Bag1–Hsp70 mediates a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth

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Survival after stress requires the precise orchestration of cell-signalling events to ensure that biosynthetic processes are alerted and cell survival pathways are initiated. Here we show that Bag1, a co-chaperone for heat-shock protein 70 (Hsp70), coordinates signals for cell growth in response to cell stress, by downregulating the activity of Raf-1 kinase. Raf-1 and Hsp70 compete for binding to Bag1, such that Bag1 binds to and activates Raf-1, subsequently activating the downstream extracellular signal-related kinases (ERKs). When levels of Hsp70 are elevated after heat shock, or in cells conditionally overexpressing Hsp70, Bag1–Raf-1 is displaced by Bag1–Hsp70, and DNA synthesis is arrested. Mutants Bag1C204A and Bag1E208A, which cannot bind Hsp70, constitutively activate Raf-1/ERK kinases but are unaffected by Hsp70; consequently neither Bag1–Raf-1 nor DNA synthesis is negatively affected during heat shock. Likewise, mutants Hsp70F245S, Hsp70R262W and Hsp70L282R, which retain chaperone activity but do not bind to Bag1, fail to repress Bag1 activation of Raf-1/ERK kinase. We propose that Bag1 functions in the heat-shock response to coordinate cell growth signals and mitogenesis, and that Hsp70 functions as a sensor in stress signalling.

To survive the challenge of environmental and physiological stress, living systems require sensing mechanisms and strategies for making critical decisions regarding cell growth and death. Cell growth is governed by precise checkpoints; but which of the myriad of regulatory proteins communicate with components of the cell stress apparatus to signal a pause or cessation of cell growth? It would seem rational to propose that biosynthetic pathways are neither initiated nor continued under suboptimal growth conditions. Furthermore, the negative effects of heat shock and other forms of stress on cell growth and differentiation are well documented[1–5], and include teratogenic effects on mammalian embryogenesis, phenocopy mutations in *Drosophila*, arrest and synchrony of the cell cycle, and inhibitory effects on DNA, RNA and protein synthesis.

A decisive molecular event that occurs during cell stress is the nearly instantaneous induction of molecular chaperones—mainly the major heat-shock proteins, Hsp104, Hsp90, Hsp70, and small heat-shock proteins[6]. Of these, Hsp104, Hsp90 and Hsp70 are ATPases regulated by co-chaperones that function in protein translocation, regulation of cell signalling and protein refolding[7–11]. Among the Hsp70 co-chaperones are Hdj-1 (Hsp40), which couples nucleotide binding and hydrolysis to the release of the substrate in a folded state; Hip and Hop, which enhance refolding activity and function in the assembly of heteromeric chaperone complexes including Hsp90 and TPR-motif containing co-chaperones; and Bag1, which binds to the ATPase domain to stimulate nucleotide exchange yet inhibits protein refolding[11–16].

Bag1 was initially identified as an anti-apoptotic protein that interacts with Bcl-2, and was shown subsequently to associate with diverse signalling molecules including Bcl-X_L, human growth factor receptor, androgen and other steroid receptors, and Siah—a p53-inducible negative regulator of cell growth proteins[17–22]. Bag1 also interacts *in vitro* with the catalytic domain of the protein kinase Raf-1 to stimulate its activity[23]. Raf-1 integrates signals from extracellular factors, acting downstream of Ras to activate mitogen-activated kinase (MAP) kinase (MEK) and ERK, which phosphorylate yet more kinases and transcription factors involved in cell growth and differentiation[24]. As Raf-1 is also modulated by many other regu1ators including 14-3-3 proteins, Hsp90, Ras-independent protein kinase C (PKC) and tyrosine kinases, here we have investigated whether the interaction with Bag1 uncovers a conduit between cell growth and the stress response[24].

Results

Identification of the Raf-1- and Hsp70-binding domains in Bag1. To assess the molecular interactions of Bag1 with Hsp70 and Raf-1, we analysed a collection of Bag1 mutants (Fig. 1). Deletion mutants were generated as glutathione *S*-transferase (GST)–Bag1 fusions for *in vitro* protein–protein interaction assays, focusing on a region (residues 90–219) of Bag1 that is sufficient for both *in vivo* and *in vitro* binding to both Raf-1 and Hsp70 (refs 14, 15, 23). Interaction was shown using two complementary binding assays of GST–Bag1-affinity chromatography and isothermal titration calorimetry (Fig. 1b; and data not shown)[15]. Residues 139–210 of Bag1 were necessary for Hsp70 binding; for example, wild-type Bag1 and deletion mutants retaining residues 90–210 and 139–210 associated with Hsp70 with a dissociation constant (K_d) of 20–45 nM using isothermal calorimetry, whereas other mutants (90–190, 90–200, 161–210) exhibited negligible interaction (Fig. 1b).

We also analysed the activity of each Bag1 mutant using an Hsp70-dependent protein refolding assay (Fig. 1c). Whereas wild-type Bag1, at a 1:1 stoichiometry with Hsp70, inhibits completely the Hsp70-dependent refolding of denatured β-galactosidase, Bag1 mutants (90–200, 161–210) that were deficient in their interaction with Hsp70 had no effect on the refolding of the unfolded substrate (Fig. 1c). As the carboxyl-terminal boundary of Bag1 required for interaction with Hsp70 corresponds to residues 200–210 (Fig. 1b, lanes 3–6), we constructed mutations of all 11 residues in this boundary to identify point mutants defective in Hsp70 interaction. Of these, mutants Bag1C204A and Bag1E208A showed a complete loss of binding to Hsp70 and did not inhibit the refolding activity

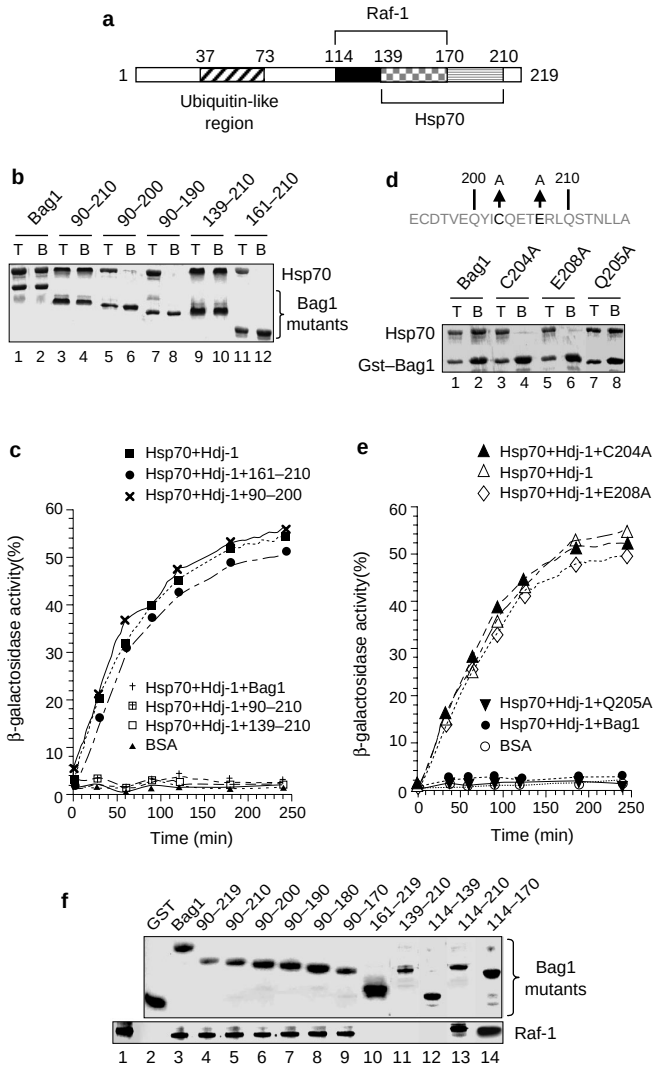

Figure 1 **Identification of the Bag1 regions required for interaction with Hsp70 and Raf-1. a**, Diagram of Bag1 (219 residues) showing the Raf-1- and Hsp70-binding motifs (black and grey boxes, respectively) and the ubiquitin-like region (striped box). Numbers indicate the boundaries of these motifs. **b**, *In vitro* binding assays of wild-type and mutant Bag1 with Hsp70. Intact or truncated GST–Bag1 protein and wild-type Hsp70 were incubated (T, total proteins; lanes 1, 3, 5, 7, 9, 11), the samples split, and protein complexes precipitated with glutathione–sepharose beads (B, bound proteins; lanes 2, 4, 6, 8, 10, 12). The proteins were analysed by SDS–PAGE and visualized with Coomassie blue stain. **c**, Hsp70-dependent refolding assay in the presence of wild-type and mutant Bag1 proteins. The refolding assay containing denatured β-galactosidase (β-gal, 3.2 nM, final concentration) was incubated with 1.6 μM Hsp70 and 3.2 μM Hdj-1 together with 3.2 μM wild-type, mutant Bag1 or bovine serum albumin (BSA). β-gal activity was measured using ortho-nitro-phenyl-galactoside (ONPG) as the substrate at intervals of 30, 60, 120, 180 or 240 min. **d**, Biochemical properties of Bag1 point mutations between residues 200 and 210. Top, sequence of Bag1 between residues 195 and 217. Alanine-scanning mutants of residues 200–210 were constructed and assayed for *in vitro* binding to Hsp70. Bottom, results of binding assays between Hsp70 and wild-type GST–Bag1 protein or point mutants Bag1C204A, Bag1Q205A or Bag1E208A. **e**, Hsp70-mediated refolding assay in the presence of 3.2 μM wild-type Bag1, Bag1C204A, Bag1Q205A, Bag1E208A or BSA. **f**, Identification of the Raf-1-binding region on Bag1. Raf-1 was transcribed and translated *in vitro* using a reticulocyte lysate system in the presence of [^{35}S]methionine. Wild-type GST–Bag1 (2 μM) and deletion mutants (2 μM) with the indicated boundaries were added to the lysate and Raf-1 precipitated using glutathione–sepharose beads. The results were visualized by autoradiography.

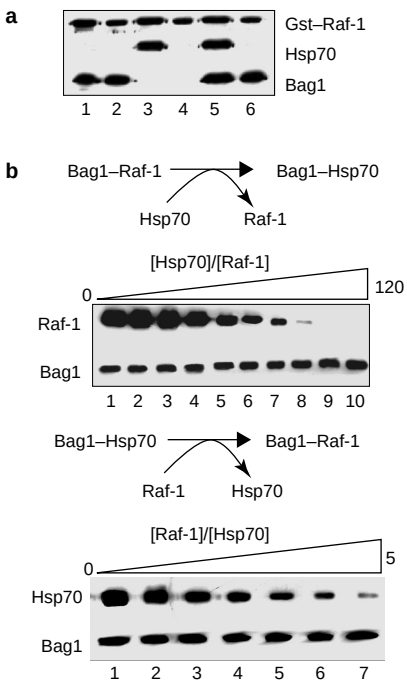

Figure 2 **Raf-1 and Hsp70 compete for binding to Bag1. a**, Bag1 forms a heterodimeric complex either with Hsp70 or Raf-1. GST–Raf-1 (0.2 μM) was incubated with Bag1 (0.2 μM, lanes 1, 2), Hsp70 (0.2 μM, lanes 3, 4), or with both Bag1 and Hsp70 (lanes 5, 6). Proteins bound to GST–Raf-1 were identified by precipitation with glutathione–sepharose beads (lanes 2, 4, 6) to detect Raf-1-associated proteins. The samples were separated by SDS–PAGE and analysed by western blot using anti-Hsp70, anti-Bag1 and anti-Raf-1 monoclonal antibodies. **b**, Bag1–Raf-1 competed by Hsp70, and Bag1–Hsp70 competed by Raf-1. Top, His–Bag1 (0.1 μM) and GST–Raf-1 (0.1 μM) were incubated for 30 min and then 0, 0.1, 0.5, 1, 2, 4, 6, 8, 10 or 12 μM Hsp70 was added. After a 30-min incubation, His–Bag1 protein complexes were precipitated with Ni^{2+}-agarose beads and detected as described in Fig. 2a. Bottom, His–Bag1 (0.2 μM) and Hsp70 (0.2 μM) were incubated for 30 min, and then 0, 0.1, 0.2, 1, 2, 3, 4 or 5 μM GST–Raf-1 was added and analysed as above.

of Hsp70 (Fig. 1d, lanes 3–6, and Fig. 1e). Mutating other residues between 200 and 210 (other than C204 or E208) retained both the Hsp70 interaction and repression of chaperone activity; for example, Bag1Q205A was indistinguishable from wild-type Bag1 (Fig. 1d lanes 7, 8, and Fig. 1e).

To identify the sequences in Bag1 required for association with Raf-1, we used a similar *in vitro* binding assay with Raf-1 expressed in reticulocyte lysates[23]. Radiolabelled Raf-1 was incubated with GST fusion proteins containing wild-type or mutant Bag1 and precipitated with glutathione–sepharose beads. This analysis identified residues 114–170 of Bag1 as necessary for interaction with Raf-1 (Fig. 1f, lanes 10–14).

Raf-1–Bag1 complexes are displaced by Hsp70. As the deletion analysis revealed that Bag1 has overlapping binding domains for Raf-1 and Hsp70 (Fig. 1a), we examined whether Hsp70 and Raf-1 compete for association with Bag1. Whereas GST–Raf-1 formed stable complexes with Bag1, no interaction was detected with Hsp70; furthermore, when all three proteins were co-incubated, heteromeric complexes containing only Raf-1 and Bag1 were observed (Fig. 2a). These results suggest that Raf-1 has a tighter interaction with Bag1 than has Hsp70. To test this hypothesis, we challenged pre-formed Bag1–GST–Raf-1 complexes *in vitro* with increasing concentrations of Hsp70 to a level of 120-fold excess, and measured the levels of Raf-1 remaining in the complex (Fig.

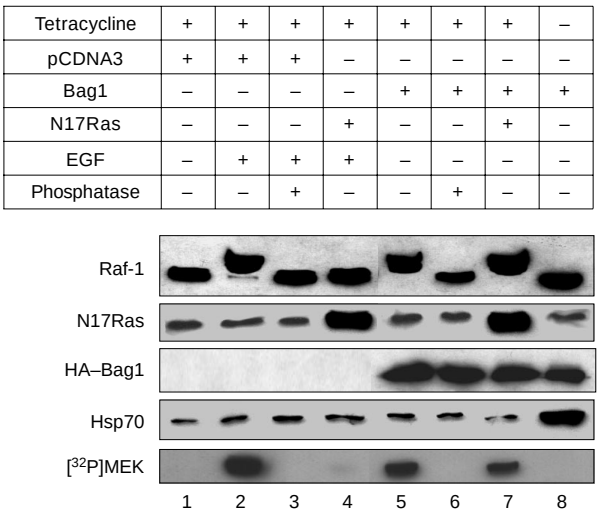

Figure 3 **Bag1 activates Raf-1 independent of Ras.** OT-tet (Hsp70) cells were transfected with pcDNA3/HA–Bag1 (wild type), pcDNA3-N17Ras or pcDNA3 in the presence (lane 9) or absence (lanes 1–8) of tetracycline (3 μM final concentration) to express Hsp70 conditionally. At 48 h after transfection, Raf-1 was immunoprecipitated from OT-tet (Hsp70) cell lysates, incubated with (lanes 3, 6) or without (lanes 1, 2, 4, 5, 7, 8) potato acid-phosphatase, and analysed by SDS–PAGE and western blot using anti-Raf-1 antibody. The activity of immunoprecipitated Raf-1 was assayed using kinase-defective GST–MEK as a substrate. The level of HA–Bag1, N17Ras or Hsp70 was determined by western blot using anti-HA, anti-Ras or anti-Hsp70 monoclonal antibodies, respectively.

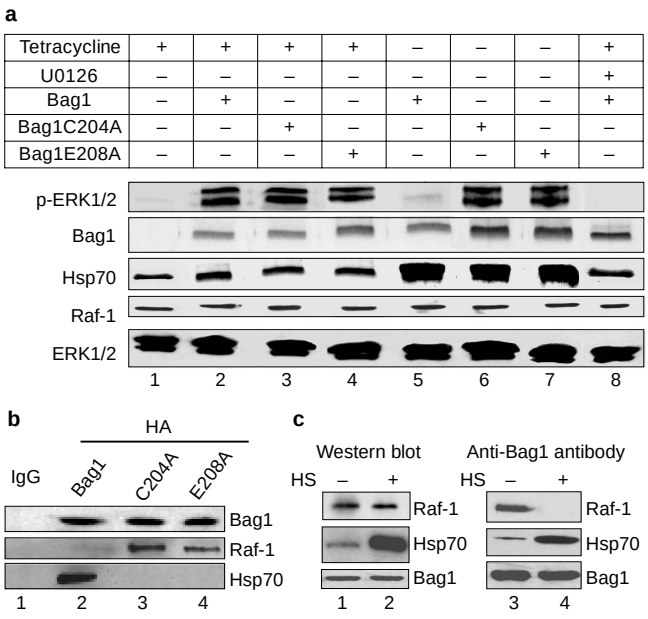

Figure 4 ***In vivo* effect of Bag1 on the Raf-1/ERK kinase pathway, and negative effect of Hsp70 on Bag1 activity. a**, Bag1 activates the Raf-1/ERK kinase pathway and is suppressed by Hsp70 overexpression. OT-tet (Hsp70) cells were transfected with pcDNA3/HA–Bag1 (wild type), pcDNA3/HA–Bag1C204A or pcDNA3/HA–Bag1E208A in the presence (lanes 2–4) or absence (lanes 5–7) of tetracycline (3 μM final concentration). Cells transfected with pcDNA3/HA–Bag1 were also incubated with U0126 (10 μM, final concentration), a specific inhibitor of MEK kinase (lane 8). At 24 h after transfection, OT-tet (Hsp70) cell lysates were analysed for the phosphorylation of ERK1/ERK2 by western blot using ERK1/2 phospho-specific monoclonal antibody, or for total levels of ERK1/2 using polyclonal antibodies. The level of Raf-1, HA–Bag1, HA–Bag1C204A, HA–Bag1E208A or Hsp70 was detected by western blot using anti-Raf-1, anti-HA or anti-Hsp70 monoclonal antibodies. Fifty micrograms of protein extract was loaded in each lane. **b**, Immunoprecipitation of Bag1-associated Hsp70 and Raf-1. The same extracts as in **a** (lanes 5–7) were immunoprecipitated with an anti-HA monoclonal antibody (lanes 2–4) or with an anti-β-gal monoclonal antibody (lane 1). Immunoprecipitates were separated by SDS–PAGE and examined by western blot for Bag1, Raf-1 or Hsp70 as indicated. **c**, *In vivo* analysis of Bag1 complexes in control and heat-shocked cells. OT23 cells were heat shocked for 1 h at 43 °C and the levels of Raf-1, Hsp70 and Bag1 were compared to those in control cells (lanes 1, 2). Lysates were immunoprecipitated using anti-Bag1 antibody (lanes 3, 4), and the levels of Raf-1, Hsp70 and Bag1 were determined by western blot.

2b). A 44-fold higher concentration of Hsp70 was required to compete 50% of Raf-1 from its complex with Bag1. We also carried out the reciprocal experiment in which Bag1–Hsp70 complexes were formed and challenged with Raf-1; these experiments showed that a 2.4-fold increase in Raf-1 was sufficient to displace Hsp70 (Fig. 2b). As Bag1–Hsp70 interactions have a K_d of 20–30 nM, these results indicate that the Bag1–Raf-1 complex must be substantially tighter, and that high concentrations of Hsp70 are required to displace Bag1–Raf-1 complexes.

Raf-1 activation by Bag1 is independent of Ras and is repressed by Hsp70. Bag1 interacts *in vitro* with the catalytic domain of Raf-1 and activates Raf-1 kinase activity[23]. To assess whether Bag1 interacts directly with Raf-1 kinase *in vivo* to stimulate downstream events, we examined activation and phosphorylation of Raf-1, ERK1 and ERK2 in cells transiently expressing epitope-tagged haemagglutinin A (HA)–Bag1 in an OT-tet cell line that conditionally expresses Hsp70 (OT-tet Hsp70 cells) under the control of a tetracycline-regulated promoter. In unstimulated cells under control conditions in which Hsp70 expression is not elevated, Raf-1 is inactive, as shown by its faster electrophoretic mobility on SDS–PAGE and by its inability when immunoprecipitated to phosphorylate *in vitro* kinase-defective MEK (Fig. 3, lane 1). On stimulation with epidermal growth factor (EGF), Raf-1 activity was induced as indicated by these two criteria (Fig. 3, lane 2); furthermore, pre-treatment of the immunoprecipitated Raf-1 with potato acid-phosphatase eliminated both activities (Fig. 3, lane 3).

We next examined Raf-1 activity in cells overexpressing Bag1 and observed that the altered electrophoretic mobility of Raf-1, whether induced by Bag1 or EGF, is due to hyperphosphorylation of Raf-1, which leads to phosphorylation of MEK (Fig. 3, lanes 5, 6). This suggests that the effects of Bag1 and EGF may be mediated by common pathways. To address this, we used a dominant-negative Ras mutant (N17Ras)[25] that blocks activation of Raf-1 in EGF-treated cells (Fig. 3, lane 4). In cells co-expressing both N17Ras and Bag1, however, Raf-1 was hyperphosphorylated (Fig. 3, lane 7), which shows that Bag1 and Ras influence Raf-1 independently. On overexpressing Hsp70 to concentrations induced by heat shock, the Bag1-mediated activation of Raf-1 was blocked completely (Fig. 3, lane 8). These results indicate that the activation of Raf-1 by Bag1 is negatively regulated by Hsp70, and independent of the Ras signalling pathway.

Repression of Raf-1/ERK activity by Hsp70 requires formation of Bag1–Hsp70 complexes. To establish the relationship between Bag1 and Hsp70 as regulators of Raf-1, we examined activation of the downstream ERK kinases after expression of Bag1 (Fig. 4a, lanes 1 and 2) and in the presence of the MEK1 inhibitor, U0126 (Fig. 4a, lane 8). As the levels of Hsp70 increase within 4–8 h after exposure of cells to stress, we also determined whether the negative effects of Hsp70 on Raf-1 were also reflected in activation of ERK kinases. We used a tetracycline-regulated cell line to increase the levels of Hsp70 4–6-fold, thus mimicking the levels of Hsp70 induced by heat shock without the complicating and pleiotropic effects of cell stress (Fig. 4A, lanes 5–7). Under these conditions, the positive effects of Bag1 on Raf-1 were repressed nearly completely

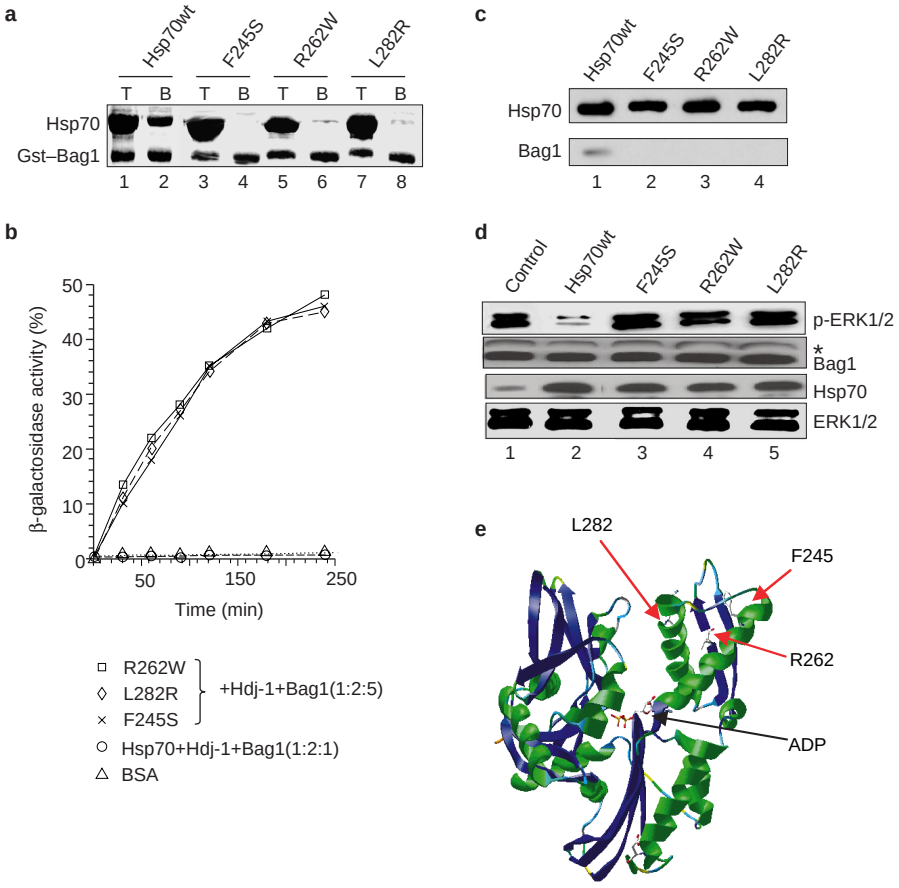

Figure 5 **Characterization of Hsp70 mutants defective in Bag1 interaction.** **a**, *In vitro* binding assays of wild-type and point mutant Hsp70 proteins with Bag1. An interaction between point mutants or wild-type Hsp70 (5 μM) and GST–Bag1 (1 μM) was determined as in Fig. 1b. **b**, Hsp70-dependent protein refolding activity of wild-type (wt) and point mutant (F245S, R262W, L282R) Hsp70, and BSA was performed as in Fig. 1c. **c**, *In vivo* association of wild-type and point mutant Hsp70 with Bag1. OT23 cells were transfected with pcDNA/Hsp70, pcDNA/Hsp70F245S, pcDNA/Hsp70R262W or pcDNA/Hsp70L282R (lanes 1–4). Cell lysates were immunoprecipitated with an anti-Hsp70 monoclonal antibody (C92) that specifically recognizes human Hsp70. Immunoprecipitates were analysed for Bag1 after western blot using an anti-Bag1 monoclonal antibody. **d**, Effects of wild-type and mutant Hsp70 on Bag1-dependent activation of Raf-1/ERK kinase activity. OT-tet (Bag1) cells were transfected with pcDNA/Hsp70, pcDNA/Hsp70F245S, pcDNA/Hsp70R262W or pcDNA/Hsp70L282R in the absence of tetracycline, and the levels of phosphorylated ERK1/2 (p-ERK1/2), Bag1, Hsp70 and total ERK1/2 were detected as in Fig. 3a. Asterisk corresponds to endogenous Bag1. **e**, Ribbon diagram showing position of point mutations in the structure of the human Hsp70 ATPase domain. Two views of Hsp70 are shown. Arrows indicate the position of residues F245, R262 and L282, and the location of ADP in the nucleotide cleft.

and the ERK kinases were not activated (Fig. 4a, lanes 2 and 5). This is in contrast to the activity of the Bag1 mutants Bag1C204A and Bag1E208A, which activated the Raf-1 signal transduction pathway (Fig. 4a, lanes 3, 4) and were not repressed by elevated levels of Hsp70 (Fig. 4a, lanes 6, 7).

What is the basis of the inhibitory effects of Hsp70 on the phosphorylation of ERK1 and ERK2? Under normal conditions of cell growth, wild-type Bag1 would be expected to associate with Raf-1. But would Raf-1 be displaced with the appearance of Bag1–Hsp70 complexes as the levels of Hsp70 increase? To address this, we examined the *in vivo* levels of Raf-1 or Hsp70 that associate with wild-type or mutant Bag1 proteins. Immunoprecipitation of HA–Bag1 from cells overexpressing Hsp70, followed by western blot analysis with antibodies to either Hsp70 or Raf-1, showed that wild-type Bag1 was associated with Hsp70 and not Raf-1 (Fig. 4b, lane 2), whereas mutants HA–Bag1C204A or HA–Bag1E208A were associated with Raf-1 with no detectable interaction with Hsp70 (Fig. 4b, lanes 3, 4).

As these results predict that Bag1 would not be associated with Raf-1 under conditions of cell stress, we next examined Bag1 complexes from extracts of control and heat-shocked cells by using immunoprecipitation with anti-Bag1 antibodies followed by western blot analysis with anti-Raf-1 and anti-Hsp70 antibodies (Fig. 4c). In control growing cells, Bag1 was detected in complexes with Raf-1 and Hsp70 (Fig. 4c, lane 3), whereas after heat shock Raf-1 was not detected and increased levels of Hsp70 were associated with Bag1 (Fig. 4c, lane 4). These results indicate that exposure of cells to stress alters the composition of Bag1 protein complexes, such that Bag1–Raf-1 interactions, detected in extracts from control cells, are displaced by Bag1–Hsp70 complexes. These results are consistent with the evidence from *in vitro* competition showing that high levels of Hsp70 displaced Raf-1 from its association with Bag1.

Point mutants of Hsp70 that cannot negatively regulate Bag1–Raf-1. To establish that Hsp70 functions directly as a negative regulator of Bag1 on Raf-1 kinase, we isolated point mutants in Hsp70 deficient for interaction with Bag1 (Fig. 5a) and assessed their effects *in vivo*. Among the various mutations identified, the mutants Hsp70F245S, Hsp70R262W and Hsp70L282R, in which the mutations lie within the Hsp70 ATPase domain IIB region (Fig. 5e), showed wild-type chaperone activity in a Hdj-1- and ATP-dependent protein refolding assay and complex formation with the co-chaperone Hip (Fig.

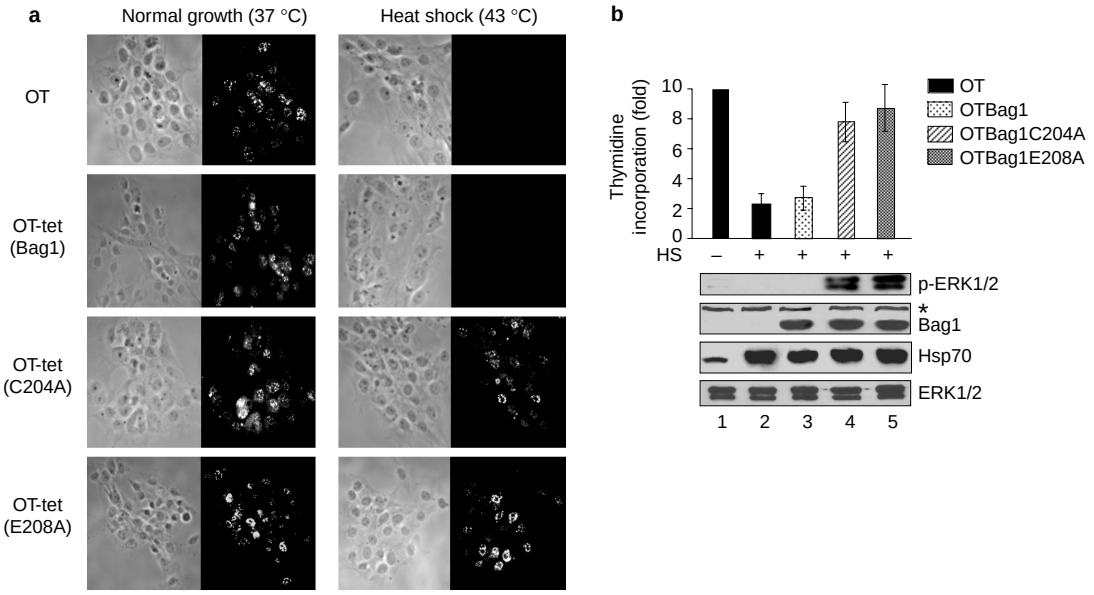

Figure 6 **Expression of Bag1 mutants defective in Hsp70 binding interferes with the arrest of DNA synthesis by heat shock. a**, Effects of heat shock on DNA synthesis in cells expressing wild-type or mutant Bag1 proteins. Wild-type (OT23 and OT-tet (Bag1)) and mutant (OT-tet (Bag1C204A) and OT-tet (Bag1E208A)) Bag1 proteins were expressed by removing tetracycline. Cells were synchronized, exposed to heat shock (43 °C), allowed to recover at 37 °C followed by addition of BrdU for 90 min, and detected using mouse anti-BrdU (Sigma) and FITC goat anti-mouse secondary antibody (Sigma). The same population of cells are shown visualized by both phase contrast and immunofluorescence. **b**, Populational analysis of the effects of heat shock on DNA synthesis in OT cells expressing wild-type or mutant Bag1. OT23, OT-tet (Bag1), OT-tet (Bag1C204A) and OT-tet (Bag1E208A) cells were grown in the absence of tetracycline for 24 h and then heat shocked at 43 °C for 1 h. The cells were incubated at 37 °C for 2 h followed by the addition of [^{3}H]thymidine for 2 h. The level of DNA synthesis was measured by TCA precipitation and scintillation counting. Extracts from each population of cells were analysed for p-ERK1/2, Bag1, Hsp70 and total ERK1/2 by western blot as in Fig. 3a. Asterisk indicates endogenous Bag1. Data are the mean ± s.e. of three independent experiments.

5b; and data not shown). However, none of these Hsp70 mutants interacted either *in vitro* or *in vivo* with Bag1 (Fig. 5a, lanes 3–8, and Fig. 5c, lanes 2–4).

We next addressed whether these Hsp70 mutants negatively regulated Bag1. We transiently co-expressed wild-type or mutant Hsp70 proteins, and assessed the activity of ERK kinase in cells overexpressing wild-type Bag1. Whereas ERK kinase activity was repressed when the levels of wild-type Hsp70 were increased (Fig. 5d, lanes 1, 2), the activity did not alter in cells overexpressing all three Hsp70 mutants defective in Bag1 binding (Fig. 5d, lanes 2–5). These results establish that Hsp70 regulates activity of the Raf-1/ERK kinase pathway through its direct interaction with Bag1.

Heat-shock induced arrest of DNA synthesis is dependent on Bag1–Hsp70 interaction. DNA synthesis is one of the downstream consequences of the Raf-1 signal transduction pathway. The induction of Hsp70 during and after heat shock, and the associated effects on DNA synthesis might be due to the sequestration of Bag1 by elevated levels of Hsp70. We therefore directly visualized DNA synthesis in populations of growing cells by 5-bromo-2′–deoxyuridine (BrdU) incorporation. Nearly all control and wild-type Bag1-expressing growing cells were actively replicating DNA at 37 °C, but there was a nearly complete arrest of DNA synthesis after heat shock at 43 °C (Fig. 6a, OT and OT-tet (Bag1)). In contrast, cells conditionally overexpressing the Hsp70-binding-deficient mutants Bag1C204A or Bag1E208A continued DNA synthesis after heat shock and were repressed from 20–40% (Fig. 6a, OT-tet (Bag1C204A) and OT-tet (Bag1E208A)).

These experiments were corroborated by the direct measure of [^{3}H]thymidine incorporation in tetracycline-regulated wild-type or mutant Bag1 cell lines transiently expressing Hsp70 (Fig. 6b). Heat shock alone inhibited DNA synthesis fivefold in the presence of wild-type Bag1 (Fig. 6b, lanes 1–3). In contrast, in cells expressing either the Bag1C204A or the Bag1E208A mutant, DNA synthesis was inhibited only slightly (10–20%) by heat shock in cells expressing equivalent levels of mutant Bag1 (Fig. 6b, lanes 4, 5).

Discussion

Our results indicate that Hsp70 has a previously unreported role as a direct negative regulator of Bag1, and consequently as an indirect regulator of downstream effectors such as Raf-1. This observation provides a molecular framework to begin to understand the negative effects of stress on cell growth and development, and offers an explanation for reports that overexpression of Hsp70 alone has negative effects on cell growth[26–28]. The properties of Bag1 as a positive regulator of DNA synthesis and as a checkpoint for cell stress are intriguing. We have shown that a function of Bag1 is to coordinate the cellular signals that determine the growth state of the cell with its environmental and physiological state; and we predict that, beyond our observations here, there may be a number of other points of regulatory crosstalk to ensure that various components of the cell growth biosynthetic apparatus are in communication with heat-shock proteins or other, unidentified, stress signalling molecules.

The role proposed for Hsp70 in cell signalling shares features in common with the role of Hsp90 as a regulator of kinases, transcription factors, and other cell-signalling molecules[8]. Hsp90 is highly abundant and has been proposed to function *in vivo* as a buffer for many regulatory activities; however, these phenotypes are revealed only when the levels of Hsp90 are reduced[29]. By contrast, in this study we have shown that the activities of enzymes such as Raf-1 are inhibited when the levels of Hsp70 increase and sequester Bag1.

Our results show that the activation of Raf-1 by Bag1 occurs in cells stimulated by EGF and expressing a dominant-negative Ras mutant. This adds to the complexity of Raf-1 regulatory molecules

and suggests that Bag1 uses a Ras-independent pathway for the Raf-1-dependent activation of the downstream MAP kinases. Separately, our results establish clearly that during heat shock, elevated levels of Hsp70 sequester Bag1 and DNA synthesis is repressed. To prove that these events are directly linked, we showed that Bag1 mutants deficient in binding to Hsp70 constitutively stimulated Raf-1/ERK activities, such that DNA synthesis was not repressed by heat shock. We also showed that Hsp70 mutants defective in binding to Bag1 had no effect on Raf-1 activity and DNA synthesis when overexpressed.

Together, these results indicate that the inhibitory effects of Hsp70 described here on the MAP kinase pathway and on DNA synthesis require Bag1. Despite the suggested pleiotropic effects of Bag1 on several signalling pathways, our studies also indicate that Bag1 is specific in its positive regulation of the MAP kinase pathway as it did not affect other stress-induced kinases p38 or Jun amino-terminal kinase (JNK) (data not shown). Hsp70 has been shown to prevent stress-induced activation of both p38 and JNK, and thus also functions as a negative regulator of a stress pathway leading to programmed cell death[30,31].

Heat-shock proteins and molecular chaperones have well-described activities in protein translocation, in the assembly of macromolecular complexes and in stress, to ensure that unfolded proteins are stabilized as intermediates such that on-pathway refolding can resume during recovery[6–12]. The role(s) of co-chaperones (Hdj-1, Hdj-2, Hip and Bag1) have been studied mainly *in vitro*, and in nearly all cases they function in molar stoichiometry to regulate chaperone activity; however, *in vivo* their concentrations relative to Hsp70 can range from 1 to 10% (C. Schmidt *et al.* personal communication). For example, the concentration of Bag1 in human tissue culture cells is roughly 1 μM, whereas that of Hsc70/Hsp70 varies up to 60 μM, therefore the ratio of Hsc70 : Hsp70–Bag1 in growing cells is about 60:1. After heat shock, even higher levels of Hsp70 accumulate. Given the relatively low levels of Bag1, and the multitude of cell signalling roles already suggested for this protein, we propose that Bag1 is negatively regulated by Hsp70. Heat-shock proteins—in particular those whose levels increase rapidly in response to cell stress—might therefore function as stress signalling molecules by binding to, sequestering or activating key events associated with cell growth and cell death.

The proposal that Bag1 function is negatively regulated by Hsp70 offers a different perspective on chaperones, in which the focus is shifted to the many regulatory properties of the co-chaperone when associated with its substrates. On the basis of these observations, we propose that other co-chaperones are likely to exhibit regulatory properties that could be either enhanced or inhibited on interaction with the partner chaperone. Such a prediction is already supported by the large number of dnaJ-related proteins that share the J domain essential for interaction with Hsp70 but that contain many other protein motifs[32]. The co-chaperone activity of Bag1 is itself intriguing as Bag1 inhibits Hsp70 refolding activity while stimulating nucleotide exchange[14,15]. Potentially, this could generate a Bag1–Hsp70 complex targeted to specific substrates that interact with Bag1 and become protected in this complex. The presence of an N-terminal ubiquitin-like motif that might target the selective degradation of Bag1–Hsp70-associated substrates has been reported[16]. As both Bag1 and Hsp70 also have anti-apoptotic activity, their effectiveness might be enhanced as a chaperone complex[17,33]. It would seem reasonable, therefore, to suggest that Bag1–Hsp70 complexes may have several roles in addition to the one shown here as a negative regulator of cell growth during stress. □

Methods

Reagents and plasmids.

Truncated mutants of Bag1 residues 90–219, 90–210, 90–200, 90–190, 90–180 and 90–170 were prepared by polymerase chain reaction (PCR) using the following primer pairs: 5′-TCCCCGGAATTCATGTTAATTGGTGAAAAG (forward), and 5′-CCGCTCGAGTTCAGCCAGGGCCAAGTTCGTA (reverse, 90–219), 5′-CCGCTCGAGCAGCCGCTCTGTCTCTTG (reverse, 90–210), 5′-CCGCTCGAGCTCCACTGTGTCACACTC (reverse 90–200), 5′-CCGCTCGAGCTGAACCTTTTTCACCAA (reverse, 90–190), 5′-CCGCTCGAGTAGCCTGCTGTCTTTAAA (reverse 90–180) or 5′-CCGCTCGAGGACCATTGTG TCAATTC (reverse 90–170). Bag1 truncated mutants 114–210, 139–210 and 161–210 were prepared using the following primer pairs: 5′-CCGCTCGAGCAGCCGCTCTGTCTCTTG (reverse), and 5′-TCCCCGGAATTCGCAGAGAAGATAGCTAACCAC (forward, 90–114), 5′-TCCCCGGAATTCGAATTGCAAGCGGAGGCTC-3? (forward, 90–139) or 5?-TCCCCGGAATTCATGAAGATCTTG GAGGAGATTG-3? (forward, 90–161). The point mutants Bag1C204A, Bag1E208A and Bag1Q205A were prepared using the following primer pairs respectively (mutation underlined): [**AUTHOR: OK?**] 5′-CCGCTCGAGTTCAGCCAGGGCCAAGTTTGTAGACTGCAGCCGCTCTGTCTCTTGGGCGATGTATTGCTC (reverse), 5′-CCGCTCGAGTTCAGCCAGGGCCAAGTTTGTAGACTGCAGCCGCTCTGTCTCGGCGCAGATGTATTGCTC (reverse) or 5′-CCGCTCGAGTTCAGCCAGGGCCAAGTTTGTAGACTGCAGCGCCTCTGTCT CTTGGCAGATG (reverse), and 5′-TCCCCGGAATTCATGGCCGAGACCGAGGAGATGGTCC (forward). The PCR products were digested with *Eco*RI/*Xho*I and cloned into the corresponding sites of plasmid of pGEX4T1, pcDNA-3 and pcDNA-3/HA. pUHD/Bag1C204A and pUHD/Bag1E208A were created by inserting the *Eco*R1 and *Xba*I fragments of pcDNA-3/Bag1C204A and pcDNA-3/Bag1E208A. Expression vectors for GST–MEK (pGEX4T1/MEK), GST–Raf-1 (pGEX5T1/Raf-1) and N17Ras (pcDNA3-N17Ras) were kindly provided by N. G. Ahn (Univ. Colorado), A. Nantel (Biotech. Res. Inst.) and J. Pessin (Univ. Iowa), respectively. The plasmid pGEX5T1/Raf-1 was digested with *Eco*RI-*Xho*I and subcloned into the corresponding sites of plasmid pGEM3zf (ref. 34).

In vitro assays.

Assays for the refolding denatured β-galactosidase using Hsp70 and Hdj-1 in the presence of Bag1 or its mutants were done as described[15]. *In vitro* protein interaction assays of Hsp70 and Bag1 or Bag1 mutants have been described[15]. For the interaction assay of Raf-1, we incubated 20 μl of *in vitro* translated Raf-1 labelled with [^{35}S]methionine with GST–Bag1 or its mutants for 30 min at 37 °C and then added glutathione–sepharose[23]. After washing three times with buffer (25 mM Tris-HCl, pH 7.2, 250 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100), proteins were eluted using glutathione solution (25 mM Tris-HCl, pH 7.2, 250 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 5 mM glutathione). We analysed the fractions by 12% SDS–PAGE and autoradiography. Raf-1 immunoprecipitation and kinase assays were done as described[35,36]. Kinase-defective GST–MEK was purified and used as a substrate for Raf-1 kinase assay[37].

Cell biology.

We carried out the expression of Hsp70 and Bag1 in OT-tet (Hsp70) and OT-tet (Bag1) cells as described[38,39]. OT cells were maintained in DMEM supplemented with 10% fetal calf serum (Life Technologies). For Raf-1 activation studies, we treated cells with EGF (100 ng ml^{-1} final concentration) for 20 min. Transient transfection was performed using Lipofectamine Plus Reagent. (Life Technologies). OT70 cells were transiently transfected with pcDNA-3/HA–Bag1, pcDNA-3/HA–Bag1C204A, pcDNA-3/HA–Bag1E208A and pcDNA-3/HA (as a control). Twelve hours after transfection, cells were washed twice with 1×PBS and incubated in medium with or without 3 μg ml^{-1} of tetracycline to induce Hsp70 expression. Cells transfected with pcDNA/HA–Bag1 were also incubated with U0126 (10 μM, final concentration) (Promega), a specific inhibitor of MEK kinase[40]. We lysed cells in solution containing 1×PBS, 1% Triton X-100, 10 μg ml^{-1} pepstatin A, 10 μg ml^{-1} leupeptin and 2.5 mM phenyl-methyl-sulphonyl fluoride. The protein concentrations of the lysates were determined by Bio-Rad protein assay. Proteins (50 or 100 μg) were resolved using SDS–PAGE gels transferred to nitrocellulose membrane and probed with anti-Hsp70 (3A3), anti-Bag1 (K56C8)[15], anti-Ras (Santa Cruz), anti-Raf-1 (E-10; Santa Cruz), anti-ERK1/2 phospho-specific monoclonal antibody (New England Biolabs) or anti-ERK1/ERK2 (C-16/C-14; Santa Cruz) antibodies.

We performed immunoprecipitation assays as follows. Cell lysates were precleared with normal rabbit antiserum bound to protein A/sepharose. Cell lysates (500 μl) were incubated with anti-Bag1-antiserum, anti-HA antiserum, anti-Raf-1 or anti-β-galactosidase-antibody (as a negative control) at 4 °C for 5 h followed by the addition of protein A/sepharose. The beads were washed five times with the lysis buffer described above and the bound proteins were eluted with SDS gel-loading buffer (50 mM Tris-HCl, pH 7.4, 10% glycerol, 100 mM dithiothreitol, 2% SDS and 0.1% bromophenol) followed by western blot.

We created the OT-tet (Bag1C204A) and OT-tet (Bag1E208A) cell lines from OT23 using pUHD/Bag1C204A and pUHD/Bag1E208A as described[36]. We cultured OT, OTBag1, OTBag1C204A and OTBag1E208A cells, and overexpressed the proteins as described[39]. Cell synchrony was achieved by treating the cells with thymidine (2 mM) for 12 h, allowing them to grow for 7 h in the normal growth medium, and then adding mimosine (400 μM).

DNA synthesis was measured by the incorporation of [^{3}H]thymidine or bromodeoxyuridine (BrdU). Synchronized or heat-shocked cells were incubated with [^{3}H]thymidine (2 μCi ml^{-1}) for 1–3 h. After collecting the cells, we counted the number of cells and determined [^{3}H]thymidine incorporation into DNA by precipitation with 10% trichloroacetic acid and measurement on a LS6500 liquid scintillation counter (Beckman Coulter). For BrdU incorporation, synchronized cells in the absence of tetracycline were heat shocked at 43 °C for 1 h, recovered at 37 °C for 1 h, and then treated with BrdU (20 μM) for 90 min. Cells were fixed using formaldehyde (4%) and DNA duplex was denatured by 2 M HCl. Incorporated BrdU was detected using mouse anti-BrdU antibody (Sigma) followed by fluorescein isothiocyanate (FITC) goat anti-mouse antibody (Sigma).

Yeast two-hybrid assay and random mutagenesis.

To create the hybrid bait protein, we fused a DNA fragment encoding the 226–383 domain of Hsp70 in-frame with the yeast LexA DNA-binding domain in the pEG202 vector. A plasmid containing full-length Bag1 was constructed in plasmid pJG4-5 (ref. 41). Random mutagenesis of the Hsp70 fragment (residues 226–383) was accomplished by PCR reaction (10 ng pEG202/Hsp70 (residues 226–383), Taq polymerase and 10× PCR buffer (Pharmacia-LKD), 0.5 mM dGTP, 2 mM dCTP, 2 mM dATP, 2 mM dTTP, 3.2 pmol of each primer) using primers corresponding to a pEG202 sequence (5′-GGGGTTGGGGTT ATTGGCAAGGGGGACTGGCTGGAATTC (forward), 5′-ATAAATCATAAG AAATTCGCCG (reverse)). We co-transformed linearized pEG202 and randomly mutagenized Hsp70 (residues 226–383) DNA fragments into EGY48 yeast cells containing pJG4-5/Bag1 and pSH18-34 *lacZ*

reporter plasmid. Transformants were grown on media lacking tryptophan, uracil and histidine. Colonies with mutant DNA encoding Hsp70 (residues 226–383) that does not interact with Bag1 are identified by their inability to transcribe the *LEU* and *lacZ* reporter genes. We performed nucleotide sequence analysis using ABI310 (PE Biosystem). Each mutant from screening was introduced into pMS119-EH/Hsp70 using site-directed mutagensis (Strategene).

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