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Formation of Subnuclear Foci Is a Unique Spatial Behavior of Mating MAPKs during Hyperosmotic Stress

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SUMMARY

The assembly of signaling components and transcription factors in ordered subcellular structures is increasingly implicated as an important regulatory strategy for modulating the activity of cellular pathways. Here, we document the inducible formation of subnuclear foci formed by two mitogen-activated protein kinases (MAPKs) in *Saccharomyces cerevisiae* upon hyperosmotic stress. Specifically, we demonstrate that activation of the hyperosmotic stress response pathway induces the mating pathway MAPK Fus3 and the filamentation pathway MAPK Kss1 to form foci in the nucleus that are organized by their shared downstream transcription factor Ste12. Foci formation of colocalized Ste12, Fus3, and Kss1 requires the kinase activity of the hyperosmotic response MAPK Hog1 and correlates with attenuated signaling in the mating pathway. Conversely, activation of the mating pathway prevents foci formation upon subsequent hyperosmotic stress. These results suggest that Hog1-mediated spatial localization of Fus3 and Ste12 into subnuclear foci could contribute to uncoupling the pheromone and osmolarity pathways, which share signaling components, under high-osmolarity conditions.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs), a family of Ser/Thr protein kinases conserved among eukaryotes, are involved in many cellular programs such as proliferation, differentiation, and death (Blenis, 1993; Kim and Choi, 2010; Wagner and Nebreda, 2009). MAPK signaling cascades are organized hierarchically into three-tiered modules. MAPKs are phosphorylated and activated by MAPK kinases (MAP2Ks), which are phosphorylated and activated by MAP2K kinases (MAP3Ks) (Chen and Thorner, 2007). MAP3Ks themselves are activated by interaction with members of small GTPase families and other protein kinases, connecting the MAPK module to cell-surface receptors or other signaling molecules at the plasma membrane. Three main families of MAPKs exist in mammalian species, grouped by their structures and functions: the extracellular signal-regulated protein kinases (ERKs), the p38 MAPKs, and the c-Jun NH2-terminal kinases (JNKs) (Graves et al., 1995; Seger and Krebs, 1995; Wagner and Nebreda, 2009). Transmission of signals to the nucleus and subsequent modulation of the activity of a number of transcription factors and chromatin remodeling proteins is a particularly important role of MAPKs. This transmission is often mediated by the physical translocation of MAPK proteins to the nucleus (Chen et al., 1992; Cohen-Saidon et al., 2009; Khokhlatchev et al., 1998; Plotnikov et al., 2011).

The budding yeast *Saccharomyces cerevisiae* possesses five genes with homology to mammalian MAPKs (*HOG1*, *SLT2*, *FUS3*, *KSS1*, and *SMK1*). These kinases, whose active forms have both cytoplasmic and nuclear targets, are important for inducing the activity of condition-specific transcriptional programs via phosphorylation of transcription factors, and in some cases by serving as transcriptional coactivators (Chen and Thorner, 2007). As in mammalian cells, activation-dependent MAPK nuclear translocation is conserved in *S. cerevisiae* and has been directly observed for Hog1, the MAPK activated in response to hyperosmotic stress (Ferrigno et al., 1998; Mettetal et al., 2008; Muzzey et al., 2009). Nuclear translocation of Fus3, the pheromone pathway MAPK, and Kss1, the filamentation pathway MAPK, has also been observed (Blackwell et al., 2003, 2007; Chen et al., 2010; Maeder et al., 2007; Slaughter et al., 2007).

Hog1 is instrumental for the specific and faithful activation of the hyperosmotic-stress-responsive genes (O'Rourke and Herskowitz, 1998; Saito and Tatebayashi, 2004). Despite the fact that the hyperosmotic stress pathway shares the same MAP3K (Ste11) with the pheromone response and filamentation pathways, hyperosmotic stress fails to activate pheromone- or filamentation-responsive genes (O'Rourke and Herskowitz, 1998; Schwartz and Madhani, 2004; Shock et al., 2009). The remarkable ability of hyperosmotic stress to exclusively activate the hyperosmotic response requires Hog1 kinase activity and has recently been attributed to Hog1-mediated feedback control, potentially through the adaptor protein Ste50 and the

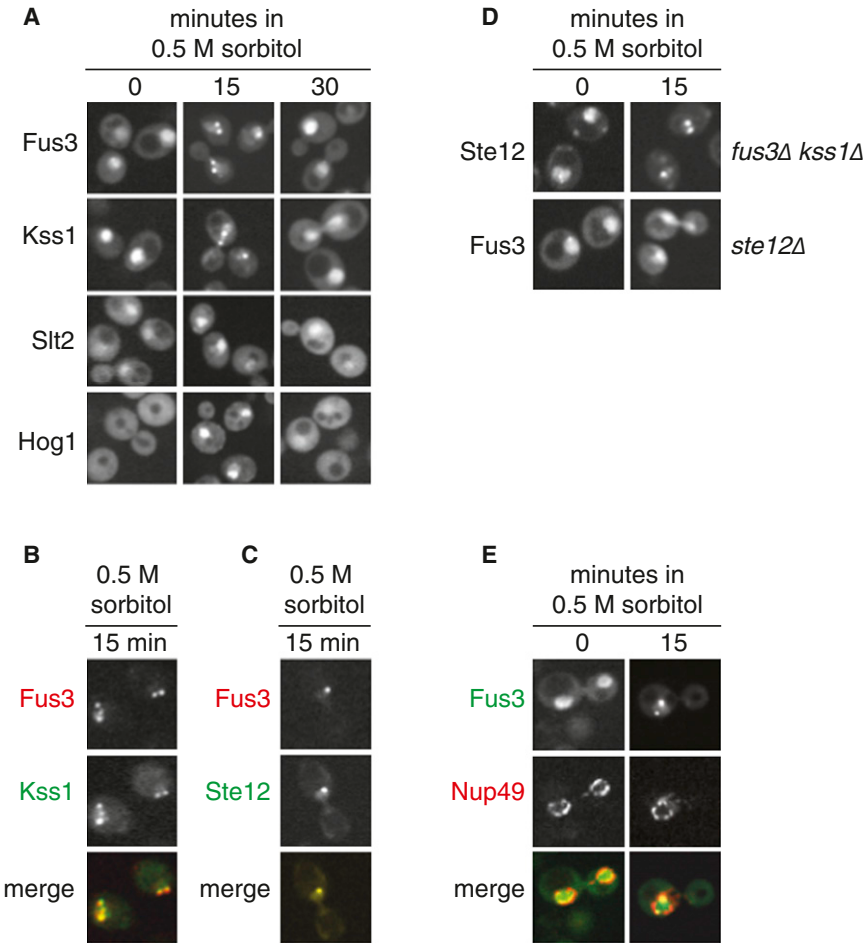

Figure 1. Ste12, Fus3, and Kss1 Colocalize in Foci under High-Osmolarity Stress

(A) The MAPKs Fus3 and Kss1, but not Slt2 and Hog1, form punctuate and transient foci under high-osmolarity stress. Endogenous Fus3, Kss1, Hog1, and Slt2 were fused on the C termini to YFP and spatial localization was followed by confocal microscopy after addition of 0.5 M sorbitol.

(B) Fus3 and Kss1 colocalize under high-osmolarity stress. Endogenous Fus3 and Kss1 were fused on the C termini to YFP and mKate, respectively. Foci formation was determined by confocal microscopy 15 min after the addition of 0.5 M sorbitol.

(C) Fus3 and Ste12 colocalize under high-osmolarity stress. Endogenous Fus3 and Ste12 were fused on the C termini to mKate and YFP, respectively. Foci formation was determined by confocal microscopy 15 min after adding 0.5 M sorbitol.

(D) Fus3 and Kss1 fail to form foci following osmotic stress in cells lacking Ste12. However, Ste12 continued to form foci following osmotic stress even in a *fus3Δkss1Δ* strain. Endogenous Ste12 was fused on the C termini to YFP. Fus3, Kss1, and Ste12 were deleted by homologous insertion. Foci formation was determined by confocal microscopy 15 min after addition of 0.5 M sorbitol.

(E) Fus3 colocalizes with the nuclear pore under high-osmolarity stress. Endogenous Fus3 and nup49 (nuclear pore marker) were tagged with YFP and mKate, respectively. Foci formation was followed by confocal microscopy in cells treated with 0.5 M sorbitol. Images were taken 10–15 min after adding sorbitol.

See also Figure S1.

kinase Rck2 (Nagiec and Dohlman, 2012). This model, however, does not fully explain the observation that hyperosmotic stress activates Fus3 and Kss1, yet the target genes of the pheromone and filamentation pathways remain off (Shock et al., 2009). As a result, some further insulation at the level of the transcription factors downstream of Fus3 and Kss1 might still occur.

In addition to simply binding to regulatory sites to control gene expression, transcription factors have been shown to organize subnuclear structures with varied functions (Brickner et al., 2012; McCullagh et al., 2010; Sutherland and Bickmore, 2009). In metazoan cells, many subnuclear foci containing transcription factors have been described, such as nuclear stress bodies, histone locus bodies, and polycomb bodies (Mao et al., 2011; Nizami et al., 2010). Rather than being nonfunctional aggregates, these structures within the nucleus implement important layers of regulation.

Here, we show that activation of Hog1 by hyperosmotic stress induces the formation of subnuclear foci containing the transcription factor Ste12 and the MAPKs of the mating and filamentation pathway. Our results are consistent with a model in which these subnuclear structures prevent pheromone and filamentation pathway target gene activation in response to hyperosmotic stress. This work uncovers a previously undocumented spatial behavior of the MAPK proteins and suggests that transcription-factor-mediated sequestration of signaling components could serve as a general mechanism to modulate the activity of signaling pathways.

RESULTS

Fus3 and Kss1 Are Unique among MAPK Proteins in Their Ability to Form Foci under High-Osmolarity Stress

To gain insight into the regulation of localization of *S. cerevisiae* MAP kinases, we fused yellow fluorescent protein (YFP) to the C termini of Fus3, Kss1, Hog1, and Slt2 and imaged these strains under a panel of metabolic and stress conditions. In vegetatively growing cells, Fus3 and Kss1 were enriched in the nucleus. Slt2 had a reduced nuclear signal compared to Fus3 and Kss1, while Hog1 was mostly cytoplasmic (Figure 1A). In cells treated with 1 μM α factor, none of the four kinases changed their localization until Fus3 partially redistributed from the nucleus to the emerging shmoo tip after 1 hr of treatment (Figure S1A), consistent with previous data (Slaughter et al., 2007).

By contrast, addition of the osmotic stressor sorbitol (0.5 M) triggered the redistribution of Hog1, Fus3, and Kss1. Hog1 transiently localized to the nucleus, within 15 min of treatment and

returned to the cytoplasm within 30 min (Figure 1A). Strikingly, following sorbitol treatment, Fus3 and Kss1 appeared in small puncta in approximately half the cells in a given confocal microscope section (Figure 1A). Each cell in which the puncta were visible contained one to three discrete foci 15 min after sorbitol addition, and these foci dispersed within 30 min; Fus3 and Kss1 returned to their prestress diffuse localization in all cells 30 min after sorbitol addition (Figure 1A).

Fus3 also formed subnuclear foci under other hyperosmotic stress conditions such as 0.5 M NaCl and 0.8 M glucose (Figure S1B). Importantly, these subnuclear foci did not form under other stress conditions such as hypoosmotic shock (50% H_2O), heat shock (40°C), oxidative stress (0.5% H_20_2), cell wall stress (100 μg/ml zymolyase), ER stress (5 mM DTT), or Tor inhibition (20 mM caffeine) (Figure S1C). These data strongly suggest that the Fus3 and Kss1 foci are a specific phenotype associated with high-osmolarity stress.

Fus3/Kss1 Foci Formation under Hyperosmotic Stress Is Dependent on the Pheromone Response Transcription Factor Ste12

We next investigated whether Fus3 and Kss1-containing foci were the same spatial structure. To do so, we measured the localization of Fus3-YFP and Kss1-mKate2 coexpressed in the same cell upon stimulation with sorbitol. Under these conditions, Fus3-YFP and Kss1-mKate2 colocalized in nuclear foci (Figure 1B), indicating that Fus3 and Kss1 are both contained within the same subnuclear structures.

The presence of nuclear foci involving the pheromone pathway transcription factor Ste12 has previously been documented. In strains deleted for *DIG1*, a gene encoding a negative regulator of Ste12, Ste12 molecules localize into discrete subnuclear foci (McCullagh et al., 2010). Because of the propensity of Ste12 to form these structures in *dig1Δ* cells, we hypothesized that Ste12 molecules might also be components of the Fus3/Kss1 foci. To test this possibility, we monitored the localization of Fus3-mKate2 and Ste12-YFP coexpressed in the same cells. Upon treatment with 0.5 M sorbitol, Ste12 indeed formed nuclear foci. Importantly, Fus3 colocalized with Ste12 in these foci (Figure 1C). Moreover, just as Ste12 is constitutively localized in foci in *dig1Δ* cells, Fus3 also appeared in constitutive foci in the absence of sorbitol in *dig1Δ* cells (Figure S1D). Furthermore, both Dig1 and its paralog Dig2 were present in sorbitol-induced foci in wild-type cells (Figure S1E). In summary, these data indicate that the MAPKs Fus3 and Kss1, along with their shared transcription factor Ste12 and its negative regulators Dig1 and Dig2, are components of hyperosmotic-stress-induced subnuclear structures.

To determine which components are required for the emergence of these structures, we monitored foci formation upon sorbitol addition in strains lacking *FUS3*, *KSS1*, *or STE12*. Interestingly, neither individual deletions of *FUS3* or *KSS1*, nor the *FUS3/KSS1* double deletion impacted the formation of Ste12 foci (Figure 1D). By contrast, Fus3 and Kss1 failed to form foci in *ste12Δ* cells under osmotic stress conditions (Figure 1D).

Since Ste12, a transcription factor, is a crucial component for the formation of these foci, we sought to ask whether DNA also associated with these foci. To test this possibility, we performed ChIP-PCR experiments using Ste12-YFP as bait in untreated, pheromone-treated, or sorbitol-treated cells (Figure S1F). In untreated cells, we found that Ste12 associated with pheromone target gene promoters (*AGA1* and *FUS1*), but not with osmo-stress target gene promoters (*HOR2* and *GPD1*) or metabolic gene promoters (*PGK1* and *TDH3*). We did not observe Ste12 binding to osmo-stress gene promoters or metabolic gene promoters in any condition. Notably, we found an increase in Ste12 binding to pheromone target promoters upon pheromone treatment, but we did not observe any depletion of Ste12 from the pheromone target gene promoters upon sorbitol treatment (Figure S1G). These data indicate that Ste12 remains specifically bound to its target gene promoters while simultaneously localizing to subnuclear foci.

Genomic loci have been reported to localize to the nuclear membrane both for activation and repression (Brickner and Walter, 2004; Green et al., 2012). Since the hyperosmotic-stress-induced foci are associated with DNA, we next asked if the foci localize to the nuclear periphery. To this end, we monitored Fus3-YFP in cells in which the nuclear pore component Nup49 was fused to mCherry. After addition of sorbitol, we observed that Fus3 foci indeed localized with or near Nup49 (Figure 1E). Taken together, these data demonstrate that the common transcription factor Ste12 is necessary to organize the MAPKs Fus3 and Kss1 into the subnuclear foci induced by high-osmolarity stress, and that these foci are associated with DNA and are localized to the nuclear periphery.

Hog1 Kinase Activity Is Required for Formation of Fus3/Kss1/Ste12 Foci in Conditions of Hyperosmotic Stress

Under high-osmolarity conditions, the effector MAPK Hog1 is phosphorylated by the MAP2K Pbs2 and translocates from the cytoplasm to the nucleus where it plays transcriptional and posttranslational roles in the hyperosmotic stress response (Ferrigno et al., 1998; Mettetal et al., 2008). Therefore, Hog1 is a prime candidate for a regulatory role in Fus3/Kss1/Ste12 foci formation. To address this notion, we first investigated whether Hog1 residence in the nucleus correlated with Fus3/Kss1/Ste12 foci formation. Fus3 and Ste12 foci form and dissipate with identical kinetics (Figures S2A and S2B), so we chose to simultaneously monitor Fus3-YFP and Hog1-mKate2 in the same cell. The kinetics of Fus3 foci formation and dissolution correlated with Hog1 nuclear residence (Figures 2A and 2B). Hog1 began to translocate to the nucleus within 2 min of 0.5 M sorbitol, and Fus3 began to form foci within 3 min. Both Hog1 nuclear enrichment and Fus3 foci formation achieved their peaks between 5 and 10 min after sorbitol addition. Within 15 min, Fus3 foci began to dissipate while Hog1 persisted longer in the nucleus, beginning to relocalize to the cytoplasm only after 20 min (Figures 2A and 2B). Due to our imaging technique, we were not able to observe Fus3 foci in all cells, likely due to the foci falling above or below the confocal plane. Nonetheless, the strong correlation between Fus3 foci and Hog1 nuclear residency held across a range of osmotic stress magnitudes that produced different but correlated Hog1 and Fus3 dynamics (Figure S2C). Moreover, Fus3, Kss1, and Hog1 are phosphorylated and dephosphorylated with similar kinetics, matching the kinetics of foci formation and Hog1 nuclear translocation (Figure S2D).

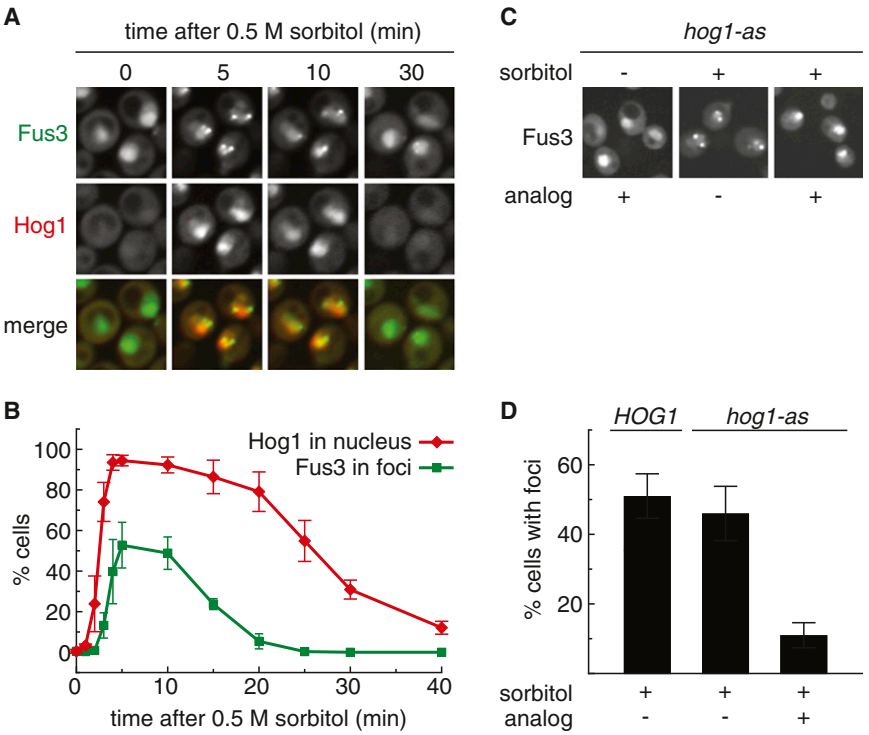

Figure 2. Fus3/Kss1/Ste12 Foci Formation Requires Hog1 Kinase Activity

(A) Hog1 residence in the nucleus correlates with Fus3/Kss1 foci formation. Endogenous Fus3 and Hog1 were fused on the C termini to YFP and mKate, respectively. Distribution of Fus3 and Hog1 was followed by confocal microscopy at different time points after the addition of 0.5 M sorbitol.

(B) Quantification of Hog1 nuclear residence and Fus3 foci formation. The percentage of cells with foci and nuclear Hog1 was calculated using ImageJ.

(C) The kinase activity of Hog1 is a prerequisite for Fus3 and Kss1 foci formation under high-osmolarity stress. Following hyperosmotic stress, Fus3-YFP failed to form foci in cells harboring a *hog1-as* allele pretreated with the cell-permeable adenine analog 1-NM-PP1 (1 μM).

(D) The percentage of cells with foci following hyperosmotic stress was calculated using ImageJ for cells with wild-type *HOG1*, *hog1-as* allele untreated with 1-NM-PP1 and cells with a *hog1-as* allele after 1-NM-PP1 treatment. Compromised Hog1 kinase activity generates dramatic reduction in foci formation. Error bars represent the mean ± SD of three independent experiments.

See also Figure S2.

To directly test if Hog1 activity is required for foci formation, we constructed an analog-sensitive allele (*hog1-as*) whose kinase activity is susceptible to inhibition by the cell-permeable ATP analog 1-NM-PP1 (Westfall and Thorner, 2006). In this mutant, and by adding 1-NM-PP1, pheromone genes are expressed under high-osmolarity stress (Westfall and Thorner, 2006). The *hog1-as* allele was inserted into the genome of a *hog1Δ* strain under the control of the native *HOG1* promoter. In *hog1-as* cells, 1-NM-PP1 alone had no effect on Fus3 localization. Moreover, in *hog1-as* cells, Fus3 formed nuclear foci at a frequency comparable to that of wild-type cells when treated with 0.5 M sorbitol alone (Figures 2C and 2D). By contrast, less than 10% of cells pretreated with 1 μM 1-NM-PP1 formed foci upon addition of 0.5 M sorbitol (Figures 2C and 2D). These data demonstrate that the kinase activity of Hog1 is required for Fus3 foci formation under high-osmolarity stress.

Preactivation of the Pheromone Pathway Inhibits the Formation of Fus3 Foci

The experiments described above show that Ste12 and Fus3, components of the pheromone response pathway, are recruited into foci in high-osmolarity conditions and that Hog1 kinase activity is required for this process to proceed. Despite the fact that the hyperosmotic stress pathway shares the same MAP3K (Ste11) with the pheromone pathway, hyperosmotic stress fails to activate pheromone-responsive genes (O'Rourke and Herskowitz, 1998; Schwartz and Madhani, 2004; Shock et al., 2009). Therefore, foci formation could constitute a spatial mechanism for attenuating signaling in the pheromone pathway when cells are exposed to high osmolarity. In this case, the presence of foci should diminish the induction of pheromone target genes. Conversely, conditions where these foci are absent should coincide with unattenuated pheromone signaling output.

To test the notion that the Fus3/Kss1/Ste12 foci represent a spatial mechanism for attenuating pheromone pathway output during hyperosmotic stress, we performed a series of experiments in which we treated cells with both α factor and sorbitol. We hypothesized that if cells treated simultaneously with sorbitol and pheromone prioritize the hyperosmotic stress response, then these cells would form foci that attenuate signaling in the pheromone pathway. On the other hand, we reasoned that pretreatment of cells with α factor would establish active signaling through the pheromone MAPK cascade that would preclude the formation of foci. The absence of foci in this situation should then correlate with unattenuated signaling and induction of pheromone-responsive gene expression.

To test these hypotheses, we first used confocal microscopy to monitor Fus3-YFP cells treated with both 1 μM α factor and 0.5 M sorbitol either simultaneously or by pretreating the cells with α factor for 10 min before sorbitol addition. When cells were exposed to α factor and sorbitol simultaneously, Fus3 formed foci to the same level as in cells treated with sorbitol alone (Figure 3A). However, when cells were preincubated in 1 μM α factor for 10 min, addition of 0.5 M sorbitol failed to induce Fus3 foci (Figure 3A). Deletion of *STE7*, the MAP2K upstream of Fus3, rescued Fus3 foci formation in cells pretreated with pheromone and subsequently treated with sorbitol, showing that signaling through the pheromone MAPK cascade is required to prevent foci formation (Figure 3A).

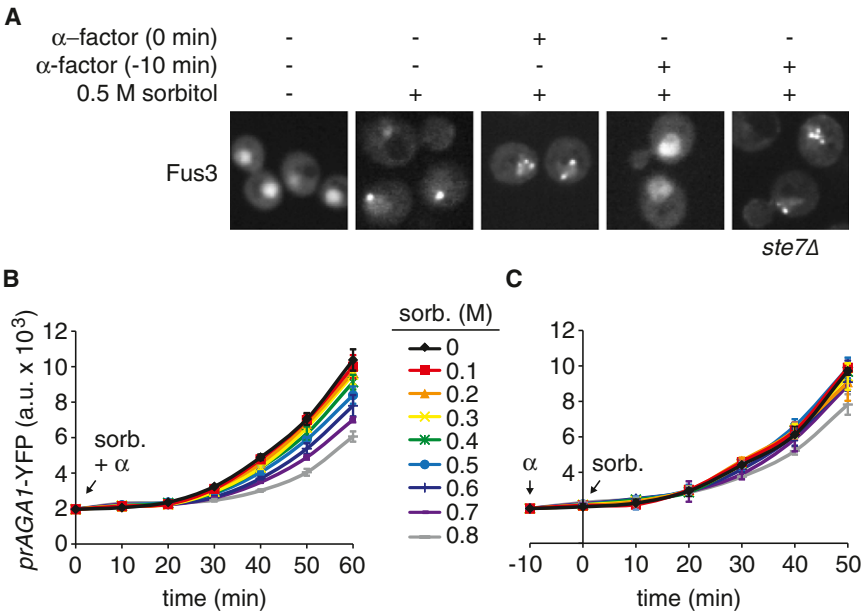

Figure 3. Preactivation of the Pheromone Pathway Inhibits the Formation of Fus3 Foci under High-Osmolarity Stress

(A) Cells harboring Fus3-YFP were treated with either 0.5 M sorbitol, 0.5 M sorbitol, and 1 μM pheromone at the same time or 1 μM pheromone for 10 min followed by 0.5 M sorbitol. While the simultaneous stimulation by pheromone and sorbitol had little qualitative effect on foci formation, foci were absent when pheromone preceded sorbitol addition. Foci formation resumed under this scenario in a *ste7Δ* strain.

(B and C) Preactivation of cells harboring a pheromone transcriptional reporter (P_{AGA1}-YFP) exhibits full induction under different concentrations of sorbitol. The time-resolved expression of a pheromone transcriptional reporter (P_{AGA1}-YFP) was recorded for cells treated at the same time (B) with 1 μM pheromone and different concentrations of sorbitol (0.1–0.8 M), or (C) with 1 μM pheromone for 10 min followed by different concentrations of sorbitol (0.1–0.8 M). YFP expression was measured by flow cytometry. While increasing amounts of sorbitol attenuate signaling in the mating pathway if cells experience sorbitol and salt simultaneously, this effect disappears if cells are pretreated with pheromone.

Error bars represent the mean ± SD of three independent experiments. See also Figure S3.

We next monitored P_{AGA1}-YFP, a transcriptional reporter of mating pathway activity, by flow cytometry to measure the output of the pheromone pathway in cells treated with both 1 μM α factor and nine different doses of sorbitol, either simultaneously or with pheromone pretreatment. When cells were simultaneously treated with 1 μM pheromone and sorbitol—conditions in which Fus3 foci form—mating pathway output was diminished in proportion to the dose of sorbitol, consistent with previous data (Nagiec and Dohlman, 2012) (Figure 3B). However, in cells pretreated with 1 μM α factor for 10 min before sorbitol addition—conditions that abolished Fus3 foci formation—P_{AGA1}-YFP exhibited full induction under all sorbitol concentrations (Figure 3C). A control promoter for a metabolic gene that is not pheromone regulated (P_{TDH3}-mCherry) remained constant over time in all conditions, demonstrating that the effects of sorbitol are specific to the pheromone pathway reporter (Figure S3). These data indicate that preactivation of Ste12 by pheromone signaling inhibits the formation of Fus3/Kss1/Ste12 foci upon subsequent hyperosmotic stress and abrogates the hyperosmotic stress inhibition of the pheromone pathway output. These results are consistent with the hypothesis that spatial localization of Fus3, Kss1, and Ste12 into foci could constitute a strategy to isolate the pheromone pathway under high-osmolarity conditions.

DISCUSSION

The activities of many signaling proteins are regulated by their physical proximity and access to substrates. A common form of this regulation in eukaryotes is controlling access of transcription factors to DNA by regulating import into the nucleus (Cyert, 2001). An extension to this emerging regulatory model is the control of subnuclear localization of transcription factors and genes to the nuclear periphery, nucleoli, or foci (Brickner et al., 2012; Mao et al., 2011; Nizami et al., 2010). Such foci of colocalized transcription factors and coordinately regulated DNA have been observed in metazoans and fungi and have been suggested to represent transcriptional factories where common transcriptional machinery can be marshaled and therefore modulate gene expression of the localized genes (Sutherland and Bickmore, 2009).

Recent work on the Ste12 transcription factor in yeast suggests that cells prevent the colocalization of genes regulated by this transcription factor through the use of a dedicated repressor, Dig1. The absence of Dig1 results in clustering of Ste12 and its target gene promoters, and a concomitant increase in gene expression noise (McCullagh et al., 2010). Since Ste12 clustering is not observed in wild-type cells upon activation by pheromone stimulation, this clustering is likely to play a role other than activation of gene expression.

Our present work reveals that Ste12 also forms subnuclear foci physiologically in wild-type cells in response to hyperosmotic stress. In these foci, Ste12 colocalizes with the upstream MAPKs Kss1 and Fus3 and the negative regulators Dig1 and Dig2. Whether and to what extent the formation of Ste12 foci in the two conditions, *dig1Δ* cells and cells experiencing hyperosmotic stress, reflects the same underlying mechanism remains to be determined. The most parsimonious model is that Ste12 clustering is a controlled process during hyperosmotic stress, but gets unspecifically triggered in the absence of Dig1. The fact that foci formation of colocalized Ste12, Fus3, and Kss1 was only observed under hyperosmotic stress and required the kinase activity of the osmotic stress MAPK Hog1 supports the hypothesis that these foci represent a specific

response to osmotic shock rather than a general response to cellular damage.

Hyperosmotic stress poses a unique challenge to the pheromone response and filamentation pathways that share a common MAP3K, Ste11. Both the MAP2K Ste7 and the MAPKs Fus3 and Kss1 become phosphorylated during high-osmolarity stress (Shock et al., 2009). Yet, downstream target genes of the pheromone and filamentation pathways remain inactive during osmotic stress. Hog1 kinase activity-mediated clustering of Ste12, Fus3, and Kss1 into subnuclear foci may represent a spatial mechanism that prevents the inappropriate activation of pheromone and filamentation genes despite the activation of their MAPKs by osmotic stress. Repression of signaling by foci formation has recently been shown to occur in the TOR pathway in yeast upon heat shock, suggesting that attenuation by spatial sequestration might be a general regulatory mechanism (Takahara and Maeda, 2012).

Despite the tight correlation of Ste12/Fus3/Kss1 foci formation with Hog1 activation and the inhibition of pheromone target gene expression, much work is still needed to elucidate the mechanisms of Ste12 foci formation and demonstrate a causal relationship between these foci and crosstalk inhibition between the hyperosmolarity and pheromone response pathways. Nonetheless, a tantalizing model suggested by our data is one in which Fus3 and Kss1 are activated by crosstalk upon osmotic stress, while the activation of downstream target genes is suppressed by sequestration of these kinases into foci. This method of inhibition would be complementary to Hog1-directed repression of upstream pheromone pathway elements such as Ste50. Both mechanisms would collaborate to prevent activation of the pheromone inducible target genes by hyperosmotic stress, which is crucial given the toxic effects of costimulation of pheromone and osmotic pathways (Patterson et al., 2010). This physical sequestration of kinases into subnuclear structures organized by their downstream transcription factors provides the overlapping pheromone, filamentation, and osmotic stress signaling pathways with a rapid and reversible way to prevent inappropriate signaling.

EXPERIMENTAL PROCEDURES

A detailed description of the experiments is included in the Extended Experimental Procedures. Briefly, standard procedure for growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used throughout the study (Tables S1 and S2). Subcellular localization of fluorescent fusion proteins was observed by spinning disc confocal microscopy. Reporter fluorescence was measured by flow cytometry. Western blots were visualized via infrared fluorescence using the Li-Cor imaging system. ChIP samples were assayed for enrichment by PCR (Table S3).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, three figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.01.022.

LICENSING INFORMATION

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REFERENCES

Blackwell, E., Halatek, I.M., Kim, H.J., Ellicott, A.T., Obukhov, A.A., and Stone, D.E. (2003). Effect of the pheromone-responsive G(alpha) and phosphatase proteins of Saccharomyces cerevisiae on the subcellular localization of the Fus3 mitogen-activated protein kinase. Mol. Cell. Biol. *23*, 1135–1150.

Blackwell, E., Kim, H.J., and Stone, D.E. (2007). The pheromone-induced nuclear accumulation of the Fus3 MAPK in yeast depends on its phosphorylation state and on Dig1 and Dig2. BMC Cell Biol. *8*, 44.

Blenis, J. (1993). Signal transduction via the MAP kinases: proceed at your own RSK. Proc. Natl. Acad. Sci. USA *90*, 5889–5892.

Brickner, D.G., Ahmed, S., Meldi, L., Thompson, A., Light, W., Young, M., Hickman, T.L., Chu, F., Fabre, E., and Brickner, J.H. (2012). Transcription factor binding to a DNA zip code controls interchromosomal clustering at the nuclear periphery. Dev. Cell *22*, 1234–1246.

Brickner, J.H., and Walter, P. (2004). Gene recruitment of the activated INO1 locus to the nuclear membrane. PLoS Biol. *2*, e342.

Chen, R.E., and Thorner, J. (2007). Function and regulation in MAPK signaling pathways: lessons learned from the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta *1773*, 1311–1340.

Chen, R.E., Patterson, J.C., Goupil, L.S., and Thorner, J. (2010). Dynamic localization of Fus3 mitogen-activated protein kinase is necessary to evoke appropriate responses and avoid cytotoxic effects. Mol. Cell. Biol. *30*, 4293–4307.

Chen, R.H., Sarnecki, C., and Blenis, J. (1992). Nuclear localization and regulation of erk- and rsk-encoded protein kinases. Mol. Cell. Biol. *12*, 915–927.

Cohen-Saidon, C., Cohen, A.A., Sigal, A., Liron, Y., and Alon, U. (2009). Dynamics and variability of ERK2 response to EGF in individual living cells. Mol. Cell *36*, 885–893.

Cyert, M.S. (2001). Regulation of nuclear localization during signaling. J. Biol. Chem. *276*, 20805–20808.

Ferrigno, P., Posas, F., Koepp, D., Saito, H., and Silver, P.A. (1998). Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. EMBO J. *17*, 5606–5614.

Graves, J.D., Campbell, J.S., and Krebs, E.G. (1995). Protein serine/threonine kinases of the MAPK cascade. Ann. N Y Acad. Sci. *766*, 320–343.

Green, E.M., Jiang, Y., Joyner, R., and Weis, K. (2012). A negative feedback loop at the nuclear periphery regulates GAL gene expression. Mol. Biol. Cell *23*, 1367–1375.

Khokhlatchev, A.V., Canagarajah, B., Wilsbacher, J., Robinson, M., Atkinson, M., Goldsmith, E., and Cobb, M.H. (1998). Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. Cell *93*, 605–615.

Kim, E.K., and Choi, E.J. (2010). Pathological roles of MAPK signaling pathways in human diseases. Biochim. Biophys. Acta *1802*, 396–405.

Maeder, C.I., Hink, M.A., Kinkhabwala, A., Mayr, R., Bastiaens, P.I., and Knop, M. (2007). Spatial regulation of Fus3 MAP kinase activity through a reaction-diffusion mechanism in yeast pheromone signalling. Nat. Cell Biol. *9*, 1319–1326.

Mao, Y.S., Zhang, B., and Spector, D.L. (2011). Biogenesis and function of nuclear bodies. Trends Genet. *27*, 295–306.

McCullagh, E., Seshan, A., El-Samad, H., and Madhani, H.D. (2010). Coordinate control of gene expression noise and interchromosomal interactions in a MAP kinase pathway. Nat. Cell Biol. *12*, 954–962.

Mettetal, J.T., Muzzey, D., Gómez-Uribe, C., and van Oudenaarden, A. (2008). The frequency dependence of osmo-adaptation in Saccharomyces cerevisiae. Science *319*, 482–484.

Muzzey, D., Gómez-Uribe, C.A., Mettetal, J.T., and van Oudenaarden, A. (2009). A systems-level analysis of perfect adaptation in yeast osmoregulation. Cell *138*, 160–171.

Nagiec, M.J., and Dohlman, H.G. (2012). Checkpoints in a yeast differentiation pathway coordinate signaling during hyperosmotic stress. PLoS Genet. *8*, e1002437.

Nizami, Z., Deryusheva, S., and Gall, J.G. (2010). The Cajal body and histone locus body. Cold Spring Harb. Perspect. Biol. *2*, a000653.

O'Rourke, S.M., and Herskowitz, I. (1998). The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in Saccharomyces cerevisiae. Genes Dev. *12*, 2874–2886.

Patterson, J.C., Klimenko, E.S., and Thorner, J. (2010). Single-cell analysis reveals that insulation maintains signaling specificity between two yeast MAPK pathways with common components. Sci. Signal. *3*, ra75.

Plotnikov, A., Zehorai, E., Procaccia, S., and Seger, R. (2011). The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. Biochim. Biophys. Acta *1813*, 1619–1633.

Saito, H., and Tatebayashi, K. (2004). Regulation of the osmoregulatory HOG MAPK cascade in yeast. J. Biochem. *136*, 267–272.

Schwartz, M.A., and Madhani, H.D. (2004). Principles of MAP kinase signaling specificity in Saccharomyces cerevisiae. Annu. Rev. Genet. *38*, 725–748.

Seger, R., and Krebs, E.G. (1995). The MAPK signaling cascade. FASEB J. *9*, 726–735.

Shock, T.R., Thompson, J., Yates, J.R., 3rd, and Madhani, H.D. (2009). Hog1 mitogen-activated protein kinase (MAPK) interrupts signal transduction between the Kss1 MAPK and the Tec1 transcription factor to maintain pathway specificity. Eukaryot. Cell *8*, 606–616.

Slaughter, B.D., Schwartz, J.W., and Li, R. (2007). Mapping dynamic protein interactions in MAP kinase signaling using live-cell fluorescence fluctuation spectroscopy and imaging. Proc. Natl. Acad. Sci. USA *104*, 20320–20325.

Sutherland, H., and Bickmore, W.A. (2009). Transcription factories: gene expression in unions? Nat. Rev. Genet. *10*, 457–466.

Takahara, T., and Maeda, T. (2012). Transient sequestration of TORC1 into stress granules during heat stress. Mol. Cell *47*, 242–252.

Wagner, E.F., and Nebreda, A.R. (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. Nat. Rev. Cancer *9*, 537–549.

Westfall, P.J., and Thorner, J. (2006). Analysis of mitogen-activated protein kinase signaling specificity in response to hyperosmotic stress: use of an analog-sensitive HOG1 allele. Eukaryot. Cell *5*, 1215–1228.

Yaffe, M.P., and Schatz, G. (1984). Two nuclear mutations that block mitochondrial protein import in yeast. Proc. Natl. Acad. Sci. USA *81*, 4819–4823.