *ste20Δ*, *ste50Δ*, *ste11Δ*, *ste7Δ*, *ste12Δ* and the *fus3Δ kss1Δ* double mutation, abolished both the basal and induced components. The third group, composed of *msb2Δ*, *opy2Δ* and *ptp2Δ*, affected only the induced component. Finally, the fourth group (*tec1Δ*, *fus1Δ*, *sho1Δ*, *hkr1Δ*, *hog1Δ*, *yps1Δ* and *ptp3Δ*) affected neither the basal

Figure 2 Signalling elements required for induction of *FUS1-lacZ* expression by glycosylation defects. **(A)** Induction of the *FUS1-lacZ* reporter gene by glycosylation defects. The indicated yeast mutant cells carrying the pFUS1-lacZ plasmid were grown exponentially, treated with (+tun) or without (–tun) tunicamycin for 8 h, and assayed for β -galactosidase activity as in Figure 1. **(B)** The indicated mutants were assayed as in (A). **(C)** Exponentially growing cells of the indicated genotypes were treated with 25 μ g/ml tunicamycin for the indicated times. Cell extracts were probed for phosphorylated (activated) forms of Fus3 (P-Fus3), Kss1 (P-Kss1) and Mpk1 (P-Mpk1) by immunoblotting using anti-phospho-p44/42 antibody.

nor the induced expression levels. Genes in groups 1 and 2, whose disruption abolishes the basal components, are known to be involved in the mating pathway. On the other hand, genes in the groups 2 and 3, whose disruption abolishes the tunicamycin-induced components, are known to be required for FG response, with the notable exception of *PTP2*. Any role of the Ptp2 protein tyrosine phosphatase (PTP) in the FG MAPK pathway has not been known or suspected. Later in this article, we will explain why inactivation of the *PTP2* gene suppresses *FUS1-lacZ* expression. Tec1 is required for the full FG response but is dispensable for induction of the *FUS1-lacZ* reporter (Madhani and Fink, 1997). Thus, in general, there is a good concordance between the genes that are required for the *FUS1-lacZ* expression induced by glycosylation defects and those that have been shown to be involved in the FG MAPK pathway.

Glycosylation defects induce characteristic FG responses

We examined whether glycosylation defects do indeed induce the FG response. A hallmark of the FG response is activation of the Kss1 MAPK, as opposed to the preferential activation of the Fus3 MAPK by the mating pathway (Chen and Thorner, 2007). As shown in Figure 2B, disruption of *KSS1* completely abolished tunicamycin-induced *FUS1-lacZ* expression, whereas disruption of *FUS3* only moderately reduced it, indicating that the Kss1 MAPK is mainly responsible for *FUS1-lacZ* expression by glycosylation defects. We then examined whether glycosylation defects activate Kss1 and/or Fus3 by probing for activation-associated phosphorylation of these kinases. The combination of a *pmt4Δ* mutation and

tunicamycin treatment activated Kss1 but not Fus3 (Figure 2C). Disruption of *STE7*, which encodes the direct activator of Kss1, completely inhibited Kss1 phosphorylation, whereas disruption of *STE4*, which encodes an upstream signalling element in the mating pathway, had no effect. The preferential activation of Kss1 by glycosylation defects is thus consistent with activation of the FG pathway.

Tunicamycin also activates the cell-wall integrity Mpk1 MAPK pathway (Bonilla and Cunningham, 2003; Cohen *et al*, 2008). Phosphorylation of Mpk1, however, occurs very slowly after tunicamycin treatment and is independent of Kss1/Fus3 (Figure 2C, upper panel). As there was no apparent connection between Mpk1 and the *FUS1-lacZ* response under study, we did not analyse Mpk1 any further.

We also examined whether glycosylation defects induce FG. As tunicamycin inhibits cell growth, however, it cannot be used to induce FG. Therefore, as an alternative to tunicamycin, we used 2-butanol that is known to accelerate FG in liquid culture (Lorenz *et al*, 2000; Andersson *et al*, 2004). When the parental (*PMT4*⁺) yeast strain was grown in SC medium containing 1% 2-butanol, no FG was observed over a 16-h period (Supplementary Figure S2). In clear contrast, *pmt4Δ* mutant cells became elongated and formed filaments. The *pmt4Δ msb2Δ* double mutant cells did not form any filaments. Thus, we conclude that O-glycosylation defects caused by *pmt4Δ* significantly lower the threshold of other stimuli for induction of the FG response.

Opy2 and Msb2 are O-glycosylated by Pmt4

Next, we investigated the potential Pmt4 target(s) whose underglycosylation might activate the FG response. Pmt4 is

unique among the protein *O*-mannosyltransferases in that it specifically glycosylates Ser/Thr-rich extracellular segments in membrane-attached proteins (Hutzler *et al*, 2007). This fact suggests that two membrane proteins required in the FG response, Opy2 and Msb2, are both potential substrates of Pmt4. Indeed, Opy2 has been shown to be glycosylated by Pmt4 (Hutzler *et al*, 2007).

Opy2 has two potential glycosylated Ser-rich (SR) regions, SR1 (aa 2–22) and SR2 (aa 75–84), in its extracellular domain (Figure 3A). The full-length, GFP-tagged Opy2 protein (Opy2-FL-GFP) isolated from *PMT4*⁺ cells was significantly larger than the same protein isolated from *pmt4Δ* cells (Figure 3B, compare lanes 1 and 5), indicating Pmt4-dependent glycosylation of Opy2. Deletion of the SR2 region did not reduce the Pmt4-dependent glycosylation (Figure 3B, lanes 3 and 7). In contrast, Opy2-ΔSR1-GFP had the same size in *PMT4*⁺ and *pmt4Δ* cells (Figure 3B, lanes 2 and 6), indicating that Pmt4 modifies only the SR1 region of Opy2.

Msb2 is also a glycosylated transmembrane protein (Cullen *et al*, 2004). Its extracellular domain contains an extended Ser/Thr rich (STR) region (aa 52–956) that is likely to be

O-glycosylated (Hutzler *et al*, 2007) (see Figure 4A). The full-length HA-tagged Msb2 (Msb2-FL-HA) isolated from *pmt4Δ* cells migrated slightly faster in SDS-PAGE than the same molecule obtained from *PMT4*⁺ cells, indicating a contribution of Pmt4-dependent glycosylation. However, both molecules migrated at positions much higher than the calculated molecular weight of Msb2-FL-HA (ca. 150 kDa), reflecting the presence of Pmt4-independent glycosylation as well (Figure 4B). To clarify the roles of individual Pmt enzymes in Msb2 glycosylation, we used Msb2-ΔSTR1-HA and Msb2-ΔSTR2-HA for further analyses (see Figure 4A). The smaller size of these constructs allowed easier evaluation of the effect of *pmt* mutations. Both proteins, when expressed in *pmt1Δ*, *pmt2Δ* or *pmt4Δ* mutant cells, were smaller than when expressed in *PMT4*⁺, *pmt3Δ*, *pmt5Δ* or *pmt6Δ* cells (Figure 4C and D), indicating that Pmt1, Pmt2 and Pmt4 contribute to Msb2 *O*-glycosylation. The fact that *pmt1Δ* and *pmt2Δ* reduced Msb2 glycosylation to a similar extent is consistent with the earlier observation that Pmt1 and Pmt2 form a functional heterodimer (Girrbach and Strahl, 2003). More important, the size reduction of Msb2 in *pmt4Δ* cells is

Figure 3 Glycosylation of Opy2 by Pmt4, and its role in the FG response. **(A)** Schematic diagrams of the Opy2 constructs used in (B–D). FL, full-length; SR, Ser-rich; TM, transmembrane segment. **(B)** C-terminally GFP-tagged Opy2 constructs were generated using a single-copy vector with the *GAL1* promoter (p414GAL1). Expression of Opy2-FL-GFP, or the indicated deletion constructs, was induced by 2% galactose for 4 h in *PMT4*⁺ or *pmt4Δ* host cells. Total cell extracts were probed with an anti-GFP antibody by immunoblotting. The horizontal arrow indicates the estimated cleavage site is shown in (A) by an upright arrow. **(C)** The full-length *OPY2* gene (with the *OPY2* promoter) carried on a single-copy vector (pRS414) or its deletion constructs were individually expressed in *pmt4Δ ste4Δ opy2Δ* host cells that also carried the pFUS1-lacZ reporter plasmid. Cells were treated with (+ tun) or without (–tun) tunicamycin (final conc. 25 μg/ml) for 8 h, before extracts were prepared for β-galactosidase assays. **(D)** GST-tagged Ste50 (GST-Ste50), or GST alone, was constitutively expressed using pP122 or the vector p426TEG1, and expression of GFP-tagged Opy2-FL (Opy2-FL-GFP), or Opy2ΔC-GFP, was induced by 2% galactose for 4 h using the expression constructs based on the p414GAL1 vector, in *pmt4Δ ste50Δ opy2Δ* host cells. GST-Ste50 (or GST) was immunoprecipitated by glutathione-Sepharose beads (bottom panel), and co-precipitated Opy2-GFP protein was detected by immunoblotting (top panel). Expression levels of the Opy2-GFP proteins are shown in the middle panel.

Figure 4 Pmt4 glycosylation of Msb2. (A) Schematic diagrams of the deletion constructs of Msb2 used in (B–F). Each construct contains two HA epitopes, one following amino acid position 48 and another at the C terminus. STR, Ser/Thr rich; HMH, Hkr1-Msb2 Homology domain; TM, transmembrane segment; FL, full-length. (B–E) Indicated Msb2-HA constructs, under the control of the *GAL1* promoter in the single-copy vector p416GAL1, were induced by 2% galactose for 3 h in *PMT*⁺ or the indicated *pmt* mutant cells. Cell extracts were probed by immunoblotting with an anti-HA antibody. (F) Endo H treatment of Msb2. The Msb2-ΔSTR2-HA construct, under the control of the *GAL1* promoter in the single-copy vector p416GAL1, was induced by 2% galactose for 3 h in *PMT*⁺ cells and in the indicated *pmt* mutant cells. Cell extracts were prepared in Buffer N, immunoprecipitated with anti-HA antibody (3F10), treated with (+) or without (–) 0.4 U/ml endoglycosidase H (Endo H) at 37°C for 15 h, applied to SDS–PAGE, and probed with another anti-HA antibody (12CA5).

quite different from that in *pmt1Δ* and *pmt2Δ* cells, clearly indicating that Pmt4 contributes to a distinct subset of Msb2 *O*-glycosylation. The Msb2-ΔSTR3-HA mutant protein, in which most of the STR region is absent, migrated closer to the calculated peptide molecular weight (ca. 50 kDa) (Figure 4E). Taken together, we conclude that Pmt4 plays a significant role in *O*-glycosylation of Msb2. Pmt1 and Pmt2 also glycosylate Msb2, but such glycosylation is either irrelevant or insufficient for the FG response.

Msb2 has a number of potential *N*-glycosylation sites in addition to its numerous *O*-glycosylation sites. As defects in Pmt4-dependent *O*-glycosylation can interfere with the *N*-glycosylation status of the same protein (Ecker *et al*, 2003), we estimated the degree of Msb2 *N*-glycosylation by endoglycosidase H (Endo H) treatment. To this end, the Msb2-ΔSTR2-HA mutant protein was expressed in *PMT*⁺, *pmt1Δ* and *pmt4Δ* host cells, and the size of the protein before and after Endo H treatment was compared by SDS–PAGE. As shown in Figure 4F, Endo H treatment reduced the size of Msb2-ΔSTR2-HA to a similar extent irrespective of the host *pmt* mutation. Thus, at least in these cases, *O*-glycosylation defects did not substantially affect the status of Msb2 *N*-glycosylation.

In some cases, *O*-glycosylation is important for targeting proteins to the plasma membrane (Proszynski *et al*, 2004). Using YFP-fusion constructs, we examined whether removal of a substantial portion of the *O*-glycosylated STR region might affect the subcellular localization of Msb2. As shown in Supplementary Figure S3, both Msb2-FL-YFP and its deletion

derivative Msb2-ΔSTR4-GFP are similarly localized on the plasma membrane, although a substantial amount of both constructs is also found in intracellular compartments, as observed earlier for Msb2-FL-YFP (Tatebayashi *et al*, 2007). Thus, removal of a substantial portion of the *O*-glycosylated STR region does not affect the cellular localization of the Msb2 protein.

Glycosylation defects of Msb2, but not of Opy2, activate the FG MAPK pathway

We then asked whether defective glycosylation of Opy2 or Msb2 induces the FG response. First, we examined the possibility that the glycosylated region in Opy2 is required for the FG response. However, an Opy2 mutant lacking the entire *O*-glycosylated domain (Opy2-ΔSR1) could fully support *FUS1-lacZ* expression (Figure 3C). In contrast, deletion of the cytoplasmic C-terminal region (aa 184–360) completely abolished *FUS1-lacZ* expression (Figure 3C). Although the full-length Opy2 protein binds to Ste50, Opy2-ΔC does not (Figure 3D). These results are consistent with the idea that the major function of Opy2 is to anchor Ste50 to the membrane, which is necessary for efficient signalling in the FG and HOG pathways (Wu *et al*, 2006; Tatebayashi *et al*, 2007). However, Opy2 glycosylation, or lack of it, may not be a factor in the FG response.

We then examined the possible role of the Msb2 glycosylation region in the FG response. When Msb2-ΔSTR4, in which a large segment of the glycosylated STR domain is deleted, was expressed in *pmt4Δ* host cells using the *MSB2* own

promoter, *FUS1-lacZ* was induced even in the absence of tunicamycin (Figure 5A and B). When less extensive deletion mutants, Msb2- Δ STR7 and Msb2- Δ STR8, were expressed, weaker induction of *FUS1-lacZ* was observed in the absence of tunicamycin. Expression of the least extensive deletion mutant, Msb2- Δ STR5, did not induce *FUS1-lacZ*. Thus, there was a clear correlation between the extent of STR deletion and the extent of *FUS1-lacZ* induction in the absence of tunicamycin. All these Msb2 STR deletion mutants supported tunicamycin-induced *FUS1-lacZ* expression to similar extent.

Figure 5 Domains of Msb2 essential for the FG response. (A) Schematic diagrams of the Msb2 deletion constructs used in (B) and (C). STR, Ser/Thr rich; HMH, Hkr1-Msb2 Homology domain; TM, transmembrane segment; FL, full-length. (B) Induction of the FG reporter gene *FUS1-lacZ* by glycosylation defects. The full-length *MSB2* gene, or its deletion constructs, carried on the single-copy vector pRS416 were individually expressed in *pmt4Δ ste4Δ msb2Δ* host cells in which the *FUS1-lacZ* reporter gene has been integrated. Cells were treated with (+ tun) or without (–tun) tunicamycin (final conc. 25 μ g/ml) for 8 h, before extracts were prepared for β -galactosidase assays. In HMH(Hkr1), the HMH domain of Msb2 was replaced by the homologous HMH domain from Hkr1. (C) Expression of the indicated Msb2 or Opy2 construct, placed under the *GAL1* promoter, was induced by galactose for 2 h. Induction of the FG reporter gene *FUS1-lacZ* was assayed.

Thus, deletion of the glycosylated STR segment constitutively activated Msb2. Indeed, overexpression of Msb2- Δ STR4 using an inducible *GAL1* promoter activated the FG pathway even in glycosylation-proficient *PMT4+* host cells, whereas expression of full-length Msb2 did not (Figure 5C). Thus, deletion of the STR region has a similar effect as glycosylation defects. In contrast, overexpression of glycosylation-defective Opy2- Δ SR1 did not activate the FG MAPK pathway at all, further supporting the earlier conclusion that glycosylation of Opy2 does not affect the FG MAPK pathway. Combining all the data, we conclude that deficiency of Pmt4-mediated O-glycosylation of Msb2 in the STR region is a part of signal that activates the FG MAPK pathway.

Msb2 has seven potential N-glycosylation sites that conform to the N-X-T/S motif (X being any aa other than P) in its extracellular domain (Asn-30, Asn-859, Asn-885, Asn-945, Asn-1049, Asn-1088 and Asn-1175). To test whether the effect of tunicamycin can be accounted for by defective Msb2 N-glycosylation, we generated a mutant termed Msb2-6N/A in which six of the potential N-glycosylation sites were mutated to Ala (Asn-30, which is outside the STR region, was left unmodified). Expression of the Msb2-6N/A construct in *pmt4Δ* host cells at the endogenous level (i.e. from the *MSB2* promoter in a single-copy plasmid) did not induce *FUS1-lacZ* expression any better than wild-type Msb2, either in the absence or presence of tunicamycin (Supplementary Figure S4). Likewise, overexpression of Msb2-6N/A in *pmt4Δ* host cells using the *GAL1* promoter did not induce *FUS1-lacZ* expression any better than wild-type Msb2 (Supplementary Figure S5). These results suggest that N-glycosylation defects in Msb2 alone are not sufficient to account for the effect of tunicamycin, and that there might be (an)other protein(s) whose N-glycosylation defects are involved in induction of *FUS1-lacZ* expression.

Activated Kss1 inhibits the Hog1 MAPK

With a reliable method to activate the FG pathway in hand, we investigated the interaction between the FG and the HOG MAPK pathways, using the HOG-specific reporter *8xCRE-lacZ* (Tatebayashi *et al*, 2006). The *pmt4Δ* defect (in the absence of tunicamycin treatment) neither constitutively activated the HOG MAPK pathway in the absence of osmotic stress, nor inhibited the HOG response in the presence of osmotic stress (Supplementary Figure S6, open bars). We found, however, that tunicamycin treatment of *pmt4Δ* mutant cells, which activates the FG MAPK pathway, did not activate the HOG MAPK pathway (Figure 6A). In clear contrast, however, if activation of the Kss1 MAPK is blocked by *ste7Δ*, the HOG MAPK pathway was activated by tunicamycin treatment in the *pmt4Δ* cells (Figure 6A). Inactivation of both Kss1 and Fus3 (*kss1Δ fus3Δ*) also activated the HOG MAPK pathway by glycosylation inhibition (Figure 6A). Expression of the Kss1 MAPK (by a plasmid-carried *KSS1*) in the *kss1Δ fus3Δ* mutant completely inhibited the HOG pathway, whereas expression of the Fus3 MAPK only weakly inhibited it (Figure 6B). Disruption of either *KSS1* or *FUS3* separately gave essentially identical results (Supplementary Figure S7).

These results suggest that activated Kss1 inhibits the HOG MAPK pathway. If so, then osmotic activation of Hog1 MAPK might be also inhibited in tunicamycin-treated *pmt4Δ* cells. Indeed, after 2 h incubation with tunicamycin, osmotic activation of the Hog1 MAPK in *pmt4Δ* cells was significantly

Figure 6 Cross-inhibition of the Hog1 MAPK by the FG MAPK pathway. (A–C) Induction of the HOG reporter gene *8xCRE-lacZ* by glycosylation defects. Yeast strains of the indicated genotypes, carrying the reporter plasmid, were stimulated with (+) or without (–) 25 µg/ml tunicamycin for 8 h, before cell extracts were prepared for β-galactosidase assay. [KSS1] and [FUS3] in (B) indicate that the strain contains a single-copy plasmid carrying the *KSS1* or *FUS3* gene with its own promoter. (D) Activation of the Hog1 MAPK by glycosylation defects. Exponentially growing cells of the indicated genotypes were treated with (+) or without (–) 25 µg/ml tunicamycin for 2 h. Cell extracts were probed for phosphorylated Hog1 (P-Hog1) and total Hog1 by immunoblotting. (E) Induction of the *FUS1-lacZ* reporter gene by glycosylation defects. The indicated yeast mutant cells carrying the pFUS1-lacZ plasmid were grown exponentially, treated with tunicamycin for 8 h (+ tun) or untreated (–tun), and assayed for β-galactosidase.

weaker than that in tunicamycin-untreated *pmt4Δ* cells, or in tunicamycin-treated *PMT4*⁺ cells (Supplementary Figure S6). The inhibition was not complete, probably because osmotic stress activated the Hog1 pathway much more intensely than glycosylation defects did. Thus, we conclude that activation of Kss1 by glycosylation defects suppresses Hog1 activation.

Mutual inhibition between the Hog1 MAPK and the Kss1 MAPK

To elucidate how activation of Kss1 inhibited the HOG pathway, we first examined the possibility that activated Kss1 induced an inhibitor of the HOG pathway. Thus, we disrupted *STE12* and *TEC1*, which are involved in Kss1-dependent gene

expression (Chou *et al*, 2006), and *FUS1*, whose expression is induced by activated Kss1 and/or Fus3. As shown in Figure 6C, however, disruption of these genes did not activate the HOG pathway by glycosylation defect. Thus, the mechanism by which Kss1 inhibits the HOG pathway is not under the control of the Ste12 or Tec1 transcription factors. Although Fus1 has been shown to bind and inhibit Sho1 (Nelson *et al*, 2004), it does not seem to have a role in the inhibition of the HOG pathway by Kss1, which is consistent with the fact that Sho1 was dispensable for the tunicamycin-dependent activation of the FG MAPK pathway (see Figure 2A).

We then examined another possibility that is suggested by the fact that yeast MAPKs are negatively regulated by PTPs.

In particular, Ptp2 preferentially inactivates Hog1, whereas Ptp3 primarily inactivates Kss1 and Fus3 (Jacoby *et al*, 1997; Wurgler-Murphy *et al*, 1997; Zhan and Guan, 1999). Therefore, we tested whether either Ptp2 or Ptp3 might be responsible for inhibition of the HOG MAPK pathway by activated Kss1. As seen in Figure 6C, tunicamycin-treatment induced the HOG-specific *8xCRE-lacZ* reporter in *pmt4Δ ptp2Δ* mutant cells, but not in *pmt4Δ ptp3Δ* mutant cells. The expression level of the *8xCRE-lacZ* reporter in *pmt4Δ ptp2Δ* treated with tunicamycin was about 30% of the reporter expression induced by osmotic stress (0.4 M NaCl) (compare Figure 6C with Supplementary Figure S6B). Furthermore, on tunicamycin treatment, phosphorylation of Hog1 was seen in *pmt4Δ ptp2Δ* mutant cells, but not in *pmt4Δ* cells (Figure 6D). The time-course of Hog1 phosphorylation in *pmt4Δ ptp2Δ* cells is comparable to that of Kss1 phosphorylation in *pmt4Δ* cells (compare Figure 2C with Supplementary Figure S8). Hog1 phosphorylation was also induced in *PMT4⁺ ptp2Δ* host cells (Supplementary Figure S8). In *PMT4⁺* host cells, tunicamycin activates the Kss1 pathway only marginally, but perhaps the signal was sufficient to phosphorylate Hog1 in the absence of Ptp2. Thus, Ptp2 is required to prevent the Hog1 MAPK from being activated by the glycosylation defects, although we cannot yet conclude that Ptp2 is the direct target of Kss1.

This finding suggested a possible explanation for the earlier observation that *ptp2Δ* mutation inhibited Kss1 activation by glycosylation defects, namely that perhaps activated Hog1 MAPK, in its turn, might inhibit the Kss1 MAPK. Indeed, when the *HOG1* gene was disrupted in *pmt4Δ ptp2Δ* mutant cells, induction of *FUS1-lacZ* expression by glycosylation defects was restored (Figure 6E). In contrast, disruption of the *PTP3* gene in *pmt4Δ ptp2Δ* mutant cells did not restore *FUS1-lacZ* expression, indicating that Ptp3 is not responsible for the suppression of Kss1 by Hog1. We tested the possibility that Hog1 directly inhibits Kss1 by phosphorylation, by mutating the only MAPK substrate motif in Kss1, namely Thr244-Pro245. Activation of the Kss1-T244A mutant, however, was also inhibited by Hog1 (data not shown). Thus, it is yet unclear how activated Hog1 inhibits Kss1 activation.

Integrating all results, we conclude that glycosylation defects activate the Ste11 MAPKKK, which can activate, if uninhibited, both the Ste7-Kss1 and the Pbs2-Hog1 kinase

cascades. However, only one kinase or the other is stably activated because of the reciprocal inhibitory loop between Hog1 and Kss1. Signal flows in several key mutants on glycosylation defects are interpreted schematically in Figure 7.

Discussion

In the first part of this report, we extended earlier observations that general glycosylation defects activate the FG response (Lee and Elion, 1999; Cullen *et al*, 2000) by showing that inhibition of *N*-glycosylation and a specific type of *O*-glycosylation, effected by a combination of tunicamycin treatment and *pmt4Δ* mutation, activates the FG MAPK pathway. In particular, we have identified a critical role for Pmt4-mediated *O*-glycosylation of the membrane protein Msb2 in FG pathway activation.

Although there are still major gaps in our knowledge, the specific role of Msb2 in the FG pathway became clearer. It is apparent that there is a considerable mechanistic similarity among the osmotic stress activation of the Hog1 MAPK (the HOG response), the activation of the Kss1 MAPK by osmotic stress in the absence of Pbs2 or Hog1 (cross-talk), and the authentic FG response. Thus, to understand the role of Msb2 in the FG response, it would be helpful to recall the known role of Msb2 in the HOG response as well as the cross-talk activation of Kss1 by osmotic stress (Tatebayashi *et al*, 2007). The SHO1 branch of the HOG pathway is initiated by an interplay between the essential HMH domain and the inhibitory STR domain in the Msb2 molecule. The extracellular HMH domain (residues 960–1113) is juxtaposed to the glycosylated STR domain and is required for activation of the HOG MAPK pathway as well as of the FG MAPK pathway (see Figure 5B). It is possible that the positively regulatory HMH domain is normally masked by the *O*-glycosylated STR domain, and that underglycosylation, or deletion of a large segment, of the STR domain unmasks the HMH domain. An analysis by deletion scanning has shown that the entire region between 979 and 1117 is required for activation of the HOG pathway (Tatebayashi *et al*, 2007), as well as for the cross-talk activation of the FG pathway by osmotic stress (KT and HS, unpublished data). It is therefore reasonable to assume that the HMH domain serves a similar positive role

Figure 7 Schematic diagrams of signal flow in the Kss1 and Hog1 MAPK pathways in three yeast mutants: (A) *pmt4Δ*, (B) *pmt4Δ ptp2Δ*, and (C) *pmt4Δ ptp2Δ hog1Δ*. Black arrows and T-shaped bars indicate, respectively, activation and inhibition. Gray arrows and bars represent lack of activation or inhibition. Crosses (X) signify disrupted genes. The precise molecular mechanisms by which Kss1 inhibits Hog1, or Hog1 inhibits Kss1, are currently unknown.

in the FG response. If so, activated Msb2 may interact with Sho1 to initiate an intracellular signal flow (Tatebayashi *et al*, 2007). As indicated in Figure 2A, however, Sho1 is not required for the glycosylation defect-dependent activation of the FG MAPK pathway. This is not contradictory, however, as we have shown that Msb2 can activate the Hog1 MAPK (and also the Kss1 MAPK by cross-talk) through two different mechanisms, or modes (Tatebayashi *et al*, 2007). The mode 1 mechanism entails the interaction between Msb2 and the ectodomain of Sho1. The mode 2 mechanism of the HOG pathway, however, involves only the Sho1 cytoplasmic domain, which is needed to activate the Pbs2 MAPKK. As the FG response does not involve Pbs2 at all, activation of the Kss1 MAPK by the mode 2 mechanism can proceed in the complete absence of Sho1.

We might then speculate that the Msb2 STR domain serves as a dual-purpose sensor for environmental conditions. Under hyperosmotic conditions, the physico-chemical changes in the oligosaccharide gel structure might unmask the essential HMH domain, whereas under poor nutritional conditions, underglycosylation of STR unmasks the HMH domain.

It was recently proposed that the aspartyl protease Yps1 activates the FG MAPK pathway by cleaving the extracellular domain of Msb2. Proteolytic degradation of overexpressed Msb2, as revealed by shedding of the Msb2 ectodomain, is nearly completely blocked in *yps1Δ* mutant (Vadaie *et al*, 2008). The primary cleavage site was mapped within the residues 1045–1145, which was termed the cleavage domain (CD). It was reported without data that *yps1Δ* mutant was only partially defective in activating the FG MAPK pathway (Vadaie *et al*, 2008), which is perhaps consistent with our result that *yps1Δ* mutation does not inhibit the induction of *FUS1-lacZ* by glycosylation defects (Figure 2A). Deleting the CD abrogated the Yps1-dependent Msb2 degradation, and completely inhibited the FG response. However, because deleting the CD also affects the essential HMH domain, it does not provide evidence that Yps1-dependent cleavage itself is essential for the FG response. Clearly, further investigations are required to elucidate the individual roles of, and possible interrelationship between, the Yps1-dependent cleavage and glycosylation defects in activating the Kss1 MAPK cascade.

The Pmt4-dependent *O*-glycosylation of the pathogenic yeast *C. albicans* has been shown to affect hyphal morphology, which is closely related to the FG of *S. cerevisiae*. Hyphal morphogenesis of a homozygous *pmt4* mutant is defective under aerobic conditions, but enhanced in embedded or hypoxic conditions (Prill *et al*, 2005). In live mouse model system, *pmt4* mutants are markedly less virulent than *PMT4* counterparts (Prill *et al*, 2005). Thus, Pmt4-dependent glycosylation serves a complex role in environment-dependent signalling that is critical for pathogenicity of *C. albicans*. It is hoped that our current results shed light on the complex role of the Pmt4-dependent *O*-glycosylation in this important pathogen.

In the second part of this report, we investigated the interaction between the FG and the HOG MAPK pathways. In the budding yeast, three functionally distinct MAPK signalling pathways share the same Ste11 MAPKKK: the mating pheromone pathway, the FG MAPK pathway, and the SHO1 branch of the osmoregulatory HOG MAPK pathway

(Chen and Thorner, 2007). Nevertheless, each pathway is only activated by cognate stimuli.

Mating pheromones activate the Ste11 MAPKKK, but Ste11 thus activated does not lead to any activation of the Hog1 MAPK (Posas and Saito, 1997). It is well established that the HOG pathway is insulated from the pheromone stimulation, because the Ste11-Ste7-Fus3 MAPK cascade of the mating pathway is tethered by the scaffold protein Ste5, which also interacts with the mating-specific upstream components (Elion, 2001). Direct inhibition of the Hog1 MAPK by activated Fus3 may also contribute to the lack of Hog1 activation by the mating pheromones (McClean *et al*, 2007). On mating pheromone stimulation, Kss1 is activated only transiently, whereas Fus3 is activated for the duration of the mating response (Sabbagh *et al*, 2001). The mechanism by which activated Fus3 inhibits Kss1 is unknown, but an involvement of a phosphatase has been suggested (Chen and Thorner, 2007). Stimulation of the FG pathway through the Msb2 sensor does not entail any Fus3 activation, probably because Fus3 is sequestered by Ste5 binding. Indeed, when binding of Fus3 to Ste5 is abrogated by nondocking mutation in Ste5 (Ste5ND), Fus3 behaves like Kss1 (Hao *et al*, 2008). Thus, there is perhaps no need for Kss1 to directly inhibit Fus3.

The relationship between the HOG and the FG MAPK pathways appears to be more subtle. These MAPK pathways use basically the same signalling machinery that includes the putative sensor Msb2, the G-protein Cdc42, the PAK-like kinase Ste20, the adaptor protein Ste50 and the Ste11 MAPKKK (Chen and Thorner, 2007). Nevertheless, osmotic stress does not activate the FG-specific Kss1 MAPK. The Kss1 MAPK is directly or indirectly inhibited by activated Hog1, because osmotic stress does potentially activate the Kss1 MAPK in *hog1Δ* or *pbs2Δ* mutant strains (O'Rourke and Herskowitz, 1998; Davenport *et al*, 1999).

It has been difficult, however, to examine the reverse situation, namely whether activated FG MAPK pathway interferes with the HOG pathway or not, because no suitable way to activate the FG pathway was available for such studies. We found that the HOG MAPK pathway was indeed inhibited when the FG MAPK pathway was activated by glycosylation defects. Furthermore, if activation of the Kss1 MAPK was prevented, for example, by *ste7Δ*, then there was a robust activation of the Hog1 MAPK by glycosylation defects. We also found that inhibition of the Hog1 MAPK was mediated by the Ptp2. It is possible that activated Kss1 affects expression or activity of Ptp2, but the alternative explanation that Kss1 and Ptp2 both inhibit Hog1 independently of each other has not been excluded. Further experiments are required to clarify the detailed mechanism of inhibition of Hog1 by Kss1.

An important finding from this study is that activation of the FG MAPK pathway and that of the HOG MAPK pathway are mutually exclusive. When either one of these MAPK pathways is activated, the second pathway is suppressed, thereby achieving bistability of the system. However, a tantalizing question remains; that is, how is the correct MAPK pathway chosen for activation by a specific stimulus to begin with?

A clue to a possible solution might be in the different kinetic properties of activating stimuli. Activation of the FG pathway by accumulation of underglycosylated Msb2

molecules is relatively weak and gradually increases in strength. In contrast, activation of the Sho1 branch of the HOG pathway by a hyperosmolarity is rapid and stronger in intensity (Tatebayashi *et al*, 2007). Perhaps, both the FG and the HOG MAPK pathways are initially activated to limited extents, but, depending on the intensity and duration of the stimulus, cross-inhibition in one or the other direction eventually dominates, resulting in activation of only one pathway.

Another clue to the problem is the observation that, even though Sho1 and Msb2 are involved in both the HOG and FG MAPK pathways, their participation in each pathway is not exactly identical. On the one hand, the Sho1 cytoplasmic SH3 domain, which binds the Pbs2 MAPKK, is essential for the HOG pathway, but is not required for the FG pathway (Maeda *et al*, 1995; Tatebayashi *et al*, 2007). On the other hand, the cytoplasmic region of Msb2, whose function is yet unknown, is essential for the FG pathway, but optional for the HOG pathway (Tatebayashi *et al*, 2007). It is possible that different stimuli might affect these cytoplasmic domains differently, so that the signal flow from activated Ste11 MAPKKK to its two potential targets, Pbs2 and Ste7, is properly steered. In this context, it will be interesting to investigate the potential role of Msb2 as a membrane anchorage protein specific to the FG MAPK pathway.

In conclusion, we showed the presence of a reciprocal inhibitory loop between the FG promoting Kss1 MAPK and the osmoregulatory Hog1 MAPK. This will serve as a basis for an integrated regulatory MAPK signalling network in yeast, as well as a model for similar networks in other organisms.

Materials and methods

Yeast strains

The yeast strains used, and their complete genotypes, are listed in Supplementary Tables S1 and S2, respectively.

Media and buffers

Yeast media YPD, YPGal, SC, SGal and CAD, and Buffers A and Z were defined earlier (Horie *et al*, 2008). Buffer N contains 50 mM Tris-HCl (pH 7.5), 15 mM EDTA, 15 mM EGTA, 2 mM dithiothreitol, 1% NP40, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, and 150 mM NaCl.

Plasmid vectors

The single-copy pRS41x series vectors and the multicopy pRS42x series vectors have been described (Sikorski and Boeke, 1991; Christianson *et al*, 1992). p414GAL1 (P_{GAL1} $TRP1^+$ ARS) and p416GAL1 (P_{GAL1} $URA3^+$ ARS) are galactose-inducible single-copy vectors (Mumberg *et al*, 1994). p424TEF (P_{TEF2} $TRP1^+$ 2 μ m) is a multicopy vector with the strong and constitutive *TEF2* promoter (Mumberg *et al*, 1995). p426TEG1 (P_{TEF2} -GST $URA3^+$ 2 μ m) is a multicopy vector that allows a constitutive expression of GST-fusion protein using the *TEF2* promoter (Posas *et al*, 1996).

Expression plasmid constructs

The C-terminally GFP-tagged Opy2 constructs used in Figure 3B are based on the galactose-inducible single-copy vector p414GAL1. The Opy2 constructs used in Figure 3C are based on the single-copy vector pRS414 ($TRP1^+$ ARS), and contains the *OPY2* promoter. Opy2- Δ SR1 is $\Delta(2-22)$ /Ser27A, which eliminates all the Ser residues between the N terminus and Ser27. Opy2- Δ SR2 is Ser67A/Ser69A/Ser72A/Ser73A/ $\Delta(75-84)$ /Ser86A/Ser91A, which eliminates all the Ser residues between Ser 67 and Ser 91 inclusive. Opy2- Δ C is $\Delta(184-360)$. pFP122 contains the full-length *STE50* ORF fused to the C terminus of GST in the p426TEG1 vector (Posas *et al*, 1998). The HA-tagged Msb2 constructs used in Figure 4B-E (Msb2-

FL HA and its Δ STR1, Δ STR2 and Δ STR3 derivatives) are based on the galactose-inducible p416GAL1 vector, and contain two HA epitopes, one following amino acid position 48 and another at the C terminus. The Msb2 expression constructs used in Figure 5B are without epitope-tag, based on the single-copy vector pRS416 ($URA3^+$ ARS), and expressed from the *MSB2*'s own promoter. In the Msb2-HMH(Hkr1) mutant, the HMH domain of Msb2 (residues 961-1117) was replaced by the homologous HMH domain of Hkr1 (residues 1210-1427) (Tatebayashi *et al*, 2007). The Msb2 and Opy2 expression constructs used in Figure 5C are based on p414GAL1.

Reporter assays

Reporter assays using the FG reporter plasmid pFUS1-lacZ (pSB231) (*CEN* $URA3^+$ *FUS1-lacZ*) and the HOG reporter plasmid pRS414-8xCRE-lacZ (*CEN* $TRP1^+$ 8xCRE-lacZ), have been described (McCaffrey *et al*, 1987; Tatebayashi *et al*, 2003, 2006). All reporter assays were at least triplicated using independent cultures. Error bars represent standard deviations.

Microscopy

Microscopic images of yeast cells were captured using a Nikon TE2000-E inverted microscope equipped with a Roper Scientific CoolSnap HQ CCD camera.

Immunoblotting analyses

Immunoblotting analyses were carried out essentially as described earlier (Takekawa and Saito, 1998; Murakami *et al*, 2008). Buffer N was used to prepare cell extracts containing HA-tagged Msb2 constructs. Images were digitally captured by LAS-1000 Plus (Fujifilm) equipped with a CCD camera. The following antibodies were used as primary antibodies in immunoblotting: the anti-Hog1 goat antibody yC-20 (Santa Cruz), anti-GST monoclonal antibody (mAb) B-14 (Santa Cruz), anti-GFP mAb B-2, anti-HA mAb 12CA5 (Roche), anti-phospho-p38 antibody (Cell Signalling) for phospho-Hog1, and anti-phospho-p44/42 antibody (Cell Signalling) for phospho-Kss1, phospho-Fus3 and phospho-Mpk1.

In vivo binding assay

Exponentially growing cells in CARaf were adjusted to 2% galactose and cultured for an additional 4 h. Cell extracts were prepared in buffer A using glass beads, essentially as described earlier (Tatebayashi *et al*, 2003). A 200 μ g aliquot of protein extract was incubated with 50 μ l of glutathione-Sepharose beads for 3 h at 4°C. Beads were washed three times in buffer A, resuspended in SDS loading buffer, boiled and separated by SDS-PAGE. Proteins were detected by immunoblotting.

Other methods

Other methods including standard genetic procedures are as described earlier (Rose *et al*, 1990; Tatebayashi *et al*, 2003; Horie *et al*, 2008; Murakami *et al*, 2008).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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