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Phosphorylation of Hsl1 by Hog1 leads to a G_2 arrest essential for cell survival at high osmolarity

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Control of cell cycle progression by stress-activated protein kinases (SAPKs) is essential for cell adaptation to extracellular stimuli. Exposure of yeast to osmostress leads to activation of the Hog1 SAPK, which controls cell cycle at G_1 by the targeting of Sic1. Here, we show that survival to osmostress also requires regulation of G_2 progression. Activated Hog1 interacts and directly phosphorylates a residue within the Hsl7-docking site of the Hsl1 checkpoint kinase, which results in delocalization of Hsl7 from the septin ring and leads to Swe1 accumulation. Upon Hog1 activation, cells containing a nonphosphorylatable Hsl1 by Hog1 are unable to promote Hsl7 delocalization, fail to arrest at G_2 and become sensitive to osmostress. Together, we present a novel mechanism that regulates the Hsl1–Hsl7 complex to integrate stress signals to mediate cell cycle arrest and, demonstrate that a single MAPK coordinately modulates different cell cycle checkpoints to improve cell survival upon stress.

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Introduction

Activation of stress-activated protein kinases (SAPKs) is essential for proper cell adaptation to extracellular stimuli (Kyriakis and Avruch, 2001). In budding yeast (*Saccharomyces cerevisiae*), the presence of high osmolarity in the extracellular environment results in the activation of the stress-activated Hog1 kinase. Activation of Hog1 is essential for cell survival in response to high osmolarity, because the MAPK elicits an extensive programme required for cell adaptation that includes regulation of gene expression, translation and cell cycle progression (de Nadal *et al*, 2002; Hohmann, 2002).

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The HOG pathway comprises a central core of kinases composed of the MAPK Hog1, the MAPKK Pbs2 and a set of three MAPKKKs (Ssk2/Ssk22 and Ste11) activated by two independent upstream mechanisms (de Nadal *et al*, 2002). The best-understood mechanism of activation involves a 'two-component' osmosensor that involves the Sln1 histidine kinase (Posas *et al*, 1998), which activates the Ssk2 and Ssk22 MAPKKKs. The Sln1 osmosensor utilizes a phospho-relay mechanism inactivated under high osmolarity conditions, thus Sln1 is a negative regulator of the HOG pathway. Mutations that inactivate the Sln1 osmosensor (i.e., *sln1*^{ts4}) are detrimental for growth and this can be prevented by disruption of the *HOG1* gene (Maeda *et al*, 1994; Posas *et al*, 1996). The HOG pathway can also be activated by expression of either constitutively active MAP3K mutants, or a constitutively active MAPKK mutant (Pbs2^{DD})(Maeda *et al*, 1995; Posas and Saito, 1997).

Recent reports have shown that exposure of yeast cells to high osmolarity or mutations that lead to activation of the Hog1 SAPK (i.e. *sln1*^{ts4}, *Pbs2*^{DD}) result in arrest of cell cycle progression (Alexander *et al*, 2001; Belli *et al*, 2001; Yaakov *et al*, 2003; Escote *et al*, 2004). It has been reported that activated Hog1 leads to G_1 arrest by a dual mechanism that involves downregulation of cyclin expression and direct phosphorylation of the CDK-inhibitor protein Sic1. This combination results in Sic1 stabilization and inhibition of cell cycle progression to prevent premature entry into S phase without proper cell adaptation (Escote *et al*, 2004; Zapater *et al*, 2005). Previous reports suggested that osmostress also induces a delay at G_2 (Alexander *et al*, 2001; de Nadal *et al*, 2002). However, the molecular mechanism mediating this effect is poorly understood (Alexander *et al*, 2001).

Entry into mitosis is controlled by the activity of the cyclin B–Cdk1 (Clb2–Cdc28) complex, which is held in check by the protein kinase Wee1, Swe1 in *S. cerevisiae*. Swe1 is thought to delay entry into mitosis until critical cell size has been reached (Rupes, 2002; Kellogg, 2003; Harvey *et al*, 2005) or defects in bud formation or cytoskeletal function are monitored by the 'morphogenesis checkpoint' (Cid *et al*, 2002; Lew, 2003).

Several requirements are critical for regulating Swe1 stability, its phosphorylation by the Clb2–Cdc28 (Asano *et al*, 2005; Harvey *et al*, 2005) and also the activity of the Hsl1 checkpoint kinase together with Cdc5 (Hanrahan and Snyder, 2003; Asano *et al*, 2005). When bound to the septins (Versele and Thorner, 2005), Hsl1 tethers Hsl7 at the bud neck, which is in turn required for recruitment of Swe1 to the bud neck to facilitate spatially controlled Swe1 phosphorylation, prior to ubiquitin-mediated degradation (Lew, 2003). Thus, timely phosphorylation and subsequent degradation of Swe1 are critical for proper activation of the Clb2–Cdc28 complex.

Here, we show that Hog1 prevents G_2 progression by direct phosphorylation of the Hsl1 kinase protein, which leads to Swe1 accumulation and decrease on Clb-associated Cdc28 activity. Failure to arrest at G_2 upon osmostress is deleterious

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for cell survival. Interestingly, we propose a mechanism to regulate G_2 progression that involves components of the morphogenesis checkpoint, such as Hsl1, but with novel regulatory properties to integrate stress signals. Taken together, it is shown that a single MAP kinase is able to modulate different cell cycle checkpoints, at G_1 and G_2, coordinately to prevent cell cycle progression in the presence of stressful conditions to allow cells to survive upon osmostress.

Results

Activation of the Hog1 MAPK results in modulation of cell cycle progression in both G_1 and G_2

Recently, we showed that Hog1 was involved in the control of G_1 progression. Briefly, we analysed the effect of Hog1 activation on cell cycle progression in cells allowed to proceed into S phase after release from α-factor arrest. Direct release of α-factor-arrested cells carrying a temperature-sensitive allele of *SLN1* (*sln1*^{ts4}) at nonpermissive temperature resulted in accumulation of a large number of cells in G_1 (unbudded cells with 1C DNA content) (Figure 1A) (Escote *et al*, 2004). This accumulation was not observed in wild-type cells and was prevented by deletion of *HOG1* and *SIC1* (Escote *et al*, 2004). Similar results were obtained when cells were treated with osmostress (Figure 1C) (Escote *et al*, 2004).

It was reported that in response to osmostress, cells arrested at G_2 (Alexander *et al*, 2001). To characterize the involvement of Hog1 in this arrest, we took advantage of the system described above in which we analysed the effect of sustained activation of Hog1 in G_2 either by mutations in the *Sln1* osmosensor or the *Pbs2* MAPKK (Maeda *et al*, 1994; Maeda *et al*, 1995; Posas *et al*, 1996; Wurgler-Murphy *et al*, 1997; Posas and Saito, 1997) and by osmostress. *sln1*^{ts4} cells were arrested in G_1 phase with α-factor then released from the arrest and monitored until they were in G_2 and incubated at nonpermissive temperature. As shown in Figure 1B, a high percentage of cells remained in G_2 phase (about 76% after 120 min were budded cells with 2C DNA content, 43% of them contained a single nuclei near the bud neck and showed short spindles as judged by tub1-GFP localization), whereas only a small proportion of wild-type cells remained in G_2 (~39% budded cells with 2C DNA content, and only 3% of them contained a single nuclei near the bud neck after 120 min). Activation of the HOG pathway can also be triggered by expression of an allele of the Ssk2 MAPKKK, which contains a deletion of the N-terminal regulatory domain (Ssk2ΔN), or expression of the active allele of Pbs2 (Pbs2^{DD}). However, the G_2 arrest caused by expression of these alleles was not as efficient as observed by the inactivation of Sln1 (Supplementary Figures S1/S4 and data not shown). Osmostress treatment of wild-type cells synchronized at G_2 also resulted in delayed G_2 exit when compared to nontreated cells (83% of cells in G_2 upon 80 min of stress versus 30% without stress) (Figure 1D). Thus, activation of Hog1 results in both G_1 and G_2 arrest of cell cycle progression.

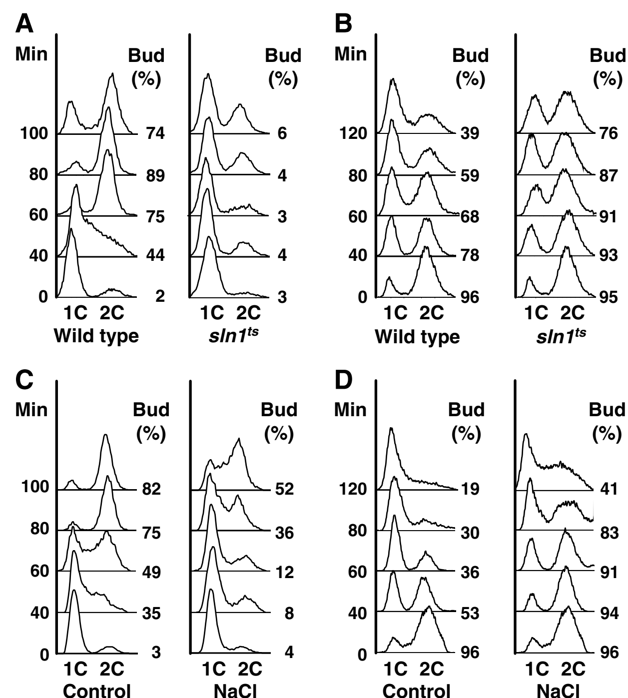

Figure 1 Activation of the HOG pathway results in G_1 and G_2 cell cycle arrest. (**A**) Cells arrest at G_1 in response to Sln1 inactivation. The *sln1*^{ts4} or wild-type strains were synchronized with α-factor for 2 h, shifted to 37°C for 1 h and then released into YPD medium at 37°C. Total DNA content was assessed by FACS analysis and presented as cell counts (*y*-axis) versus 1C and 2C DNA content (*x*-axis). The percentage of budding cells is depicted on the right-hand side of each graph. (**B**) Cells arrest at G_2 in response to Sln1 inactivation. The *sln1*^{ts4} or wild-type strains were synchronized with α-factor for 3 h, released into fresh media at 25°C and shifted to 37°C after 50 min in YPD (time 0). Total DNA content was analysed as in (A). (**C**) Cells arrest at G_1 in the presence of osmostress. Wild-type cells were synchronized in G_1 phase with α-factor and released into YPD medium containing 0.4 M NaCl. Total DNA content was assessed as in (A). (**D**) Cells arrest at G_2 in the presence of osmostress. Wild-type cells were synchronized with α-factor and released into YPD medium. After 50 min, NaCl was added (0.4 M) (time 0). Total DNA content was assessed as in (A).

Deletion of SWE1 prevents the G_2 arrest caused by activation of the Hog1 MAPK

Activation of the HOG pathway at G_2 resulted in accumulation of cells with an elongated bud, which is reminiscent of altered Clb2–Cdc28 activity (Figure 2A). The phosphorylation that regulates Cdc28–Clb2 activity is driven by the activities of the Swe1 kinase and the Mih1 phosphatase (Morgan, 1997). Previous results indicated that deletion of *SWE1* prevented G_2 arrest in response to osmostress (Alexander *et al*, 2001); however, osmostress causes profound cytoskeleton defects which could be monitored by Swe1 to arrest cell cycle at G_2 independently of Hog1. To test whether *SWE1* was mediating the G_2 arrest caused by Hog1 activation, *sln1*^{ts4}, *sln1*^{ts4} *hog1* and *sln1*^{ts4} *swe1* were synchronized at G_2 and incubated at nonpermissive temperature. As shown in Figure 2A, upon Hog1 activation, deletion of *SWE1* resulted in a clear decrease of cells arrested in G_2 when compared to cells carrying wild-type *SWE1* (65 versus 93% of cells in G_2, respectively, after 80 min at nonpermissive temperature). Correspondingly, deletion of *SWE1* prevented bud enlargement. It is worth noting that deletion of *SWE1* causes already some morphological defects in cells growing under normal conditions in w303 strain background (Buscemi *et al*, 2000)

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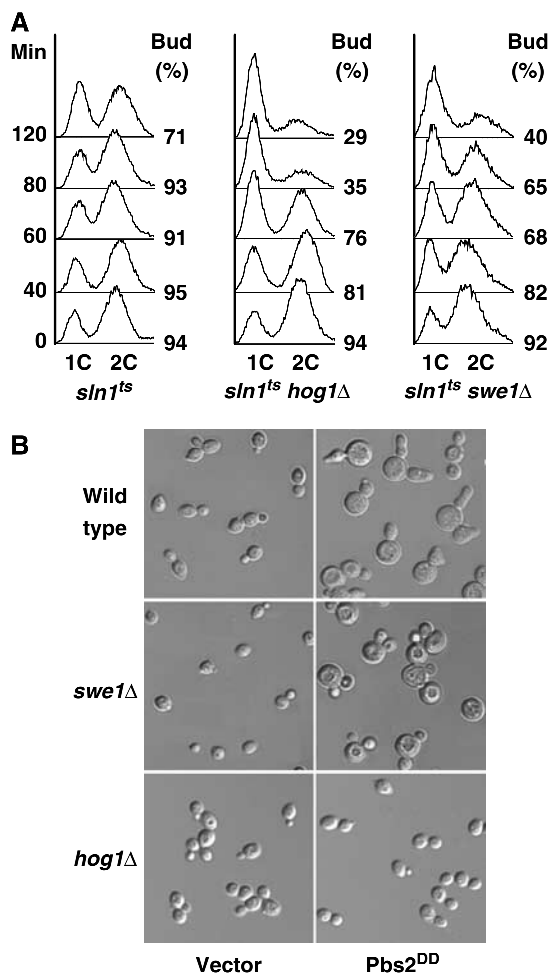

Figure 2 Swe1 mediates the G_2 arrest caused by Hog1 activation. (**A**) Deletion of *HOG1* or *SWE1* abolishes cell cycle arrest at G_2 caused by Sln1 inactivation. *sln1^{ts4}*, *sln1^{ts4} hog1*Δ, and *sln1^{ts4} swe1*Δ strains were synchronized with α-factor for 3 h, released into fresh media at 25°C and shifted to 37°C after 50 min in YPD (time 0). Total DNA content was analysed as in Figure 1A. (**B**) Wild-type, YPC89 (*hog1*Δ) and YPC166 (*swe1*Δ) cells containing an empty vector or P_{GAL1}-Pbs2^{DD} were grown for 7 h in SD-Ura plus galactose and visualized by differential interference contrast.

(Figure 2B). In contrast, deletion of *MIH1* only enhanced the effect of Hog1 activation (not shown). Therefore, Swe1 plays an important role in the G_2 arrest caused by activation of the Hog1 MAPK.

Activity of Cdc28 at G_2 can be controlled by its state of phosphorylation and by the levels of Clb2. Clb2 mRNA and protein levels were diminished upon expression of the Pbs2^{DD} (Figure 3A and B). Similar results were observed when Clb2 mRNA levels were monitored in the *Sln1ts* mutant (Supplementary Figure S1). In addition, the relative levels of Clb2–Cdc28 activity diminished upon expression of the Pbs2^{DD} (Figure 3A and B), as reported to occur in response to osmostress (Alexander *et al*, 2001). Actually, Cdc28–Clb2 activity was almost not downregulated when the Cdk1 complex was purified from *swe1* cells (Supplementary Figure S2). Taken together, Hog1 activation results in a decrease of the activity of the Clb–Cdc28 complex and this modulation is mediated by the downregulation of the Clb2 levels and the activity of the Swe1 kinase.

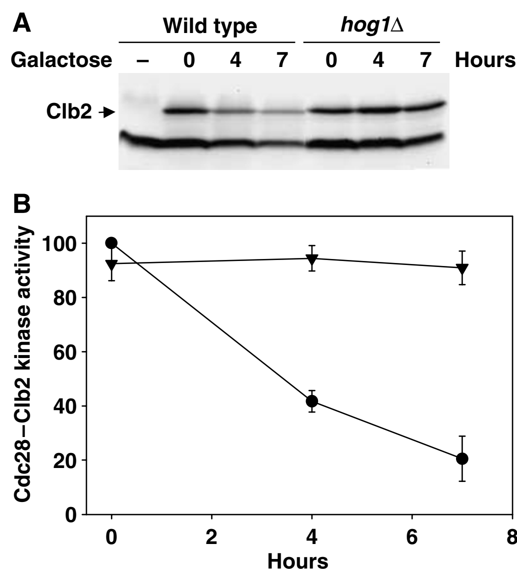

Figure 3 Activation of Hog1 causes a decrease on Clb2 levels and Clb2–Cdc28 activity. (**A**) The levels of Clb2 protein decreases upon Hog1 activation. Wild-type and YPC89 (*hog1*Δ) cells containing P_{GAL1}-Pbs2^{DD} were transformed with a plasmid expressing HA-tagged Clb2 under its own promoter. Cells were grown in raffinose and then galactose was added (time 0). Cells were collected at the indicated times and Clb2 was visualized by immunoblotting with monoclonal antibody 12CA5 to HA. Wild-type cells without the Clb2-HA plasmid are shown as negative control (−). (**B**) Hog1 activation leads to a reduction on the relative levels of Clb2–Cdc28 activity. Cells as in (A); wild type (●) and *hog1*Δ (▼) were grown in the presence of galactose and collected at the indicated time points. Protein extracts were prepared and Clb2–Cdc28 kinase activity was assessed by an *in vitro* kinase assay of immunoprecipitated Clb2–Cdc28 using Histone H1 (HH1) as a substrate. Kinase activity was normalized to that of wild type, time 0. Data ± s.d. from three independent experiments are shown.

Sustained activation of the Hog1 MAPK pathway results in stabilization of the Swe1 kinase

We therefore decided to investigate how Swe1 was regulated upon Hog1 activation. It was reported that stability of Swe1 is critical to regulate its activity towards Cdc28–Clb2 (Sia *et al*, 1996; Sia *et al*, 1998). Swe1 degradation is stimulated by protein phosphorylation. We therefore analysed the phosphorylation of Swe1 in synchronized cells in the presence or absence of stress (Figure 4A). Osmostress induced a delay in Swe1 phosphorylation, suggesting that Swe1 stability could be affected by Hog1 activation. We then analysed whether Swe1 accumulates in response to Hog1 activation. Expression of Pbs2^{DD} or the addition of NaCl (as reported previously (Sia *et al*, 1998)) resulted in a strong accumulation of Swe1 protein (Figures 4B and C). Therefore, activation of Hog1 results in accumulation of the CDK regulator, Swe1.

Failure of *sic1*Δ cells to arrest at G_1 upon osmostress causes premature entry into S phase and cells become partially osmosensitive (Escote *et al*, 2004). Because Swe1 was required for Hog1-mediated G_2 arrest and Swe1 was stabilized upon stress, we analysed whether the lack of *SWE1* also resulted in cells unable to adapt to osmostress. As shown in Figure 4D, *swe1*-deficient cells also become partially osmosensitive. It is worth noting that *swe1*-deficient cells do not arrest at G_2 in the presence of high osmolarity and this results in a high percentage of binucleated cells due to a premature entry into mitosis (Figure 2A and data not shown). Synthetic

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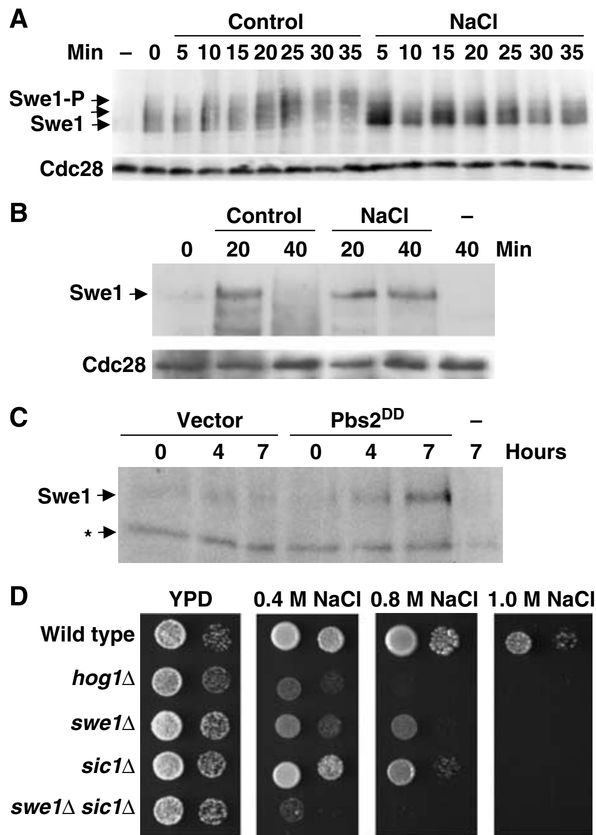

Figure 4 Swe1 stabilization is critical for cell survival upon stress. (**A**) Swe1 phosphorylation is affected by osmostress. Cells expressing epitope-tagged Swe1 from its chromosomal locus were synchronized by pheromone treatment and 50 min after release, cells were subjected (NaCl) or not (control) to 0.4 M NaCl. Swe1 and Cdc28 (loading control) were detected from cell extracts by immunoblotting using specific antibodies. Control strain (without tagged Swe1) was wild type W303 (−). (**B**) Swe1 accumulates in response to osmostress. Tagged Swe1 was expressed from its chromosomal locus in wild-type cells (YPC130). Cells were synchronized as in (A) and subjected (NaCl) or not (control) to 0.4 M NaCl. Swe1 was detected from yeast extracts using monoclonal anti-myc-specific antibodies. Cdc28 is presented as loading control. Control strain was wild type W303 (−). The percentage of acrylamide on (A) was lower than in (B) to increase the separation between the phosphorylated forms of Swe1. (**C**) YPC130 strain (which expresses Swe1-myc) containing either control vector (vector) or P_{GAL1}-Pbs2^{DD} plasmid were grown in SD-Ura plus raffinose and after addition of galactose cells were collected at the indicated times. The presence of myc-tagged Swe1 was detected as in (C). The asterisk indicates an unspecific protein detected by the myc antibodies that is kept constant along the culture. It is presented as a loading control. (**D**) Deletion of *SWE1* or *SIC1* results in osmosensitive cells. The wild-type (W303) strain and its derivatives *hog1*Δ, *sic1*Δ, *swe1*Δ and *sic1*Δ *swe1*Δ mutants were spotted on YPD plates or YPD plates containing 0.4 M, 0.8 M or 1 M NaCl. Growth was scored after 3 days at 30°C.

sic1 and *swe1* mutations render cells even more osmosensitive than the single mutations, resulting in sensitivity similar to the *hog1* mutation (Figure 4D). Therefore, modulation of cell cycle progression at different phases of the cell cycle, in G_1 and G_2, is required for cell survival upon stress.

Hsl7 delocalizes from the bud neck in response to Hog1 activation

It is known that localization of the septin cytoskeleton, the activity of the checkpoint protein kinase Hsl1 and the presence of its interacting protein Hsl7 in the bud neck regulate Swe1 stability. Actually, Hsl7 interacts directly with both Hsl1 and Swe1 and mutations that impair either of these interactions, or change the localization of Hsl1 or Hsl7, stabilize Swe1 protein (Barral *et al.*, 1999; McMillan *et al*, 1999; Shulewitz *et al*, 1999; Cid *et al*, 2001; Hanrahan and Snyder, 2003; Lew, 2003; Theesfeld *et al*, 2003). We then followed the localization of septins (i.e., Cdc11 and Cdc12), of the Hsl1 kinase and the Hsl7 protein fused to GFP. Localization of the septins Cdc11 and Cdc12 (not shown) and Hsl1 kinase in the bud neck were not affected by Hog1 activation, nor by osmostress or expression of Pbs2^{DD} (Figure 5A and C). However, Hsl7 localization dramatically changed upon Hog1 activation. Cells exposed to osmostress showed a dramatic delocalization of Hsl7 from the bud neck. Hsl7 delocalization was transient and correlated to the level of Hog1 activation (Figure 5A and B). In addition, whereas Hsl7 was recruited at the bud neck in more than 70% of control cells, only 15% of these cells displayed Hsl7 in the bud neck when expressing Pbs2^{DD} (Figure 5C). It was reported that when delocalized from the septin ring, Hsl7 is rapidly dephosphorylated (Theesfeld *et al*, 2003; Sakchaisri *et al*, 2004). Correspondingly, activation of Hog1 results in rapid Hsl7 dephosphorylation (not shown). Thus, activation of the MAPK results in the delocalization of Hsl7 from the bud neck, which promotes Swe1 stabilization.

Hog1 interacts and directly phosphorylates the Hsl1 checkpoint kinase

To analyse whether the regulation of Hsl7 localization at the bud neck was a direct event regulated by Hog1, we tested whether Hog1 was able to interact with and phosphorylate Hsl1 or Hsl7. *In vivo* co-precipitation experiments were performed with yeast cells containing a chromosomally HA-tagged Hsl1 or Hsl7 transformed with plasmids that expressed GST-tagged full-length Hog1 or GST control. As shown in Figure 6A, Hog1 is able to interact with Hsl1 but not with Hsl7 (not shown). Therefore, we investigated whether Hsl1 was phosphorylated upon osmostress by Hog1. Yeast cells were exposed to a brief osmotic stress (10 min, 0.4 NaCl) after 50 min of release from pheromone arrest. Osmostress induced a rapid phosphorylation of Hsl1, as seen by a shift on Hsl1 mobility in an SDS–polyacrylamide gel. Interestingly, stress-induced Hsl1 phosphorylation was not observed in a *hog1*Δ strain (Figure 6B).

We then tested whether Hsl1 was directly phosphorylated by the Hog1 MAPK. Several fragments of Hsl1 were expressed and purified from *Escherichia coli,* and then incubated with activated recombinant Hog1 in presence of radioactive ATP. Those experiments showed that, whereas Hsl7 or Swe1 were not phosphorylated by the MAPK (not shown), Hsl1 was phosphorylated in its C-terminal region (amino acids (aa) 711–1517) but not in the N-terminal domain (aa 1–900) (Figure 6C). It was described that Hsl7 recruitment to the bud neck requires the binding of Hsl7 to the Hsl1 C-terminal region (Shulewitz *et al*, 1999; Cid *et al*, 2001). *In vitro* kinase assays testing several Hsl1 fragments for direct phosphorylation pointed at the Ser1220 as the Hog1 phosphorylation site in the Hsl1 C-terminal region. Mutation of Ser1220 to Ala abolished phosphorylation of Hsl1 by Hog1 (Figure 6D). Correspondingly, *in vivo* phosphorylation assays showed

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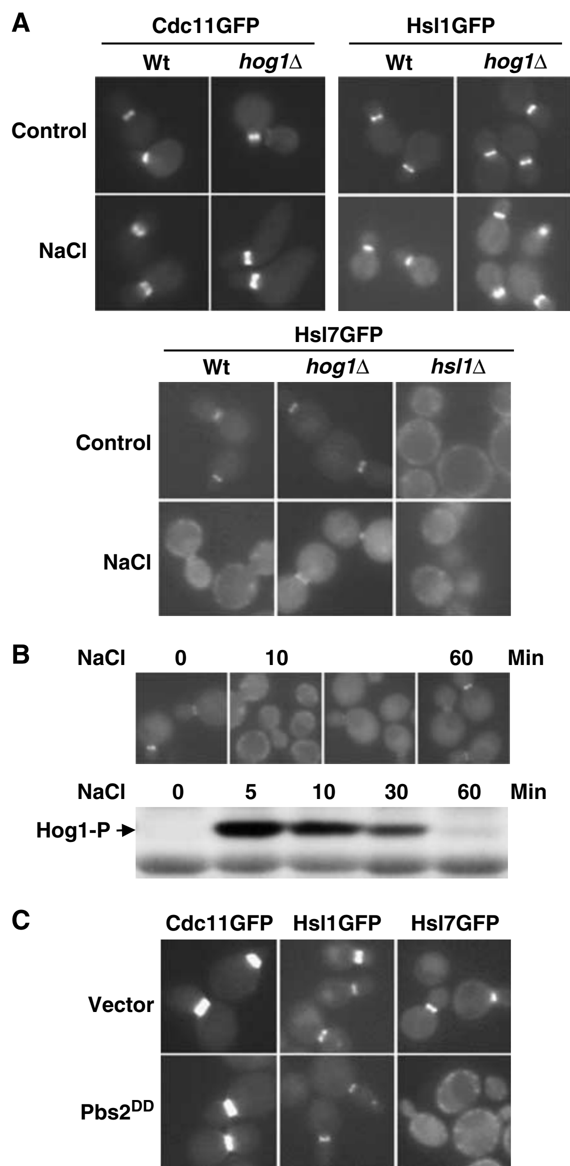

Figure 5 Localization of Hsl7 is affected by activation of the HOG pathway. (**A**) Localization of Hsl7 but not Cdc11 or Hsl1 is affected by osmostress. Wild-type, *hog1*Δ or *hsl1*Δ cells containing chromosomal GFP-tagged Cdc11, Hsl1 or Hsl7 were grown in YPD and subjected (NaCl) or not (control) to a brief osmostress (10 min at 0.4 M NaCl). GFP proteins were visualized by direct fluorescence. Numbers represent percentage of cells with GFP fluorescence at the bud neck and is the result of the measurement in triplicate of 200 cells each. (**B**) Time course of Hsl7GFP localization upon osmostress. Wild-type cells containing Hsl7GFP were subjected to 0.4 M NaCl at the indicated times. Hog1 phosphorylation was followed using specific antibodies against phosphorylated p38 in whole-cell extracts. Numbers represent percentage of cells with GFP fluorescence at the bud neck and is the result of the measurement in triplicate of 200 cells each. (**C**) Localization of Hsl7 is affected by Hog1 activation. Wild-type cells expressing P_{GAL1}-Pbs2^{DD} or a control vector and GFP-tagged Cdc11, Hsl1 or Hsl7 were grown in minimal media containing galactose. Numbers represent percentage of cells with GFP fluorescence at the bud neck and is the result of the measurement in triplicate of 200 cells each.

that the Hsl1S1220A mutant was not phosphorylated upon osmostress (Figure 6B). Interestingly, Ser1220 is situated in the middle of the Hsl7-binding domain in Hsl1 (Shulewitz *et al*, 1999; Cid *et al*, 2001). Therefore, the Hog1 MAPK is able to interact and directly phosphorylate a single residue within the Hsl7-binding domain of the Hsl1 kinase.

Phosphorylation of Hsl1 S1220 by the Hog1 MAPK is critical for Hsl7 localization, G_2 arrest and proper adaptation to osmostress

To assess the relevance of the phosphorylation of Hsl1 Ser1220 in the localization of Hsl7 in response to Hog1 activation, we analysed Hsl7 localization in cells expressing wild-type Hsl1 or the unphosphorylatable mutant of Hsl1, Hsl1 S1220A (Hsl1^{SA}). Whereas Hsl7 localization changed dramatically in response to osmostress or in response to Pbs2^{DD} expression in cells containing wild-type Hsl1, it did not change in cells expressing the unphosphorylatable Hsl1^{SA} protein (Figure 7A and B). The amounts of Hsl7-GFP were similar in a wild-type and the Hsl1^{SA} strain (not shown). It is worth noting that in cells carrying the Hsl1 S1220E allele (Hsl1^{SE}, which cannot be phosphorylated by Hog1, but the acid residue mimics the phoshorylated state), the amount of Hsl7 present in the bud neck under normal conditions was already half of that observed in wild-type cells (72% wild type versus 35% *HSL1*^{SE}). Correspondingly, they showed an abnormal morphology reminiscent to *hsl1*-deficient cells (data not shown).

To obtain direct evidence for the role of Hsl1 phosphorylation in Hsl7 binding, we tested by two-hybrid analysis whether binding of Hsl7 to Hsl1 was affected by osmostress and whether this association was altered by mutations in the Hsl1S1220 phosphorylation site. Wild-type Hsl1 or Hsl1S1220A and Hsl1S1220E mutants (from aa 729 to the stop) were fused to the *GAL4* activator domain and their interaction with the Hsl7 fused to the LexA-DNA-binding domain was tested. As shown in Figure 7C, binding of Hsl7 to Hsl1 decreases in response to osmostress. Interstingly, the interaction of Hsl7 to Hsl1 did not change when Hsl1 was unable to be phosphorylated by Hog1 (Hsl1^{SA}). Furthermore, the binding of Hsl7 to Hsl1 was reduced when Hsl1 contained a mutation that mimics Hog1 phosphorylation (Hsl1^{SE}). It is worth noting that Hsl1 phosphorylation by Hog1 was much more stronger than Hsl1 autophosphorylation and did not alter *in vitro* Hsl1 kinase activity (Supplementary Figure 3). Thus, phosphorylation of Hsl1 by Hog1 is likely to be the key determinant for Hsl7 localization in response to high osmolarity.

We showed that in response to osmostress, Swe1 was stabilized (Figure 4B). We therefore tested whether mutation of Hog1 phosphorylation site in Hsl1 was important for Swe1 accumulation. As shown in Figure 7D, whereas Swe1 accumulated upon osmostress in wild-type cells, it failed to accumulate in cells containing the *HSL1*^{SA} mutation.

We then analysed the relevance of Hsl1 S1220 phosphorylation in the G_2 arrest mediated by Hog1. *sln1*^{ts4} or *sln1*^{ts4} *HSL1*^{S1220A} cells were synchronized at G_2 and incubated at nonpermissive temperature. As shown in Figure 7E, mutation of Hsl1^{S1220A} resulted in a clear decrease of cells arrested in G_2 when compared to cells carrying wild-type Hsl1 (48 versus 85% of cells in G_2, respectively, after 50 min at nonpermissive temperature). Thus, phosphorylation of S1220 in Hsl1 is a critical event for G_2 arrest upon Hog1 activation. Correspondingly, cells containing the Hsl1^{SA} allele were more sensitive to osmostress than cells carrying the wild-type Hsl1 (Figure 7F).

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Discussion

Activation of the Hog1 SAPK is a key step for the generation of adaptive responses that allow for cell survival upon osmostress. Modulation of cell cycle progression is essential for adaptation to stress. Previous reports have shown that activation of Hog1 modulates G_1 by the direct targeting of the CDK inhibitor Sic1 (Escote *et al*, 2004) and that, in response to osmostress, cells arrested at G_2 by an unknown molecular mechanism involving somehow the product of the *SWE1* gene (Alexander *et al*, 2001). Here we show that, in response to stress, Hog1 controls G_2 progression by downregulation of the cyclin B levels, as well as the direct phosphorylation of the Hsl1 kinase, which leads to the stabilization of the Swe1 kinase and the decrease of Clb–Cdc28 activity.

It is known that Swe1 stability is regulated by Clb2–Cdc28 (Asano *et al*, 2005; Harvey *et al*, 2005) and Hsl1 kinases (Hanrahan and Snyder, 2003; Lew, 2003). The Hsl1 checkpoint kinase is part of the so-called 'morphogenesis checkpoint' which monitors cytoskeleton alterations or delayed bud formation and regulates Swe1 stability. Under normal growth conditions, Hsl1 binds to the septin ring at the bud neck which triggers the recruitment of Hsl7 and phosphorylation and degradation of Swe1 prior to entry into mitosis (Lew, 2003). In response to morphogenetic defects, the Hsl1 kinase is inhibited and this prevents Hsl7 and Swe1 recruitment (Lew, 2003). Here, we have shown that osmostress induces Swe1 stability by the direct regulation of components of the 'morphogenesis checkpoint'. However, whereas the lack of Hsl1 localization at the bud neck or the inactivation of the kinase activity is the leading signal for Swe1 accumulation, a novel mechanism of regulation seems to modulate the Hsl1–Hsl7 complex formation in response to osmostress. Upon stress, Hsl1 localization is maintained and it is the phosphorylation of Ser1220 at the Hsl7-binding domain that promotes Hsl7 delocalization from the bud neck and Swe1 accumulation. Thus, we propose a novel regulatory mechanism of the Hsl1 morphogensis checkpoint kinase that allow cells to integrate stress signals to modulate cell cycle.

Hog1 controls G_1 transition by a dual mechanism that involves regulation of cyclin expression and the targeting of the cell cycle regulatory protein Sic1. It is worth noting that in G_2, Hog1 is also controlling cell cycle progression by downregulation of Clb2 transcription, and the phosphorylation of the cell cycle regulatory protein Hsl1. Again, it seems that the coordinated action of the MAPK over cyclin transcription and components of the cell cycle machinery mediated cell cycle progression in different steps of cell cycle. If the direct phosphorylation of Sic1 or Hsl1 were to

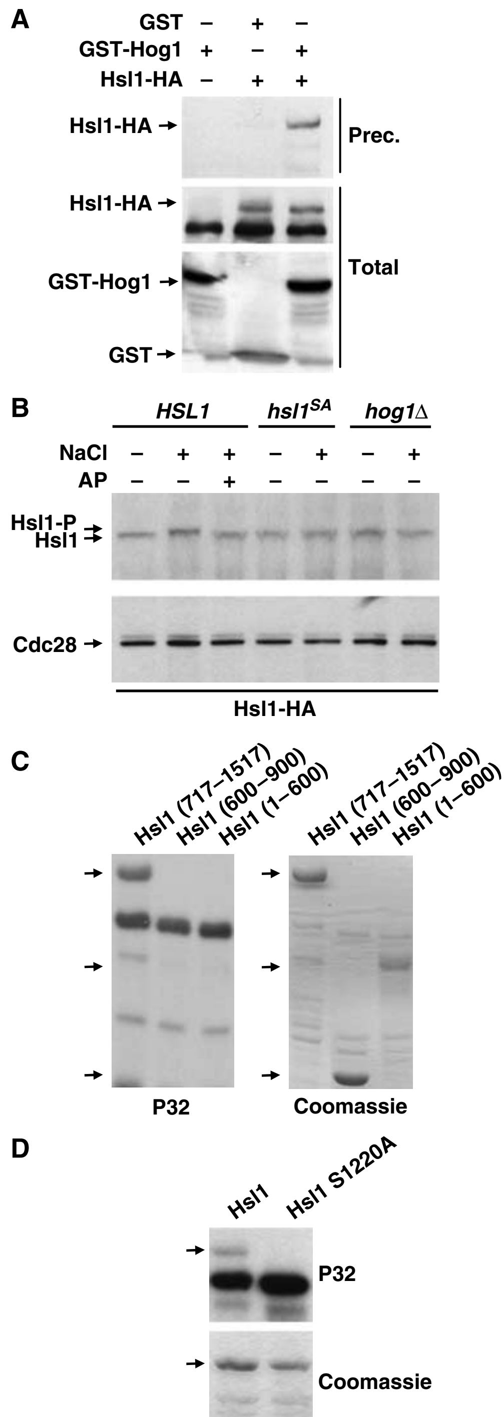

Figure 6 Hog1 phosphorylates Ser1220 within the Hsl7-binding site in Hsl1. (**A**) Hog1 interacts with Hsl1 *in vivo*. Yeast extracts containing GST or GST-Hog1 and untagged or tagged HA-Hsl1 (from its chromosomal locus) were precipitated using glutathione beads and precipitated Hsl1 was probed using anti-HA antibodies. GST proteins were detected using anti-GST antibodies. (**B**) Hsl1 is phosphorylated upon osmostress in a HOG1-dependent manner. *hsl1* cells or *hog1* cells were transformed with a plasmid expressing a catalytically inactive Hsl1, contains the K110A mutation (*HSL1*) or in addition the S1220A mutation (*hsl1^{SA}*) under the *GAL1* promoter. Cells were synchronized with pheromone treatment in the presence of galactose and 50 min after release, cells were subjected (+) to a brief osmotic shock (0.4 M NaCl, 10 min). Extracts were treated (+) with alkaline phosphatase (AP). The effect of AP was prevented by the addition of phosphatase inhibitors (not shown). The presence of Hsl1 and Cdc28 was probed. (**C**) Hog1 directly phosphorylates the C-terminal region of Hsl1 *in vitro*. Hog1 and the constitutively activated Pbs2 allele (Pbs2^{EE}) purified from *E. coli* were incubated in the presence of kinase buffer and ATP. Then, catalytically inactive Hsl1 kinase domain (1–600), an Hsl1 fragment (from aa 600 to 900) or the C-terminal domain of Hsl1 that contains aa 717–1517, purified from *E. coli*, were added in the presence of radioactive ATP. Phosphorylated proteins were detected by autoradiography (P32) or Coomassie staining. Position of Hsl1 fragments is indicated on the left. (**D**) Hog1 directly phosphorylates the Ser1220 of Hsl1 *in vitro*. The wild-type Hsl1 and the Hsl1 S1220A mutant were expressed in *E. coli* and assayed as in (C). Phosphorylated proteins were detected by autoradiography (P32) or Coomassie staining. Position of Hsl1 proteins is indicated on the left.

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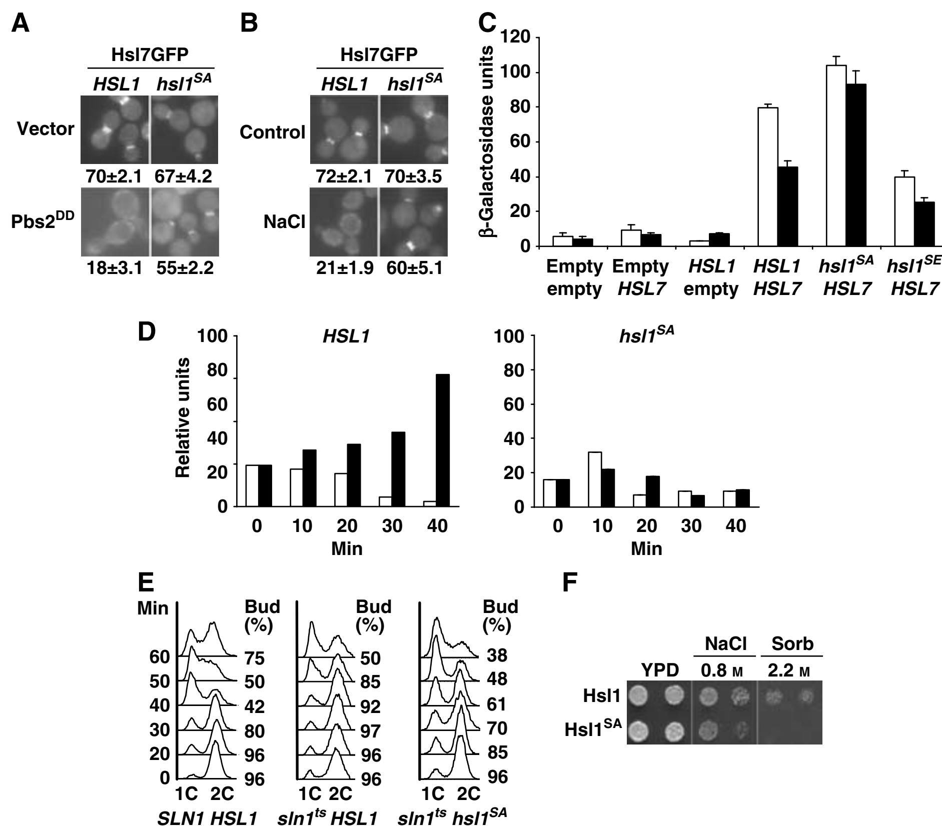

Figure 7 Phosphorylation of Hsl1 Ser1220 by Hog1 determines Hsl7 localization and is essential for G_2 arrest and survival upon stress. (**A**) Mutation of Hsl1 Ser1220 affects Hsl7 localization. The *hsl1Δ HSL7GFP* strain transformed with plasmids containing wild-type Hsl1 or the mutated alleles Hsl1 Ser1220A ($Hsl1^{SA}$) or Hsl1 Ser1220E ($Hsl1^{SE}$) and P_{GAL1}-$Pbs2^{DD}$ or a control vector were grown as in Figure 5C. Hsl7GFP was visualized by direct fluorescence. Numbers represent percentage of cells with GFP fluorescence at the bud neck and is the result of the measurement in triplicate of 200 cells each. (**B**) Cells as in (A) were grown in minimal media and subjected (NaCl) or not (control) to a brief osmostress (10 min, 0.4 M NaCl). Hsl7GFP was visualized as in (A). Numbers represent percentage of cells with GFP fluorescence at the bud neck and is the result of the measurement in triplicate of 200 cells each. (**C**) The interaction between Hsl7 and Hsl1 decreases upon osmostress depending on Hog1 phosphorylation. Two-hybrid analyses were performed in liquid assays with cells carrying wild-type Hsl1 and mutant Hsl1 ($Hsl1^{SA}$ or $Hsl1^{SE}$) fused to the GAL4 activator domain together with Hsl7 fused to the LexA BD. β-Galactosidase activity was assayed in cells grown to mid-log phase that were subjected (NaCl, filled bars) or not (control, open bars) to hyperosmotic stress (0.4 M NaCl for 60 min). β-Galactosidase activity is given in nmol/min/mg and is the result of the measurement in duplicate of three independent transformants. Error bars indicate standard error of the mean. (**D**) Accumulation of Swe1 by osmostress depends on phosphorylation of Hsl1 S1220. *hsl1* cells carrying HA-tagged wild-type (Hsl1) or the Hsl1S1220A mutant ($Hsl1^{SA}$) were grown as in Figure 4A and collected after the indicated time points under osmostress (filled bars). Swe1 was detected by using anti-HA antibodies. Cdc28 was detected by specific antibodies on the same membranes and used as a loading control. Data were quantified by phosphoimager and is the mean of two independent experiments. (**E**) Cells with the $Hsl1^{SA}$ mutant fail to arrest at G_2 in response to Sln1 inactivation. Wild-type cells (*SLN1 HSL1*) or an $sln1^{ts}$ *hsl1* strain containing wild-type *HSL1* or $Hsl1^{SA}$ in a plasmid were synchronized with α-factor for 3 h, released into fresh media at 25°C and shifted to 37°C after 75 min in YPD (time 0). Total DNA content was assessed by FACS analysis and presented as cell counts (*y*-axis) versus 1C and 2C DNA content (*x*-axis). The percentage of budding cells is depicted on the right-hand side of each graph. (**F**) Cells containing an unphosphorylatable allele of Hsl1 become osmosensitive. *hsl1Δ* cells as in (E) were spotted on YPD plates containing NaCl or Sorbitol. Growth was scored after 3 days.

permanently stabilize Sic1 or Swe1, an extra decrease in cyclin–Cdc28 activity to further stabilize Sic1 or Swe1 would be unnecessary. Nevertheless, the sole phosphorylation of Sic1 or Hsl1l by the MAPK cannot totally account for the G_1 and G_2 arrest observed upon stress, which suggests that there must be a selective advantage for maintaining such a complex regulatory mechanism. An obvious advantage of the coordinated effect over the inhibitors could be the increase of the efficiency of the G_1 and G_2 arrests by establishing two converging (additive) mechanisms over Sic1 and Swe1, neither of them too strict to interfere with the normal cell cycle progression without stress.

Here, we show that multiple checkpoint activation by the MAPK is required to transiently arrest cell cycle and generate the required responses for cellular adaptation. This observation is coherent with the idea that cells might be subjected to stress at any stage of cell division and thus, they have to be able to adapt before progressing into the sensitive phases of cell cycle. As stated before, in response to stress, the MAP kinase is able to regulate both, G_1 and G_2, through its coordinate action over two-independent checkpoints, the control of Sic1 at G_1, and Hsl1 at G_2, to allow cells to recover before they progress into S phase and mitosis. Exposure of mammalian cells to osmotic imbalances results in the activation of the p38 SAPKs. As observed in yeast, mammalian cells also respond to high osmolarity by modulating cell cycle progression. Actually, different reports and our own unpublished observations indicate that different type of mammalian cells arrest at several stages of the cell cycle (G_1–S, G_2 and mitosis) upon osmostress (Ambrosino and Nebreda, 2001; de Nadal *et al*, 2002; Dmitrieva *et al*, 2002; Mikhailov *et al*, 2004; Sheikh-Hamad and Gustin, 2004). Different mechanisms

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have been proposed for the control of cell cycle progression by the p38 SAPKs and several targets have been defined (Ambrosino and Nebreda, 2001; Pearce and Humphrey, 2001; Goloudina *et al*, 2003; Xiu *et al*, 2003; Todd *et al*, 2004). In such a complex scenario, where several targets for the SAPKs have been described, it is still not clear whether specific mechanisms are used to respond to different stimuli or if different cell types use different mechanisms to cope with stressful situations. From the yeast studies we propose that in response to stress, the SAPKs might coordinate different mechanisms, probably involving modulation of cyclin levels together with the targeting of specific cell cycle regulators, to promote transient cell cycle arrest at several steps of cell cycle allowing for proper cellular adaptation to extracellular stimuli.

Materials and methods

Yeast strains and plasmids

Strains used: W303 (*MATa, his3 leu2 trp1 ura3 ade2 can1*) and its derivatives YPC88 (*hog1::LEU2*), YPC65 (*sic1::KanMX*), YPC166 (*swe1::KanMX*), YPC343 (*sic1::KanMX swe1::URA3*), YPC410 (*SWE1-HA::TRP1*), YPC253 (*HSL1-GFP::KanMX*), YPC251 (*hog1::LEU2 HSL1-GFP::KanMX*), YPC361 (*HSL7-GFP::KanMX*), YPC357 (*hog1::LEU2 HSL7-GFP::KanMX*), YPC425 (*hsl1::URA3 HSL7-GFP::KanMX*). TM141 (*MAT***a** *ura3 leu2 trp1 his3*) and its derivatives YPC38 (*sln1-ts4*), YPC29 (*sln1-ts4 hog1::LEU2*), YPC60 (*sln1-ts4 swe1::KanMX*), YPC130 (*SWE1-9xMyc::TRP1*). L40 (*MAT***a** *trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*). The P_{GAL1}-$Pbs2^{DD}$ expresses a *PBS2* allele that contains two mutations that replace both phosphorylation sites required for Pbs2 activation (Ser514 an Thr518 to Asp). Plasmid expressing Cdc11::GFP under the control of its own promoter was a generous gift from Drs A Casamayor and M Snyder. The last 800 aa from wild type Hsl1 (pJCE 1060) or the corresponding mutant S1220A (pJCE 1096) were cloned into pGEX-4T plasmid. Centromeric plasmid pYB117 expressing HSL1-HA from its own promoter was a generous gift from Dr Y Barral. To express haemaglutinin (HA)-tagged mutated versions of Hsl1 in yeast, an S1220A or S1220E substitutions were introduced in pYB117 generating pJCE1090 and pJCE1099, respectively. A plasmid expressing GST-Hsl7 was a generous gift from Dr J Thorner.

Growth conditions, cell synchrony and cytometry analyses

Cells were grown in YPD or SC medium without uracil (URA) supplemented with either 2% dextrose, or 2% raffinose, when indicated. Galactose induction was accomplished by initial growth in URA plus raffinose, followed by addition of galactose to 2%. Cell synchrony was accomplished by treatment of cells with 40 μg/ml of pheromone for the indicated times. Pheromone was added to the cultures after 10 min upon osmostress or after 15 min that cells were shifted to 37°C (*sln1ts*) to avoid re-entry of the cells into S phase (Figures 1 and 2). For flow cytometry analyses, cells were fixed in ethanol, treated with RNAse A, stained with propidium iodide and analysed in a FACScan flow cytometer (Becton Dickinson). A total of 10 000 cells were analysed for each time point.

Binding assays

In vivo interaction of GST-Hog1 with chromosomal HA-tagged Hsl1 was determined by co-precipitation. Exponential growing cells were subjected to a brief osmotic shock (0.4 M NaCl, 10 min). Yeast extracts (3 mg) were prepared as by Barral *et al* (1999) and incubated with glutathione-sepharose beads. Beads were washed extensively and proteins precipitated were detected using anti-HA and GST antibodies.

Kinase assays

The Hsl1 proteins were expressed in *E. coli* and purified using glutathione-sepharose beads, mixed with 1 μg of purified GST-Hog1 or Hog1(KN) activated with GST-$PBS2^{DD}$ and radioactive ATP. Clb2-associated Cdc28 kinase activity assays were performed on immunoprecipitated Clb2-HA. Clb2–Cdc28 complexes were immunoprecipitated using anti-HA antibodies from 1 mg of total cellular protein and assayed essentially as described by Belli *et al* (2001), using histone H1 as substrate. Phosphorylated histone H1 was assessed by using a Phosphoimager and referenced to the time 0 wild-type activity.

Two-hybrid analysis

The two-hybrid analysis was carried out essentially according to that given by Durfee *et al* (1993), using pACTII and pBTM116, as the activation domain (AD) plasmid and the LexA DNA-binding domain (DB) plasmid, respectively. LexA-Hsl7 plasmid (containing the full-length Hsl7) was cotransformed with the pACTII plasmid containing the c-terminal region of Hsl1 (aa 729 to stop) either wild type, S1220A or S122E mutations, using the L40 reporter strain. Positive clones were selected and further tested for β-galactosidase activity. β-Galactosidase activity was quantified in liquid media before or after 60 min of osmotic stress (0.4 M NaCl) (Durfee *et al*, 1993).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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