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The Sch9 protein kinase regulates Hsp90 chaperone complex signal transduction activity *in vivo*

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Basal and stress-induced synthesis of the components of the highly conserved heat shock protein Hsp90 chaperone complex require the heat shock transcription factor (HSF); *Saccharomyces cerevisiae* cells expressing the HSF allele HSF(1-583) reversibly arrest growth at 37°C in the G_2/M phase of the cell cycle due to diminished expression of these components. A suppressor mutant capable of restoring high-temperature growth to HSF(1-583) cells was identified, harboring a disruption of the *SCH9* protein kinase gene, homologous to the protein kinase A and protein kinase B/Akt families of mammalian growth control kinases. Loss of Sch9 in HSF(1-583) cells derepresses Hsp90 signal transduction functions as demonstrated by restoration of transcriptional activity by the mammalian glucocorticoid receptor and the heme-dependent transcription factor Hap1, and by enhanced pheromone-dependent signaling through the Ste11 mitogen-activated protein kinase (MAPK). Moreover, Sch9-deficient cells with normal levels of Hsp90 chaperone complex components display hyperactivation of the pheromone response MAPK pathway in the absence of pheromone. These results demonstrate that the evolutionarily conserved function of the Hsp90 chaperone complex as a signal transduction facilitator is modulated by a growth regulatory kinase.

Keywords: chaperone/heat shock/Hsp90/protein kinase/signal transduction

Introduction

All cells counter the potentially deleterious effects of environmental stress via the coordinated synthesis of a battery of proteins called heat shock proteins (Hsps), which both protect the cell and facilitate rapid recovery from stress-induced cellular damage (Parsell and Lindquist, 1993). Many Hsps are also maintained at high levels in unstressed cells and are required for a number of cellular processes from protein biosynthesis, processing and transport to signal transduction (Craig *et al.*, 1994; Pratt, 1998). Hsp90, a ubiquitous and abundant Hsp essential for viability in eukaryotic cells (Borkovich *et al.*, 1989; Cutforth and Rubin, 1994), is an essential component of steroid hormone receptor heterocomplexes, where it plays both negative and positive roles: restraining transcriptional activation by the receptor in the absence of ligand, and potentiating receptor function upon hormone binding (for review, see Smith and Toft, 1993; Pratt and Toft, 1997). Hsp90 chaperone complex client proteins include transcription factors, protein kinases, reverse transcriptases and signaling enzymes (reviewed in Csermely *et al.*, 1998; Caplan, 1999). A key feature of many of these client proteins is the capacity to switch between on and off states depending on cellular input; Hsp90 is thought to stabilize these conformationally flexible molecules and poise them for subsequent activation (Toft, 1998). For example, the activity and levels of two cell cycle regulatory kinases, mammalian v-src expressed in yeast (Xu and Lindquist, 1993) and endogenous Wee1 in the yeast *Schizosaccharomyces pombe* (Aligue *et al.*, 1994), are strictly dependent on Hsp90 function. Given the emerging role of Hsp90 as a critical component of cell cycle regulation and signal transduction pathways, it would be surprising if the function of this central protein-folding machine was not itself regulated.

Hsp90 is found associated with a number of partner proteins in dynamic complexes, with distinct subunits binding to Hsp90–substrate heterocomplexes at various stages of the chaperoning/folding cycle (Chang and Lindquist, 1994; Pratt and Toft, 1997). Nearly all of the Hsp90-associated proteins are conserved in the yeast *Saccharomyces cerevisiae*, including the DnaK/DnaJ protein chaperone pair encoded by the *SSA/SSB* (Chang and Lindquist, 1994; Bohen, 1998) and *YDJ1* (Kimura *et al.*, 1995) genes, the p60 (Hop) ortholog *STI1* (Chang *et al.*, 1997), the Cyp-40 cyclophilin orthologs *CPR6* and *CPR7* (Duina *et al.*, 1996), *CDC37* (Kimura *et al.*, 1997) and the p23 ortholog *SBA1* (Bohen, 1998; Fang *et al.*, 1998). In addition, genetic and biochemical studies in yeast have led to the identification of new Hsp90 co-chaperones, such as Cns1, a protein with strong homology to Hop/Sti1 (Dolinski *et al.*, 1998; Marsh *et al.*, 1998; Nathan *et al.*, 1999), and Sse1, a member of the Hsp110 protein chaperone family (Liu *et al.*, 1999). The basal and stress-induced expression of Hsp90 and many of its partner proteins in eukaryotes is coordinated by the heat shock transcription factor (HSF). Yeast HSF has both N- and C-terminal transcriptional activation domains (Wu, 1995; Morano and Thiele, 1999), and deletion of either domain alone has little effect on cell growth at normal temperature but truncation of the C-terminal domain renders cells temperature-sensitive for growth at 37°C (Sorger, 1990; Morano *et al.*, 1999). This phenotype was recently characterized as a reversible arrest in the G_2/M phase of the cell cycle using the C-terminal truncation allele HSF(1-583) (Morano *et al.*, 1999). We have recently demonstrated that the temperature-sensitive phenotype of the HSF(1-583) mutant is due to deficient expression of Hsp90 and most of the heat-inducible Hsp90 chaperone complex genes (Liu *et al.*, 1999; Morano *et al.*, 1999). These findings are corroborated by another recently characterized HSF mutant

allele *hsf1-82*, which is specifically defective in expression of the two yeast Hsp90 genes *HSC82* and *HSP82*, and also exhibits cell cycle arrest at elevated temperatures (Zarzov *et al.*, 1997).

To uncover regulators of Hsp90 complex function in yeast, we took advantage of the temperature-sensitive phenotype of HSF(1-583) cells to select for mutants capable of restoring high-temperature growth. Inactivation of the *SCH9* gene, encoding a putative growth regulatory serine/threonine protein kinase, derepresses Hsp90 chaperone function as indicated by increased tolerance to the Hsp90 inhibitors geldanamycin and macbecin in HSF(1-583) cells, restoration of transcriptional activity by the mammalian glucocorticoid receptor and the heme-dependent transcription factor Hap1, and pheromone-dependent signaling through the Ste11 mitogen-activated protein kinase (MAPK). Moreover, Sch9 is required to restrain MAPK pathway activity in the absence of inducer in wild-type cells. These results demonstrate that the evolutionarily conserved function of the Hsp90 chaperone complex as a signal transduction facilitator is modulated by a growth regulatory kinase.

Results

Inactivation of the SCH9 protein kinase gene suppresses HSF(1-583) G_2/M cell cycle arrest

A genetic selection was undertaken to isolate mutants capable of reversing the temperature sensitivity of the HSF(1-583) strain to identify genes involved in regulation of Hsp90 function. One isolate identified by transposon mutagenesis, designated Tn3-13, was capable of growth at 37°C and exhibited a growth rate at 30°C approximately half that of both wild-type and HSF(1-583) cells (~108 versus 225 min per doubling) (Figure 1A).

HSF(1-583) cells grown at 37°C undergo cell cycle arrest in the G_2/M transition phase of the cell cycle. This arrest is manifested by the appearance of large-budded cells with a single nucleus (Morano *et al.*, 1999). Two models could be envisaged to explain the growth of strain Tn3-13 at 37°C: rapid recovery from cell cycle arrest or bypass of the G_2/M block altogether. To differentiate between these mechanisms, wild-type HSF, HSF(1-583) and suppressor Tn3-13 strains were grown to mid-logarithmic phase at 30°C and then shifted to 37°C for 6 h. As shown in Figure 1B, DAPI-stained HSF wild-type cells retained their ability to grow and segregate nuclei to daughter buds, while, as reported previously, HSF(1-583) cells exhibited a large-budded morphology and failed to properly orient or segregate nuclei. In contrast, strain Tn3-13 cells more closely resembled wild type, as shown by the presence of small buds with segregated nuclei. Microscopic analysis of Tn3-13 cells from earlier or later time points failed to detect G_2/M-arrested cells (data not shown). These data suggest that the Tn-13 suppressor mutant overcame the Hsp expression defect in HSF(1-583) cells leading to cell cycle arrest at 37°C.

Mating the Tn3-13 strain to an otherwise isogenic *MAT*α HSF(1-583) strain demonstrated that the HSF(1-583)/Tn3-13 diploid was unable to grow at 37°C, indicating that the mutation was recessive (data not shown). The diploid was sporulated and five representative four-spore tetrads were tested for growth at 30 and 37°C

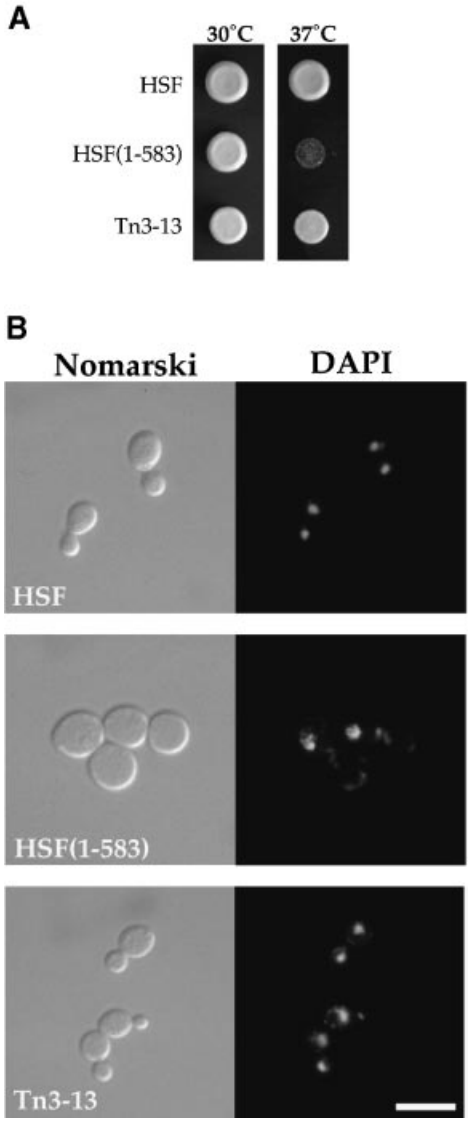

Fig. 1. The transposon-mutagenized suppressor strain Tn3-13 suppresses cell cycle arrest of HSF(1-583) cells at 37°C. The strains indicated were grown at 30°C to logarithmic phase and plated on solid media at both 30 and 37°C for 3 days in a dilution series as described in Materials and methods (**A**), or shifted to growth at 37°C for 6 h and stained with the nuclear stain DAPI (**B**). Bar, 10 μM.

(Figure 2A). In all five cases, growth at 37°C segregated in a 2:2 fashion, indicative of a mutation in a single nuclear locus. Additionally, all spore-derived colonies that grew at 37°C exhibited a slight slow-growth phenotype characteristic of suppressor strain Tn3-13 at 30°C. Genetic linkage between the suppressor locus and the transposon insertion was verified using PCR analysis with transposon cassette-specific primers (Figure 2A). The suppressor locus was isolated and identified, and the transposon was found to have integrated within the open reading frame (ORF) of the *SCH9* gene on chromosome VIII through sequence analysis using the *Saccharomyces* genome database (Cherry *et al.*, 1997).

SCH9 was first isolated as a multicopy suppressor of a temperature-sensitive allele of *CDC25*, encoding a guanine nucleotide exchange factor for yeast Ras proteins (Toda *et al.*, 1988). The *SCH9* gene is predicted to encode an 824 amino acid protein with significant homology in the

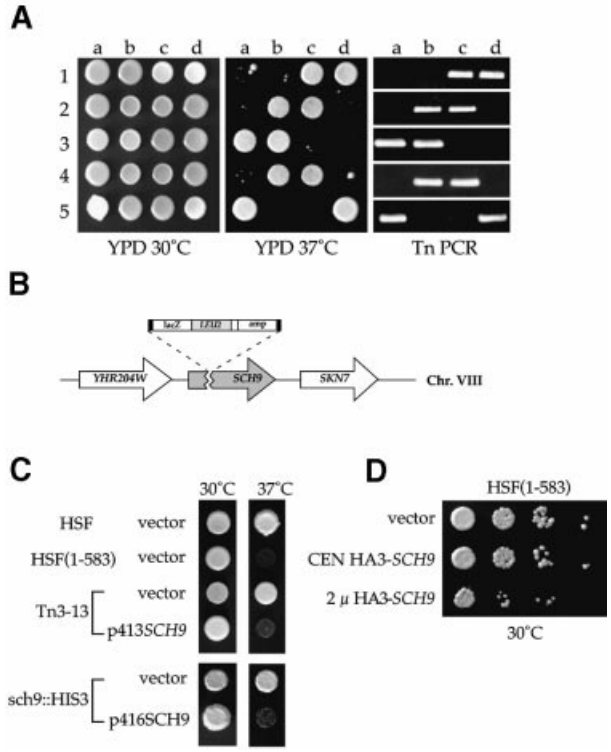

Fig. 2. Temperature-sensitive growth suppression in strain Tn3-13 is due to inactivation of the Sch9 protein kinase. (**A**) Five four-spore tetrads were isolated by tetrad dissection of an HSF(1-583)/Tn3-13 [HSF(1-583) *sch9::mTn*] diploid and spotted onto YPD plates for growth at the temperatures indicated for 3 days. PCR analysis of genomic DNA from each spore verified co-segregation of Tn3 with growth suppression. (**B**) A diagram illustrating the nature of *SCH9* gene inactivation by the transposon cassette. (**C**) The indicated strains were transformed with low-copy *SCH9*-expressing plasmids or the corresponding empty vectors alone, plated in a dilution series, and incubated at 30 and 37°C for 3 days. (**D**) HSF(1-583) cells transformed with the indicated plasmids (CEN, low-copy; 2μ, high-copy) were grown in plasmid-selective medium to logarithmic phase and plated in a dilution series at 30°C for 3 days.

C-terminal half of the protein to the serine/threonine class of protein kinases, specifically the AGC family whose members include protein kinases A, C and the recently characterized protein kinase B/Akt group (Coffer and Woodgett, 1991; Hunter, 1991; Jones *et al.*, 1991). The Sch9 N-terminus also contains a C2 phospholipid and calcium binding motif found in a number of signal transduction proteins (Nalefski and Falke, 1996). The transposon integration resulted in disruption of the locus after amino acid residue 359, which, if expressed, would be expected to generate a truncated form of Sch9 containing the putative C2 motif but entirely lacking the protein kinase domain (Figure 2B). Because genetic analysis of the diploid test cross demonstrated that the suppressor allele was recessive, and because the slow growth of the strain was consistent with growth of an *sch9::ADE8* null mutant generated in a previous report (Toda *et al.*, 1988), we reasoned that introduction of the wild-type locus on an episomal plasmid should reverse the high-temperature, growth-suppressor phenotype. A genomic fragment encompassing the *SCH9* gene was subcloned into a low-copy vector and transformed into HSF(1-583) cells. Whereas suppressor strain Tn3-13 carrying an empty vector allowed growth at 37°C, the same strain expressing *SCH9* (Tn3-13/p413SCH9) was unable to grow at the same temperature, effectively demonstrating that loss of *SCH9* gene expression was responsible for suppression (Figure 2C). This was further established by generating a traditional *SCH9* gene knockout mutant in the HSF(1-583) background, replacing residues 150–629 with the *HIS3* cassette (*sch9Δ::HIS3*). This strain also exhibited a slightly reduced growth rate at 30°C, which was reversed by ectopic expression of *SCH9* (data not shown), as well as *sch9*Δ-dependent suppression of HSF(1-583) temperature sensitivity, as shown in Figure 2C.

Because loss-of-function of the Sch9 protein kinase through gene inactivation suppressed the temperature-sensitive phenotype of HSF(1-583) cells, overexpression of the *SCH9* gene in the same genetic background might produce the opposite effect and debilitate cell growth under non-stress conditions. HSF(1-583) cells were transformed with low- (CEN) or high-copy (2μ) plasmids expressing a functional *SCH9* gene containing three copies of the hemagglutinin (HA) epitope immediately after the initiator ATG codon (HA_3-Sch9), or an empty vector alone, and were grown under plasmid-selective conditions, as shown in Figure 2D. Overexpression of *SCH9* has previously been demonstrated to cause heat shock sensitivity in wild-type cells, with no detectable effects at normal growth temperatures (Toda *et al.*, 1988; data not shown). In contrast, the growth of HSF(1-583) cells, as measured by colony-forming units on solid medium, was markedly reduced in the presence of high-copy versus single-copy HA_3-Sch9 (Figure 2D). Moreover, this effect was recapitulated in liquid culture as a slower growth rate coupled with growth cessation at approximately half the culture density of HSF(1-583) cells bearing low-copy HA_3-Sch9 (data not shown). Taken together, the findings that loss of Sch9 kinase restores high-temperature growth to HSF(1-583) cells and that Sch9 overproduction restricts viability of HSF(1-583) even under non-stress growth conditions support a role for Sch9 in negative regulation of HSF or an HSF-dependent function.

Point mutations predicted to abolish activity of the Sch9 protein kinase suppress HSF(1-583) growth arrest

Overexpressed Sch9 functionally substitutes for loss of all three of the catalytic subunits of protein kinase A (Toda *et al.*, 1988) and coupled with the resemblance of the carboxyl half of the Sch9 protein to serine/threonine protein kinases this strongly suggests that Sch9 functions as a protein kinase *in vivo*. To assess the potential role of Sch9 kinase activity in suppression of HSF(1-583) temperature sensitivity, point mutations predicted to inactivate the kinase were introduced within the putative kinase domain. Two specific residues critical for catalytic function and strictly conserved in other protein kinases were altered: the lysine residue at position 441 was substituted with alanine, and aspartic acid at position 556 was substituted with arginine as illustrated in Figure 3A. Mutations in these residues have been demonstrated to eliminate protein kinase function completely in other protein kinases without substantially altering the stability or expression of the proteins (Bryant and Parsons, 1984). Both mutations were introduced in the HA-epitope-tagged

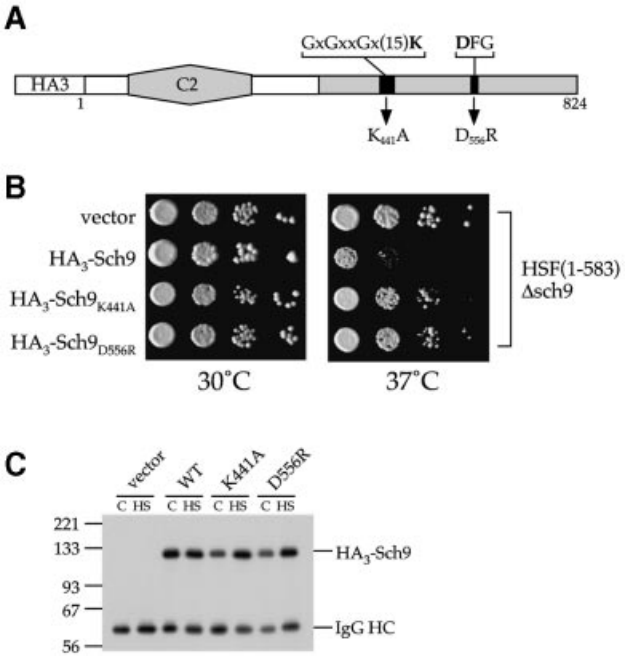

Fig. 3. Kinase-inactivating mutations in *SCH9* suppress the cell cycle block in HSF(1-583) cells. (**A**) Diagram depicting the domain architecture of functional HA-tagged Sch9 kinase and kinase-inactivating mutations. The putative C2 lipid/calcium binding domain stretches from position 182 to 356, and the serine/threonine protein kinase catalytic domain encompasses the C-terminal half of the protein, from residues 400 to 824. The conserved kinase subdomains mutagenized are shown above the protein and the specific alterations are shown below. (**B**) Strain KMY6 [HSF(1-583) *sch9*Δ*::HIS3*] was transformed with the indicated low-copy plasmids or the vector alone, and plated in a dilution series at 30 and 37°C for 3 days of growth. (**C**) Transformed strains shown in (B) were grown to logarithmic phase in plasmid-selective medium, and protein extracts were generated and subjected to immunoprecipitation. Immune complexes were resolved by SDS–PAGE and immunoblotted using 12CA5 antibody. The band labeled IgG HC is the mouse monoclonal heavy-chain immunoglobulin detected by the secondary antibody. We note that the tagged kinase migrates at ~125 kDa, despite a predicted mol. wt of 91 kDa.

SCH9 allele and expressed from a low-copy vector in strain KMY6, [HSF(1-583) *sch9*Δ*::HIS3*]. As shown in Figure 3B, KMY6 cells bearing the vector alone exhibited suppression of HSF(1-583) temperature-sensitive growth at 37°C. Introduction of wild-type HA_3-Sch9 into this strain reversed the *sch9*Δ slow-growth phenotype at 30°C, as demonstrated by small colony size and growth rates observed from liquid cultures (data not shown) and abrogated growth at 37°C. In contrast, expression of HA_3-$Sch9_{K441A}$ or HA_3-$Sch9_{D556R}$ resulted in slow-growing cells at 30°C and failed to reverse *sch9*Δ suppression of HSF(1-583) temperature sensitivity. The proteins produced from the mutant *SCH9* alleles were not compromised for stability at either growth temperature, as shown in Figure 3C. These data strongly support a protein kinase function for Sch9, and directly implicate this activity in genetic interactions with HSF or downstream targets of HSF.

Loss of Sch9 does not hyperactivate the HSF or Msn2/Msn4 stress response pathways

Given the genetic interactions between HSF(1-583) and *SCH9* observed, we tested whether Sch9 modulates the activity of HSF itself, first by examining whether the reduced levels of Hsp90 chaperone complex subunits caused by the HSF(1-583) mutation were elevated in the Tn3-13 suppressor strain. Wild-type HSF, HSF(1-583) and Tn3-13 cells were held at the control temperature (30°C) or incubated at 39°C for 1 h to induce the heat shock response. Protein extracts were immunoblotted with antiserum against the Hsp90 chaperone complex components Hsp90, Sti1 and Cpr6, all dependent on HSF for basal and heat-induced expression, and phosphoglycerate kinase (PGK) as a control, as shown in Figure 4A. As previously demonstrated (Liu *et al.*, 1999; Morano *et al.*, 1999), the HSF(1-583) strain exhibited diminished levels of all three Hsps, as quantitated for heat shock expression relative to wild-type cells in Figure 4B. These protein levels remained essentially unchanged in the Tn3-13 strain, suggesting that suppression of HSF(1-583) by loss of the *SCH9* kinase was not via restoration of chaperone complex synthesis in response to heat shock. Furthermore, we observed no changes in either the transient or sustained transcriptional activities previously ascribed to yeast HSF (data not shown) (Sorger, 1990). Together, these data argue against a direct effect of Sch9 on HSF function in activating the heat shock response. Consistent with these findings, we examined the effect of the *sch9*Δ mutation on wild-type HSF function, and again found no alteration in the transcriptional response (data not shown).

In yeast, a second transcriptional response system governed by the Msn2/4 proteins is responsive to a variety of stress signals including heat, oxidative stress, nutrient depletion and osmotic imbalance (Ruis and Schuller, 1995). This system has also been shown to contribute to the heat-induced expression of a number of Hsp genes also activated by HSF, including *HSP104*, *HSP26*, *HSP12* and *SSA3* (Treger *et al.*, 1998). To test whether *sch9*Δ suppression of HSF(1-583) temperature sensitivity might operate through this pathway, *MSN2* and *MSN4* were deleted in the Tn3-13 suppressor background. An analysis of the four possible allele combinations generated from a cross between strain Tn3-13 [HSF(1-583) *sch9*Δ] and an otherwise isogenic *msn2*Δ *msn4*Δ strain is shown in Figure 5A. Strains carrying either a single *msn* mutation or deletions in both *MSN2* and *MSN4* in addition to the HSF(1-583) and *sch9*Δ alleles grew as well at 37°C as the *MSN2/4* wild-type strain, indicating that *sch9*Δ suppression does not require an intact general stress response pathway. The loss of the respective Msn proteins was verified by immunoblot analysis, as shown in Figure 5B. Furthermore, no changes in HSF(1-583) levels were observed in these strains. Inactivation of the general stress response pathway was verified by RNA blot analysis of transcripts derived from the Msn-dependent catalase gene, *CTT1*, in response to a 30 min heat shock treatment as shown quantitatively in Figure 5C. Heat stress resulted in robust activation of *CTT1* transcription in the *MSN2 MSN4* background and also in the strain carrying a deletion in *MSN4* only, consistent with previous reports (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996). Loss of Msn2 alone or in combination with Msn4 drastically reduced *CTT1* gene activation, confirming disruption of this stress response mechanism. Additionally, all four strains were defective in heat-shock-inducible *CUP1* gene expression, as expected for the HSF(1-583) mutation.

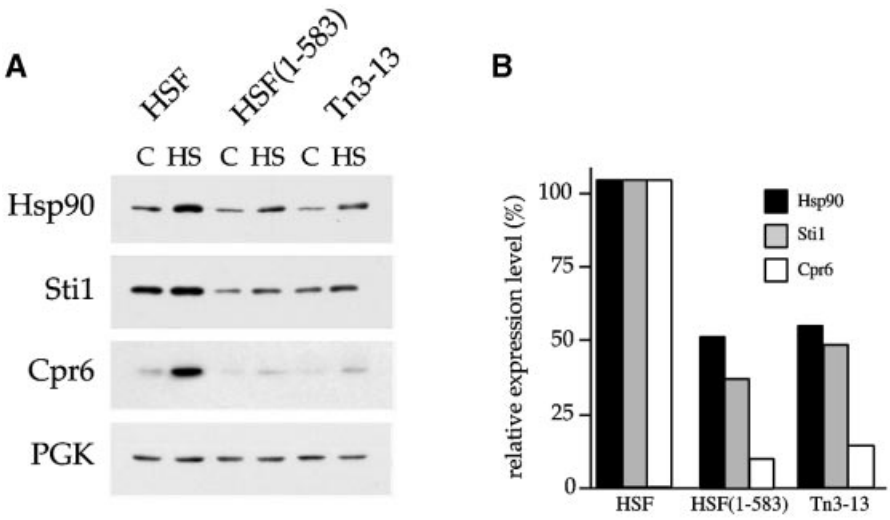

Fig. 4. The *sch9*Δ mutation does not restore the heat shock transcriptional response to HSF(1-583) cells. (**A**) The strains indicated were grown at 30°C to logarithmic phase, and the cultures were divided in half and heat shocked for 1 h at 39°C or maintained at 30°C. Protein extracts were made from these cells and resolved by SDS–PAGE, followed by immunoblotting to detect cellular levels of Hsp90, Sti1 and Cpr6, with PGK as a load control. (**B**) Quantitation of Hsp levels after heat shock shown in (A) via densitometric analysis of multiple scanned exposures.

Together, these experiments demonstrate that although HSF(1-583) cells are defective in certain aspects of the heat shock response, inactivation of the *SCH9* locus does not restore full transcriptional competency to HSF nor does this mutation override HSF regulation of chaperone synthesis by hyperactivation and recruitment of a parallel stress response pathway.

Sch9 modulates Hsp90 chaperone activity in HSF(1-583) cells

A major cellular defect observed in the HSF(1-583) mutant is impaired transcription of Hsps, resulting in lower steady-state- and heat-stress-induced levels of key components of the Hsp90 chaperone system such as Hsp90 itself, Sti1/Hop and the cyclophilin Cpr6. Consistent with the temperature-sensitive nature of the HSF(1-583) allele, it has been established that cells have a greater demand for Hsp90 chaperone complex activity at higher temperatures, where conformationally flexible proteins are likely to require stabilization (Borkovich *et al.*, 1989; Nathan *et al.*, 1997). Our results suggest that sufficient Hsp90 chaperone activity has been restored to allow growth at elevated temperatures despite no increase in the levels of Hsp90 chaperone complex components in HSF(1-583) *sch9*Δ cells. These data support a model whereby inactivation of Sch9 suppresses the temperature-sensitive phenotype of HSF(1-583) cells by enhancing Hsp90 chaperone complex activity. To test this hypothesis, we ascertained whether deletion of *SCH9* would ameliorate the demonstrated sensitivity of HSF(1-583) cells to geldanamycin and macbecin, two compounds that selectively bind to and inhibit the chaperone activity and signal transduction functions of Hsp90 (Whitesell *et al.*, 1994; Prodromou *et al.*, 1997; Stebbins *et al.*, 1997; Bohen, 1998). These compounds are cytotoxic to cells compromised for Hsp90 expression or function, such as the HSF(1-583) mutant (Morano *et al.*, 1999), or strains carrying deletions of Hsp90-associated co-chaperones such as Sti1, Sse1 (Liu *et al.*, 1999), Cns1 and Cpr6/7 (Dolinski *et al.*, 1998). HSF, HSF(1-583) and Tn3-13 cells were serially diluted and grown on rich medium containing 35 μM macbecin or geldanamycin, or no applied drug. As shown in Figure 6, the growth of wild-type cells was unaffected by the compounds, while HSF(1-583) cell growth was drastically inhibited by geldanamycin and to a lesser extent macbecin. Deletion of the *SCH9* gene in the Tn3-13 strain, in contrast, provided a significant level of tolerance to both drugs, relative to HSF(1-583). These data support a model where loss of Sch9 function leads to 'derepression' of Hsp90 chaperone complex activity, thereby countering the inhibitory effects of the drugs.

To test further the hypothesis that Sch9 functions as a regulator of Hsp90 activity, we assayed the activities of three known Hsp90 chaperone complex-dependent proteins *in vivo*, chosen to illustrate the broad spectrum of Hsp90 signal transduction function in yeast: mammalian glucocorticoid receptor (GR) (Picard *et al.*, 1990), the yeast heme-responsive Hap1 transcription factor (Zhang *et al.*, 1998), and the yeast MEK kinase Ste11 (Louvion *et al.*, 1998). The activity of all three proteins has been demonstrated to require the Hsp90 chaperone system in yeast through the use of Hsp90-deficient mutants harboring mutations in Hsp90 or some of its associated co-chaperones. In addition, we have demonstrated that HSF(1-583) cells display reduced GR activity consistent with the marked reduction in chaperone protein expression in this strain (Liu *et al.*, 1999; Morano *et al.*, 1999). Wild-type HSF, HSF(1-583) and Tn3-13 cells were treated with the synthetic hormone deoxycorticosterone (DOC) in ethanol, or ethanol alone (Figure 7A). An ~7-fold hormone-dependent activation of GR was obtained in wild-type cells, which was reduced by >70% in HSF(1-583) cells. Remarkably, DOC-induced GR function in strain Tn3-13 was increased 5-fold relative to HSF(1-583) cells and 1.5-fold relative to wild-type cells. Additionally, DOC-independent basal GR activity was also restored to wild-type levels in strain Tn3-13.

The Hap1 heme-responsive transcription factor was recently found to exist in a multiprotein complex *in vivo* containing Hsp90 and the co-chaperone Ydj1 (Zhang

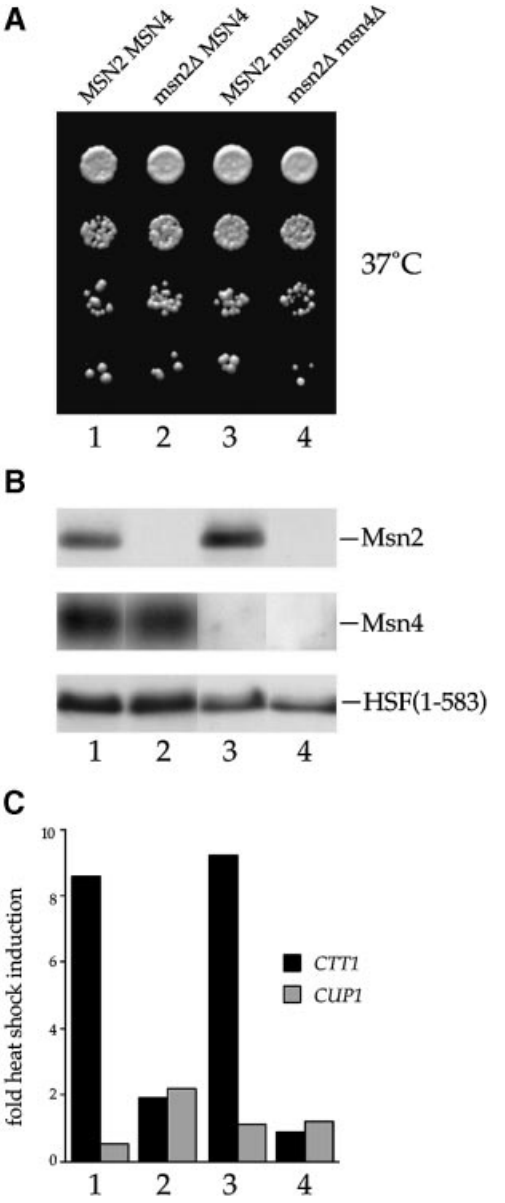

Fig. 5. The *sch9*Δ mutation does not deregulate the general stress response. (**A**) Haploid HSF(1-583) *sch9*Δ*::mTn* strains carrying all four allele combinations of *MSN2* and *MSN4* were generated using standard genetic techniques. These strains were grown at 30°C to logarithmic phase and plated on solid media at both 30 and 37°C for 3 days in a dilution series, demonstrating no loss of suppression ability. (**B**) Protein extracts from the strains indicated were resolved by SDS–PAGE and immunoblotted using polyclonal antisera recognizing Msn2, Msn4 or HSF. Numbered lanes correspond to strains in (A). (**C**) Heat shock gene expression from the HSF pathway (*CUP1*) or the general stress response pathway (*CTT1*) was measured using Northern blot analysis. Band intensities were obtained, normalized to *ACT1* for load control, and are presented relative to non-shocked cells as fold heat shock induction. Numbered lanes correspond to strains in (A).

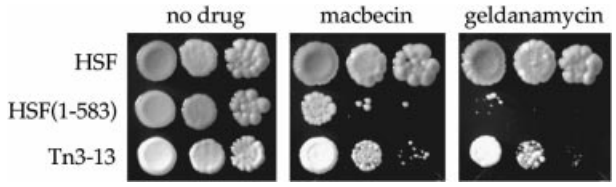

Fig. 6. Inactivation of Sch9 kinase restores resistance of HSF(1-583) to the Hsp90 inhibitors macbecin and geldanamycin. The strains indicated were grown at 30°C to logarithmic phase and plated in a dilution series on solid media containing no added drug, 35 μM macbecin or 35 μM geldanamycin, and grown at 30°C for 4 days.

et al., 1998). Strains expressing low levels of Hsp90 exhibit dramatically impaired transcription from a Hap1-dependent *CYC1-lacZ* reporter gene, establishing the dependence of Hap1 on Hsp90 chaperone complex activity. The *CYC1-lacZ* reporter was transformed into wild-type HSF, HSF(1-583) and Tn3-13 cells, and β-galactosidase activity was measured from exponentially growing cultures. As shown in Figure 7B, this reporter system mirrored the results obtained with GR; HSF(1-583) cells displayed an ~80% reduction in Hap1 activity, which was completely reversed in suppressor strain Tn3-13. Hap1 was in fact hyperactivated in this strain, producing β-galactosidase levels 3-fold greater than in wild-type cells.

It was recently demonstrated that Hsp90 is also required for full stability and function of the yeast Raf homolog Ste11 (Louvion *et al.*, 1998), which plays crucial roles in signal transduction MAPK cascades controlling cellular responses to pheromone and nutrient depletion (reviewed in Gustin *et al.*, 1998). Cells expressing low (~10% of wild type) Hsp90 levels or mutant yeast Hsp90 proteins exhibit many of the defects associated with Ste11-deficient cells, such as failure to arrest the cell cycle in G_1 phase in response to α-factor, and reduced transcriptional activation of a synthetic pheromone-responsive reporter gene by the transcription factor Ste12, which is downstream of Ste11 (Louvion *et al.*, 1998). To ascertain the status of Ste11-dependent pheromone signaling in HSF(1-583) and Tn3-13 cells, a synthetic reporter chimera consisting of *lacZ* driven by a minimal promoter containing three repeats of the pheromone response element was utilized (PRE-*lacZ*), as shown in Figure 7C (Hagen *et al.*, 1991). Wild-type cells exhibited robust α-factor-dependent induction of this reporter that was reduced by ~80% in the HSF(1-583) background. Analysis of pheromone induction in strain Tn3-13 revealed complete restoration of pheromone-dependent activation.

To investigate this role of Sch9 in Hsp90 regulation in wild-type cells, *HSF1 SCH9* and *HSF1 sch9*Δ cells (see Materials and methods) were transformed with the PRE-*lacZ* reporter plasmid and assayed for pheromone responsiveness. No reproducible increase in pheromone-dependent signaling was observed in *sch9*Δ cells compared with the wild type (~1000 U of β-galactosidase activity). However, as shown in Figure 7D, a dramatic 5-fold increase in activity of the reporter in the absence of exogenous pheromone was detected, similar to that observed for the Hsp90-compromised strain Tn3-13 in Figure 7C. The MAPK module governing pheromone response is known to exhibit a low degree of basal activity, thought to keep the system primed for rapid induction upon exposure to pheromone, and prevent inappropriate G_1 phase arrest that is known to be triggered by mating pheromone and required for mating to occur (Hagen *et al.*, 1991). This low basal activity has recently been demonstrated to rely upon Hsp90 as well (Louvion *et al.*, 1998). Taken together, these assays unambiguously demonstrate that inactivation of Sch9 in the HSF(1-583) strain restores Hsp90 chaperone function in diverse signal transduction pathways.

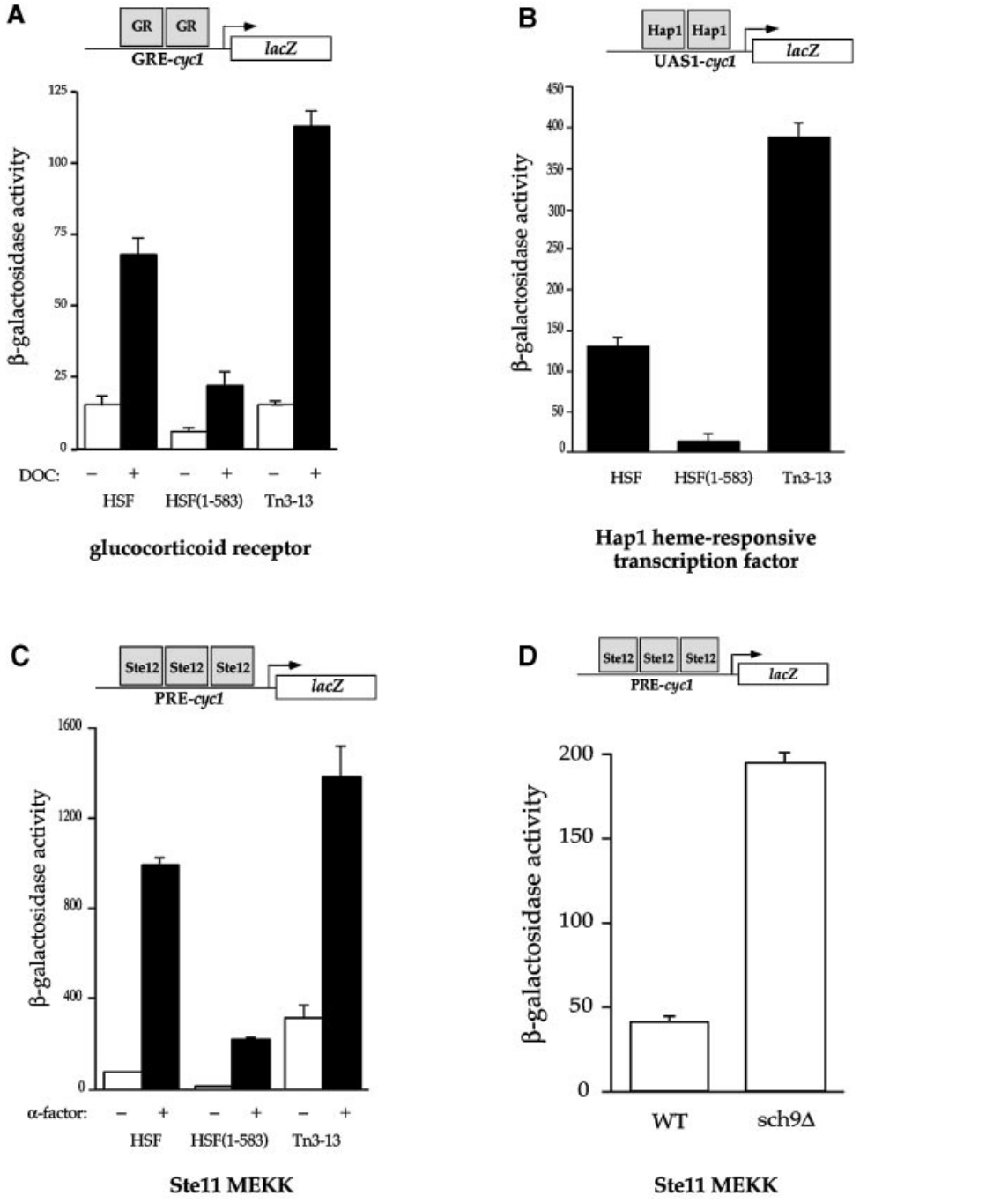

Fig. 7. Loss of Sch9 kinase restores Hsp90 chaperone function. (**A**) Glucocorticoid receptor. The strains indicated were grown to logarithmic phase at 30°C, treated with the synthetic hormone analog DOC (10 μM) in ethanol or ethanol alone for 1 h, and harvested for β-galactosidase assay. (**B**) Hap1 transcription factor. The strains indicated were grown to logarithmic phase at 30°C and harvested for β-galactosidase assay. (**C**) Ste11 kinase. The strains indicated were grown to logarithmic phase at 30°C, treated with 5 μM α-factor or not for 3 h, and harvested for β-galactosidase assay. (**D**) The strains indicated were grown to logarithmic phase in the absence of pheromone at 30°C and harvested for β-galactosidase assay. All reporter gene activities were calculated in Miller units as described in Materials and methods and represent three independent experiments.

Discussion

Modulation of Hsp90 chaperone complex function by a growth control kinase

In this report we present evidence for cellular regulation of the Hsp90 chaperone complex by a protein kinase previously implicated in cellular growth control. Given the powerful activation potential of the Hsp90 chaperone machine on cellular targets that regulate gene expression and cell proliferation, a mechanism for modulating this activity is not unexpected. The need for regulation is made more apparent by the fact that Hsp90 is one of the most abundant proteins in eukaryotic cells (Borkovich *et al.*, 1989). Association with Hsp90 restrains the activation of many client proteins until the appropriate conditions arise requiring their action. One example is HSF, which can acquire DNA binding or transcriptional activation potential in the absence of stress induction if Hsp90 is dissociated or inactivated via drug inhibition or if Hsp90 co-chaperones are absent (Ali *et al.*, 1998; Duina *et al.*, 1998;

Zou *et al.*, 1998). Additionally, removal of the Hsp90 binding domain from steroid receptor family members renders them constitutively active, and insensitive to both Hsp90 repression and ligand stimulation (Picard *et al.*, 1990). The role of Hsp90 in Ste11 signaling operates in a similar manner: the chaperone complex is required both for low basal activity of the pheromone pathway and for pheromone induction (Louvion *et al.*, 1998). The elevated basal activity of the MAPK pathway in the absence of pheromone, which we observed in *sch9*Δ cells, is an important demonstration of inappropriate activation of an Hsp90 client protein resulting from an increase in specific activity of the chaperone complex, a potential consequence of recruiting a powerful protein chaperone to facilitate signal transduction.

A common feature of many Hsp90 client proteins is the capacity to switch reversibly between distinct functional modes (on/off) in response to cellular input. Overlaying this regulation with modulation of Hsp90 chaperone activity would provide rheostatic control over client protein function, perhaps tied to overall cellular stress status indicators, such as nutrient availability or cytotoxic environmental conditions including thermal and oxidative stress. Indeed, although little is known about the cellular roles of the Sch9 kinase in yeast, it has been implicated in nitrogen sensing, potentially through a serpentine receptor-G-protein-linked pathway involving the heterotrimeric G-protein α subunit Gpa2 (Crauwels *et al.*, 1997; Xue *et al.*, 1998). This G-protein is also directly involved in glucose signaling (Colombo *et al.*, 1998). Together, these observations support a model whereby disparate cell growth and signaling pathways might be coupled to nutritional status and metabolic potential by recruitment of Hsp90, and subsequent regulation through Sch9 and perhaps other kinases and phosphatases.

Potential Sch9 regulatory mechanisms

How does Sch9 regulate Hsp90 complex function? Hsp90 is a phosphoprotein in higher eukaryotic cells (HeLa; Legagneux *et al.*, 1991), and its level of phosphorylation increases immediately following heat shock. The chaperone is probably multiply phosphorylated *in vivo*, as Hsp90 immunoprecipitated from NIH 3T3 cells contains both phosphoserine and phosphothreonine, as determined by phosphoaminoacid analysis (Mimnaugh *et al.*, 1995). Moreover, Hsp90 phosphorylation may have biological relevance, as treatment of cells with the serine phosphatase inhibitor okadaic acid both increased the levels of phosphorylation and coincided with the release of the associated substrate protein v-src (Mimnaugh *et al.*, 1995). Regulation of Hsp90 chaperone complex activity may also be accomplished through post-translational modification of the co-chaperones. For example, the FK506-binding protein FKBP52 is phosphorylated *in vivo* and *in vitro* by casein kinase II, and phosphorylated FKBP52 exhibits reduced binding to Hsp90 (Miyata *et al.*, 1997). Because subunits such as FKBP52 play important roles in steroid receptor maturation and trafficking (Czar *et al.*, 1995), modulation of co-chaperone association and dissociation may provide a means to regulate distinct Hsp90–substrate interactions. It was recently demonstrated that the weak ATPase activity of Hsp90 is potently inhibited upon addition of purified Sti1 to Hsp90, while the addition of purified Cpr6, which binds to the same tetratricopeptide repeat (TPR) acceptor domain on Hsp90, had no effect, suggesting that subunit association may provide a level of control in yeast (Prodromou *et al.*, 1999). Although these intriguing findings prompt speculation for similar protein kinase-mediated regulation of Hsp90 complex composition in yeast, it is not yet known whether any of these specific co-chaperones are phosphoproteins *in vivo*. Efforts are currently underway to determine precisely the phosphorylation status of Hsp90 chaperone complex components in wild-type and *sch9*Δ cells. It will be of considerable interest to elucidate the mechanism by which signal transduction mediated by the ubiquitous Hsp90 chaperone complex is regulated.

Materials and methods

Strains, plasmids, media and reagents

Strains NSY-A [*MAT***a** *ade2-1 trp1 can1-100 leu2-3,-112 his3-11,-15 ura3 hsf1*Δ*::LEU2* (pRS314-HSF)] and NSY-B {*MAT***a** *ade2-1 trp1 can1-100 leu2-3,-112 his3-11,-15 ura3 hsf1*Δ*::LEU2* [pRS314-HSF(1-583)]} were described previously and are referred to throughout the text as 'HSF' and 'HSF(1-583)' for simplicity (Morano *et al.*, 1999). Strain KMY3 {*MAT*α *ade2-1 trp1 can1-100 leu2-3,-112 his3-11,-15 ura3 hsf1*Δ*::LEU2* [pRS314-HSF(1-583)]} was created using the HO recombinase mating type-switching technique (Herskowitz and Jensen, 1991). Strain Tn3-13 (NSY-B *hsf1*Δ*::leu2::KANMX2 sch9::mTnLEU2*) was obtained via transposon mutagenesis. Strain KMY55 (relevant genotype *HSF1, sch9::mTnLEU2*) was constructed from Tn3-13 by plasmid shuffling, replacing pRS314-HSF(1-583) with pRS314-HSF. Strain KMY6 (NSY-B *sch9::HIS3*) was constructed by cloning both 5′ and 3′ gene fragments from the *SCH9* ORF into plasmid pGEM-3Z-HIS3, flanking a 1.76 kb *Bam*HI DNA fragment containing the *HIS3* gene. PCR was used to generate a 415 bp fragment encompassing nucleotides 41–456, and a 593 bp fragment containing nucleotides 1882–2475, numbered relative to the first nucleotide of the initiator codon. The template for this and all subsequent *SCH9* gene manipulations was plasmid pHV1SCH9, containing a genomic DNA fragment with the entire *SCH9* ORF and flanking sequences kindly provided by Dr Anne Vojtek. The PCR products were cloned into the plasmid pGEM-3Z-HIS3, and the gene disruption fragment (5′-*SCH9-HIS3*-3′ *SCH9*) was generated by restriction endonuclease digestion using unique flanking sites. Plasmids p413SCH9, p416SCH9 and p426HA_3-Sch9 were derived from plasmid pHV1SCH9. To construct an epitope-tagged version of *SCH9*, a *Not*I restriction endonuclease site was introduced after the second codon using the Chameleon site-directed mutagenesis kit (Stratagene, La Jolla, CA). A DNA fragment encoding three tandem repeats of the HA epitope was subcloned into the *Not*I site. The epitope-tagged gene was judged to be fully functional. The Chameleon kit was also used to individually change the lysine at residue 441 to alanine and the aspartic acid at residue 556 to arginine to create kinase-dead alleles. To reduce the chance of extraneous mutation, internal 0.8 kb *Msc*I–*Stu*I DNA fragments encompassing the mutated regions were subcloned after sequencing to verify that no additional mutations were introduced.

All DNA manipulations were performed using standard techniques. All yeast and molecular genetic techniques were carried out as described except where indicated (Kaiser *et al.*, 1994). Transposon mutagenesis and allele rescue were perfomed as described (Ross-Macdonald *et al.*, 1999) using reagents provided by Michael Snyder, Yale University. Yeast complete media, yeast extract/peptone/dextrose (YPD), and synthetic complete media (SC) were prepared as described (Kaiser *et al.*, 1994). Macbecin was kindly provided by the Drug Synthesis and Chemistry branch of the National Cancer Institute, and geldanamycin was a gift of Dr William Pratt (University of Michigan Medical School, Ann Arbor, MI).

Heat shock protein expression analysis, immunoprecipitation and antibodies

All cultures were grown at 30°C to mid-logarithmic phase prior to heat shock treatment. To amplify the heat shock response, cells were heat shocked at low density (OD_{600} <0.4) at 39°C. Analysis of Hsp protein levels was carried out as described (Morano *et al.*, 1999). The following antisera were used for immunodetection: monoclonal anti-HA antibody

12CA5 (Roche Molecular, Basel, Switzerland); anti-Hsp90 and anti-Cpr6 (kind gift of Dr Susan Lindquist, University of Chicago, IL); anti-Sti1 (kind gift of Dr David Toft, Mayo Graduate School, MN); anti-Msn2, Msn4 (kind gift of Dr Francisco Estruch, University of Valencia, Spain); anti-PGK (Molecular Probes, Eugene, OR).

In some cases, HA_3-Sch9 was immunoprecipitated prior to immunoblotting as follows. Cells were glass-bead lysed as described above, equalized for protein concentration and diluted with 1 ml of lysis buffer with 0.1% Triton X-100. Four microliters of 12CA5 antibody (corresponding to ~20 μg of monoclonal IgG, demonstrated to be in excess of antigen levels), 5 μl of 10 mg/ml bovine serum albumin and 25 μl of Pansorb (Calbiochem, San Diego, CA) suspension were added and the mixture was rocked for 2 h at 4°C. Immune complexes were washed four times in the same buffer and eluted into SDS–PAGE sample buffer.

For analysis of general stress response pathway function, Northern analysis was used to measure transcript levels of *CUP1*, which is not under control of this pathway, and *CTT1*, whose heat shock induction is HSF-independent, using radiolabeled probes. Gene expression is reported as fold induction relative to non-heat-shocked cells, normalized to internal *ACT1* levels.

Hsp90 chaperone assays

Glucocorticoid receptor. A GR reporter system was created by co-expressing rat GR from plasmid pRS413GPDGR and plasmid pYRP-GRElacZ carrying a *lacZ* reporter gene driven by glucocorticoid response elements (Morano *et al.*, 1999). Logarithmic phase cells were divided in half, and one culture was treated for 1 h with DOC dissolved in ethanol to a final concentration of 10 μM, while the other received ethanol alone. Cells were harvested by centrifugation and processed for β-galactosidase assay as described (Liu *et al.*, 1999).

Hap1 transcription factor. For assay of Hsp90 chaperoning of Hap1, the plasmid pAx (*CEN*, *URA3*) (kind gift of Dr Li Zhang, NYU Medical Center, NY), which contains the minimal UAS1/*CYC1-lacZ* reporter construct, was used. This reporter construct is responsive to cellular heme levels via transcriptional activation from the Hap1 transcription factor. In cells that are wild-type for heme biosynthesis, this reporter exhibits robust expression, which is nearly eliminated in strains deficient in Hsp90 function (Zhang *et al.*, 1998). Cells carrying pAx were grown to logarithmic phase in selective media and processed for β-galactosidase assay.

Ste11 kinase. To assay pheromone-dependent gene expression, a reporter plasmid was constructed containing three consensus binding sites for the Ste12 transcription factor driving the *lacZ* gene as previously described (Hagen *et al.*, 1991). Complementary oligonucleotides with three tandem repeats were synthesized, hybridized and cloned into the minimal *CYC1-lacZ* expression vector pCM64 (*CEN*, *URA3*) digested with *Bgl*I and *Xho*I to create plasmid pPRE-lacZ. Cells transformed with this plasmid were grown to logarithmic phase and the cultures were divided in half. α-factor (Sigma, St Louis, MO) in water was added to one culture to a final concentration of 5 μM, while a parallel culture was left untreated, and the cells were grown for an additional 3 h. Cells were then harvested and processed for β-galactosidase assay.

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