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BAG3 affects the nucleocytoplasmic shuttling of HSF1 upon heat stress

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ABSTRACT

Bcl2-associated athoanogene (BAG) 3 is a member of the co-chaperone BAG family. It is induced by stressful stimuli such as heat shock and heavy metals, and it regulates cellular adaptive responses against stressful conditions. In this study, we identified a novel role for BAG3 in regulating the nuclear shuttling of HSF1 during heat stress. The expression level of BAG3 was induced by heat stress in HeLa cells. Interestingly, BAG3 rapidly translocalized to the nucleus upon heat stress. Immunoprecipitation assay showed that BAG3 interacts with HSF1 under normal and stressed conditions and co-translocalizes to the nucleus upon heat stress. We also demonstrated that BAG3 interacts with HSF1 via its BAG domain. Over-expression of BAG3 down-regulates the level of nuclear HSF1 by exporting it to the cytoplasm during the recovery period. Depletion of BAG3 using siRNA results in reduced nuclear HSF1 and decreased Hsp70 promoter activity. BAG3 in MEF($hsf1^{-/-}$) cells actively translocalizes to the nucleus upon heat stress suggesting that BAG3 plays a key role in the processing of the nucleocytoplasmic shuttling of HSF1 upon heat stress.

1. Introduction

Heat shock transcription factor 1 (HSF1) is responsible for the activated expression of a large class of heat shock proteins (Hsps) that protect cells from damage incurred from cellular insults [1–3]. HSF1 is activated in response to various pathophysiologic stresses. Normally, HSF1 largely localizes to the cytoplasm. Upon exposure to stressful conditions, HSF1 translocalizes to the nucleus where it activates the transcription of target genes, including Hsps [4–6]. Hsps contribute to protection against conditions that generate cellular damage by functioning as molecular chaperones that accelerate refolding of denatured proteins and promote the degradation of damaged proteins during stressful conditions [6–8]. However, precise molecular mechanisms by which HSF1 translocalizes to the nucleus are poorly understood.

Bcl2-associated athanogene 3 (BAG3) is a member of BAG family of co-chaperones, and it interacts with Bcl2 and Hsp70 through its BAG domain [9]. In addition, BAG3 contains a WW domain, two IPV motifs, and a proline-rich repeat (PXXP) through which BAG3 interacts with other partners, such as Hsp22 and phospholipase C [9,10]. BAG3 is the only member of the family to be induced by stressful stimuli, including heat shock and heavy metals [11,12]. Interestingly, it has been recently reported that the induction of BAG3 in response to stress is mainly through the activation of HSF1 [13]. Growing evidence has shown that BAG3 regulates cellular adaptive responses against stressful stimuli by regulating apoptosis, development, cytoskeleton organization and autophagy [10,14–19]. However, the underlying molecular mechanisms are still largely unknown.

Considering the association of BAG3 with cellular stress, we hypothesized that BAG3 might be associated with HSF1 regulation in response to cellular stress. Therefore, in this study, we investigated the molecular action mechanism of BAG3 under stress conditions. We first demonstrated that under heat stress, BAG3 rapidly co-translocalized to the nucleus with HSF1. Down-regulation of BAG3 reduced the level of nuclear HSF1. The response of $hsf^{-/-}$ mouse embryonic fibroblast (MEF) cells showed that the translocation of BAG3 upon heat stress is not affected by the absence of HSF1, suggesting that BAG3 may act as an HSF1 regulator by nucleocytoplasmic shuttling upon heat stress.

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2. Materials and methods

2.1. Cell culture and treatment

HeLa and MEF cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin was used as a growth medium for all cell lines. MEF cells derived from wild-type mice and *hsf1*$^{-/-}$ mice were a gift from Dr. Ivor Benjamin (Southwestern Medical Center, University of Texas). For the heat-shock treatment, culture plates were wrapped with Parafilm™ and immersed in a 42 °C water bath for 1 h.

2.2. Plasmids and transfection

The open reading frame of BAG3 (1728 nucleotides) corresponding to GenBank® accession number NM_004281 was amplified by PCR using human skeletal muscle cDNA as a template (Clontech). The mammalian expression vectors for the N-terminally FLAG-tagged full length BAG3 (BAG3-WT, amino acids 1–575), Δ-BAG (BAG3-ΔBAG, amino acids 1–423), Δ-PXXP/BAG (BAG3-ΔPXXP/BAG, amino acids 1–309), Δ-WW (BAG3-ΔWW, amino acids 100–575), and Δ-WW/IPV (BAG3-ΔWW/IPV, amino acids 212–575) BAG3 proteins were created by inserting cDNA fragments into the 5′ *KpnI* and 3′ *XbaI* sites of pCDNA3.1-NF. Cells were transiently transfected using FuGENE® HD (Promega) according to the manufacturer's instructions. All constructs were confirmed by DNA sequencing.

2.3. Subcellular fractionation

To obtain cytoplasmic and nuclear fractions, cells were washed with PBS, resuspended in solution A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and incubated on ice for 5 min. Nonidet P-40 was added to a final concentration of 0.6% and the mixture was centrifuged at 4 °C, 12,000 rpm for 1 min. The supernatant (cytosolic fraction) was removed and the pellet (nuclear fraction) was resuspended in buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). After 15 min incubation on ice, samples were centrifuged at 4 °C, 13,000 rpm for 5 min, and the supernatant was used as the nuclear extract.

2.4. Immunoprecipitation and immunoblotting

For immunoprecipitation, HeLa cells were transiently transfected using FuGENE® HD (Promega) as described above. After 30 h, cells were treated heat shock at 42 °C for 1 h and lysed in RIPA buffer (PBS supplemented with 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM PMSF, 1 μg/ml aprotinin, and 1 mM sodium orthovanadate). Cell lysates were harvested, incubated at 4 °C for 30 min and then centrifuged at 10,000 × g for 10 min. The supernatant was incubated with agarose-conjugated anti-FLAG antibody (Sigma–Aldrich) for 3 h. Immunoprecipitates were washed 4 times with RIPA buffer containing 0.05% SDS and boiled in SDS-PAGE sample buffer. For immunoblotting, proteins were resolved by SDS-PAGE and immunoblotted using the following antibodies: anti-FLAG (Sigma–Aldrich), anti-HSF1 (Santa Cruz Biotechnology), anti-BAG3 (Abcam), anti-Hsp70 (Santa Cruz Biotechnology), anti-actin (Sigma–Aldrich), and anti-TBP (Abcam).

2.5. Luciferase reporter gene assay

To assess the effects of BAG3 on HSF1 activity, HeLa cells were co-transfected with pGL3-Hsp70-Luc, pCDNA3.1-FLAG-BAG3, and pCH110 using FuGENE® HD (Promega) as described above. After 30 h, the cells were lysed with the Reporter Lysis Buffer (Promega) and luciferase activity was measured using the Luciferase Activity Assay kit (Promega) according to the manufacturer's instructions. β-galactosidase activities were determined to normalize the luciferase activities. All experiments were performed in triplicate, and the mean ± SD values were determined.

2.6. siRNA experiment

HeLa cells (2×10^5 cells/ml) were seeded on 12-well plates and transfected with 1.5 μg/ml of siGENOME BAG3 siRNA (Dharmacon) using DharmaFECT transfection reagent (Dharmacon) according to the manufacturer's instruction. siGENOME Non-Targeting siRNA Pool was used as control. After 48 h, cells were harvested and cell lysates were separated by SDS-PAGE. The expression level of BAG3 was analyzed by immunoblotting as described above.

2.7. Immunofluorescence microscopy

HeLa cells were seeded on 35 mm plates at a density of 1×10^5 cells/plate. The cells were cultured overnight and given a heat shock at 42 °C for 1 h, followed by recovery at 37 °C for 1 h. The cells were then washed twice with PBS and fixed with 4% paraformaldehyde for 10 min. After being washed twice with PBS, the cells were incubated with methanol for 2 min. For immunostaining, the cells were pretreated with 5% BSA for 30 min and then incubated with anti-BAG3 antibody or anti-HSF1 antibody in 2.5% BSA containing PBS for 1 h. After being washed with PBS for 10 min, the cells were incubated for 30 min with FITC-conjugated anti-rabbit secondary antibody (Vector Laboratories) or Alexa Fluor 594-conjugated anti-mouse secondary antibody (Invitrogen) in 2.5% BSA containing PBS. Finally, the cells were washed twice with PBS for 10 min and then mounted using crystal/mount (Biomeda Corporation). Nuclei were stained with 4′,6-diamidino-2-phenylindole (Invitrogen). The fluorescence analysis was performed by conventional fluorescence microscopy (Axio Observer D1, Carl Zeiss) and by confocal microscopy (LSM700, Carl Zeiss).

3. Results

3.1. BAG3 translocates to the nucleus upon heat stress

To investigate whether heat stress can modulate BAG3 expression, HeLa cells were given a heat shock, and the expression level of BAG3 was examined. When the cells were treated with heat shock for 1 h, HSF1 phosphorylation, an indicator of heat activation, was detected (Fig. 1A). Following the activation of HSF1, Hsp70 expression began to be induced after approximately 3 h of recovery. Like Hsp70 expression, BAG3 expression was induced by heat stress (Fig. 1A). Previous studies have shown that the expression of BAG3 is stimulated to protect cells from stressful stimuli, including heat shock, heavy metals, and proteasome inhibitors [11,12,20]. Our data also showed that BAG3 is induced by heat stress in HeLa cells.

To assess the distribution of BAG3 upon heat stress, HeLa cells were given a heat shock at 42 °C for 1 h and allowed to recover for the indicated time periods. The cytosolic and nuclear proteins were fractionated, and the localization of BAG3 was detected by Western blot analysis. HSF1 mainly localized to the cytosolic fraction under normal conditions and rapidly translocated to the nucleus upon heat treatment. Under non-stressed conditions, BAG3 was also predominantly found in the cytosolic fraction. Interestingly, the heat shock treatment also induced BAG3 translocation to the nucleus (Fig. 1B). To confirm the translocation of BAG3 to the nucleus, we examined the subcellular localization of endogenous BAG3 and

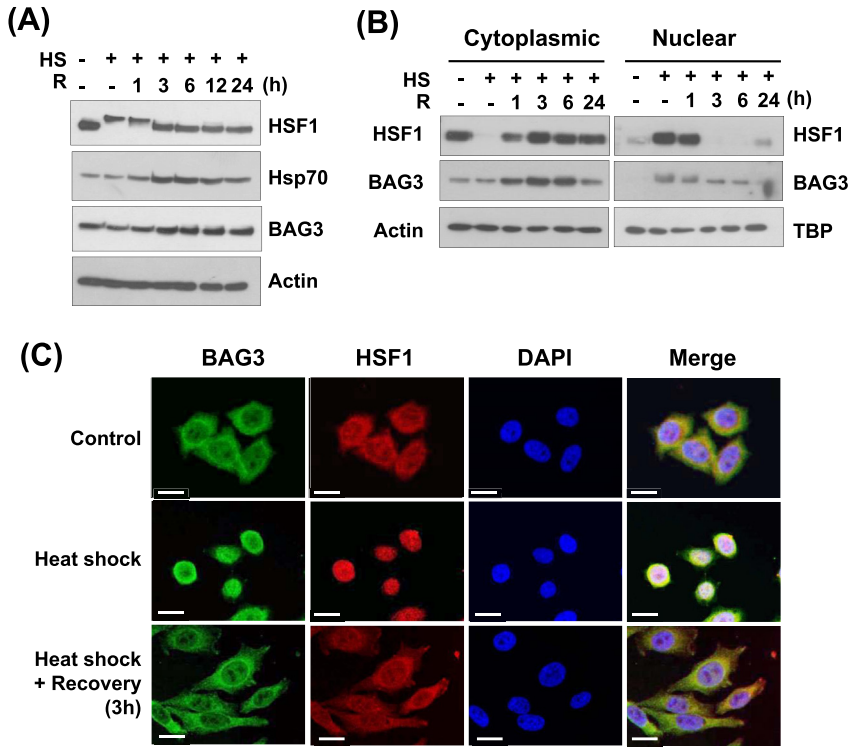

Fig. 1. BAG3 translocates to the nucleus upon heat stress. HeLa cells were treated with heat shock at 42 °C for 1 h, followed by recovery at 37 °C for the indicated time periods. (A) Total cell lysates were obtained, and the expression levels of HSF1, Hsp70 and BAG3 were detected by Western blot analysis. (B) Cytoplasmic and nuclear fractions were prepared and the levels of BAG3 and HSF1 were detected by Western blot analysis. Actin and TBP were used as loading controls. (C) HeLa cells were treated with heat shock at 42 °C for 1 h and allowed to recover at 37 °C for 3 h. For immunostaining, cells were incubated with the primary antibodies against BAG3 or HSF1 and then incubated with FITC- or Alexa Fluor 594-conjugated secondary antibodies, respectively. Nuclei were stained with DAPI. The subcellular localization of BAG3 and HSF1 were observed by confocal microscopy.

HSF1 by confocal microscopy. As shown in Fig. 1C, both BAG3 and HSF1 mainly localized to the cytoplasm under normal conditions. However, heat treatment induced the translocation of these proteins to the nucleus, and they re-localized to the cytoplasm during the recovery period.

3.2. BAG3 interacts with HSF1 and co-localizes to the nucleus

To examine whether BAG3 translocates from the cytoplasm to the nucleus through an interaction with HSF1, HeLa cells were transiently transfected with N-terminally FLAG-tagged BAG3 expression vectors. The nuclear and cytosolic proteins were prepared, and immunoprecipitation assays were performed. Interestingly, BAG3 interacted with HSF1 in the cytoplasm under normal conditions and also interacted with activated HSF1 in the nucleus under heat stressed conditions (Fig. 2A).

BAG3 contains several protein motifs, including the BAG domain, WW (Trp—Trp) domain, PXXP (Proline-rich repeat) region, and IPV (Ile-Pro-Val) motif, and through these domains, BAG3 can interact with other proteins (Fig. 2B) [9,10]. To identify the region mediating the BAG3/HSF1 interaction, FLAG-tagged BAG3 and its deletion mutants were transiently transfected into HeLa cells. BAG3 was immunoprecipitated, and the associated HSF1 was analyzed by Western blot analysis. As shown in Fig. 2C Upper, HSF1 showed interaction with the full length BAG3 (BAG3-WT), the WW domain deleted mutant (BAG3-ΔWW), and the WW/IPV domain deleted mutant (BAG3-ΔWW/IPV). However, the BAG domain deleted mutant (BAG3-ΔBAG) did not associate with HSF1, suggesting that HSF1 interacts with BAG3 through the BAG domain. Protein levels were verified by Western blot analysis (Fig. 2C Lower).

3.3. BAG3 regulates nuclear shuttling of HSF1

Our finding that BAG3 interacts with HSF1 both in the cytoplasm under normal conditions and in the nucleus under heat stressed conditions raised the possibility that BAG3 might be involved in HSF1 regulation. Therefore, we next examined whether BAG3 can affect the nuclear shuttling of HSF1 upon heat stress. Nuclear and cytoplasmic fractions were prepared from control cells and cells that overexpressed BAG3, and the localization of HSF1 was examined. As expected, heat shock induced the nuclear translocation of HSF1, and the increased level of nuclear HSF1 was sustained for 1 h of recovery at 37 °C. The cells that overexpressed BAG3 also showed nuclear translocation of HSF1 after heat treatment. However, HSF1 was quickly exported to the cytoplasm during the 1 h recovery period (Fig. 3A). These results suggest that BAG3 induces HSF1 export from nucleus to cytoplasm. To evaluate the effect of BAG3 on HSF1 translocation, we next performed the reporter gene assay. HeLa cells were co-transfected with the pGL3-Hsp70-Luc luciferase reporter vector and pcDNA3.1-FLAG-BAG3, and the promoter activity of Hsp70, a main target of HSF1, was examined. As shown in Fig. 3B, the heat shock induced Hsp70 promoter activity was significantly decreased by BAG3 in a dose-dependent manner, suggesting that BAG3 plays a role in the HSF1 export from the nucleus.

To further investigate the role of BAG3 in HSF1 translocation, cells were transfected with BAG3-specific siRNA. As shown in Fig. 3C, the level of BAG3 was appreciably reduced by the BAG3 siRNA. Consistent with the result shown in Figs. 1B and 3A, heat stress induced nuclear HSF1 and BAG3. However, the level of nuclear HSF1 was decreased in BAG3 siRNA transfected cells confirming that BAG3 affects HSF1 translocation to the nucleus

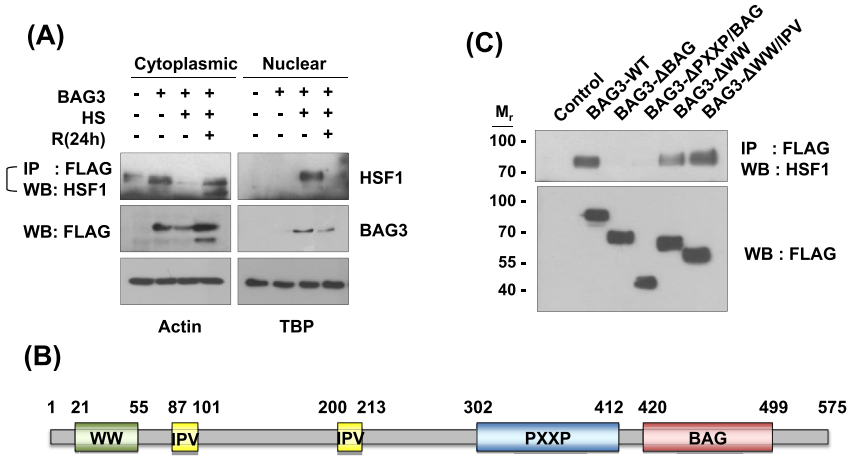

Fig. 2. BAG3 interacts with HSF1. (A) HeLa cells were transfected with FLAG-tagged BAG3 expression vectors. After 30 h, cells were treated with heat shock at 42 °C for 1 h, followed by recovery at 37 °C for 24 h. Total cell extracts, cytosolic and nuclear extracts were subjected to immunoprecipitation by using anti-FLAG antibody. Co-precipitated endogenous HSF1 was analyzed by Western blot analysis using anti-HSF1 antibody. The expression level of BAG3 in the transfected cells was monitored by Western blot analysis. (B) A schematic diagram of the BAG3 domain organization. BAG3 is composed of WW domain, IPV motif, PXXP motif, and BAG domain. Domain boundaries were obtained from the SMART program. (C) HeLa cells were transfected with the indicated expression vectors. Thirty hours after transfection, cell extracts were immunoprecipitated with anti-FLAG antibody. Co-precipitated endogenous HSF1 was analyzed by Western blot analysis (Upper). The expression levels of proteins in the transfected cells were monitored by Western blot analysis (Lower).

(Fig. 3D). Hsp70 promoter activity also decreased in BAG3 siRNA transfected cells (Fig. 3E).

3.4. BAG3 is a key regulator for HSF1 nuclear shuttling

We next examined whether HSF1 also can affect the nuclear translocation of BAG3. For this purpose, mouse embryonic fibroblast (MEF) cells derived from congenic wild-type mice and *hsf1*$^{-/-}$ mice were used. In accordance with the results from HeLa cells, heat treatment induced HSF1 activation and the subsequent expression of BAG3 and Hsp70 in wild type MEF cells (Fig. 4A). To further investigate the relationship of BAG3 and HSF1 in nuclear translocation, cells were given a heat shock, and the level of nuclear BAG3 was examined in wild type and *hsf1*$^{-/-}$ cells. In wild type cells, the levels of nuclear BAG3 and HSF1 were increased by heat stress and decreased during the recovery period (Fig. 4B). Interestingly, BAG3 translocation was not affected by the absence of HSF1. In the *hsf1*$^{-/-}$ cells, nuclear BAG3 increased upon heat stress and decreased during the recovery period (Fig. 4B). Consistent with this result, *hsf1*$^{-/-}$ cells showed rapid nuclear translocation of BAG3 upon heat stress (Fig. 4C). These results suggest that BAG3 is a key player in the nuclear translocation of HSF1 by heat stress.

4. Discussion

BAG3, a co-chaperone protein, is induced in cells by heat shock and exposure to heavy metals through the activation of HSF1, and it plays diverse cellular roles in proliferation, apoptosis, cytoskeleton organization and autophagy [10–19]. Several studies have shown that BAG3 is overexpressed in cancer cells, including glioblastoma, prostate and pancreatic cancer, and it promotes tumor cell survival [16,21–23]. Considering that HSF1 enhances the survival of cancer cells under various stresses, it would be of great interest to investigate the molecular inter-relationship between BAG3 and HSF1.

Here, we demonstrated that BAG3 is a nuclear-cytoplasmic shuttling protein that interacts with HSF1 and regulates HSF1 nuclear translocation. Although several chaperone proteins that regulate HSF1 activity have been identified, the molecular mechanism of HSF1 translocation to the nucleus remains to be elucidated [24,25]. In this study, we showed that BAG3 interacts with HSF1 through its BAG domain and co-localizes with HSF1 both in the cytoplasm under the normal conditions and in the nucleus under the heat stressed conditions (Figs. 1 and 2). Although BAG3 is known to be involved in various cellular functions, our data clearly demonstrates BAG3 undergoes a dynamic translocation between the nucleus and the cytoplasm. In fact, we identified a putative nuclear localization signal (NLS) and three nuclear export signals (NES) within the c-terminal region of BAG3 [26–28].

When overexpressed, BAG3 accelerated the export of nuclear HSF1 from the nucleus to the cytoplasm during the recovery period after heat stress. Furthermore, a knock down of BAG3 protein using BAG3 specific siRNA decreased the import of cytoplasmic HSF1 into the nucleus (Fig. 3A and D). It is unclear how overexpressed BAG3 accelerate the export of nuclear HSF1. BAG3 and HSF1 have multiple binding partners, which regulate their roles. However, all the known interacting proteins are restricted to the cytoplasm. It is possible there are other interacting proteins that regulate nuclear shuttling.

Several studies have shown that HSF1 regulates the BAG3 gene expression induced by a cellular stimulus [13,15,20,29]. Additionally, the autoactivation of the BAG3 gene in human glial cells, but not in other non-glial cell lines has led to a suggestion for the possible role of nuclear BAG3 [30]. We showed that BAG3 overexpression decreased the transcriptional activity of HSF1 by increasing the export of nuclear HSF1 (Fig. 3A and B). A knock down of the level of BAG3 also decreased HSF1 transcriptional activity by decreasing the level of nuclear HSF1, suggesting that BAG3 effectively affects the transcriptional activity of HSF1 by controlling its nuclear shuttling (Fig. 3D and E). The physiological significance of the nuclear shuttling of BAG3 is presently unclear. However, our data demonstrated that BAG3 could regulate the expression level of heat shock responsive proteins, including BAG3 itself, by regulating the nuclear shuttling of HSF1 by interaction.

Although the activation of HSF1 by interacting chaperons has been widely studied, there was no information about the regulatory proteins involved in its nuclear translocation. In this study, we identified BAG3 as an HSF1 interacting protein that regulates HSF1 nuclear translocation and subsequent target gene expression. Although the level of BAG3 affects the nuclear shuttling of HSF1, the absence of HSF1 did not affect the nuclear translocation of BAG3,

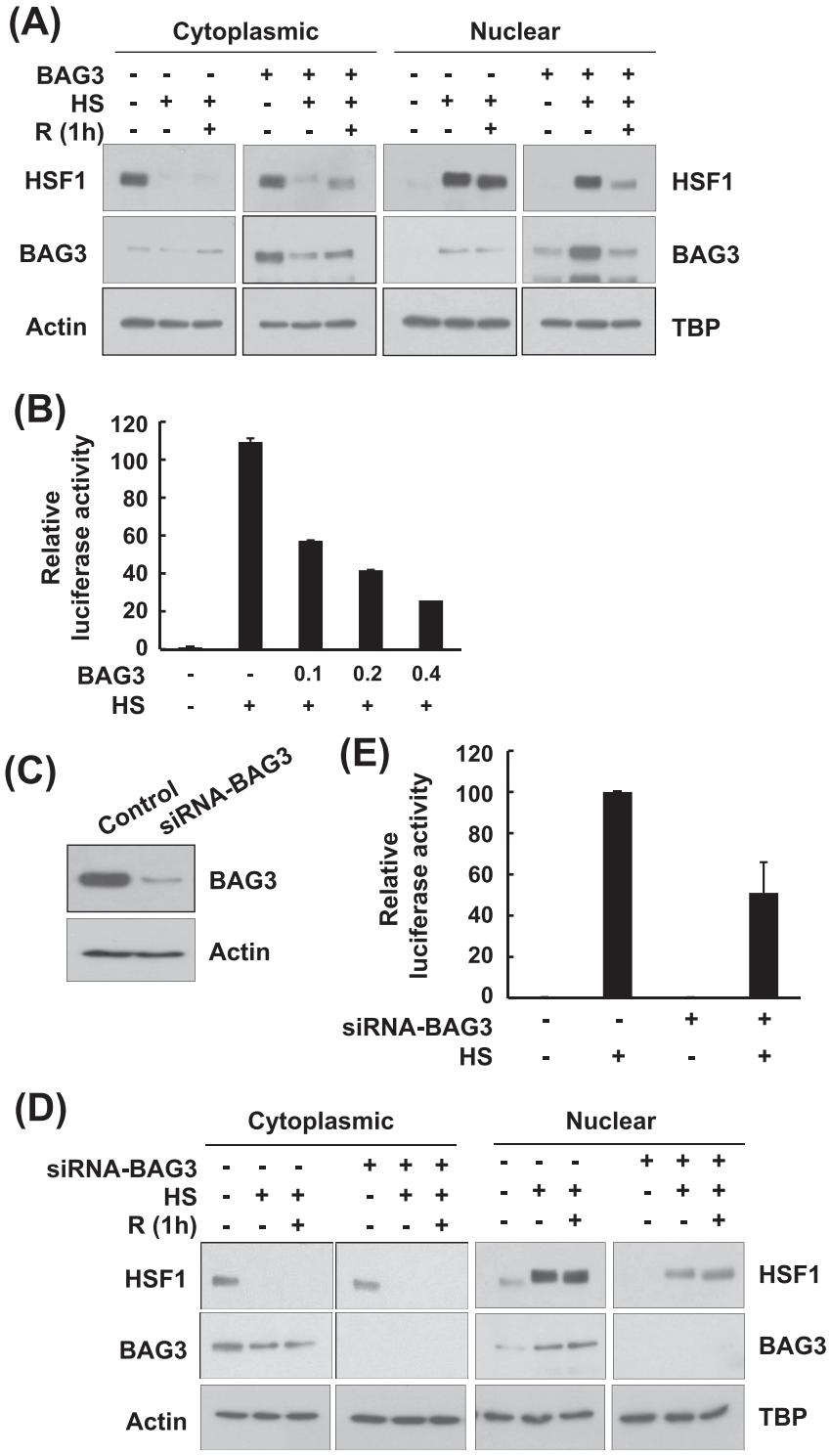

Fig. 3. BAG3 affects the nucleocytoplasmic shuttling of HSF1. (A) HeLa cells were transfected with FLAG-tagged BAG3 expression vectors. After 30 h, cells were treated with heat shock at 42 °C for 1 h, and allowed to recover at 37 °C for 1 h. Cytosolic and nuclear fractions were prepared, and the levels of BAG3 and HSF1 were detected by Western blot analysis. (B) HeLa cells were co-transfected with the pGL3-Hsp70-Luc reporter vector and the pcDNA3.1-FLAG-BAG3 expression vector. After 30 h, cells were treated with heat shock and allowed to recover for 24 h. Total cell extracts were assayed for luciferase. Luciferase activities were normalized for transfection efficiency by co-transfection with the β-galactosidase-expressing vector, pCH110. The data were expressed as the means ± SD of three separate experiments. (C) HeLa cells were transfected with BAG3 siRNA constructs. After 48 h, the protein level of BAG3 was detected by Western blot analysis. (D) HeLa cells were transfected with BAG3 siRNA constructs. After 48 h, cells were treated with heat shock at 42 °C for 1 h and allowed to recover at 37 °C for 1 h. Cytoplasmic and nuclear fractions were prepared, and the levels of BAG3 and HSF1 were detected by Western blot analysis. (E) HeLa cells were transfected with BAG3 siRNA constructs. After 6 h, cells were co-transfected with the pGL3-Hsp70-Luc reporter vector. Cells were treated with heat shock 48 h after BAG3 siRNA transfection and allowed to recover for 24 h. Total cell extracts were assayed for luciferase activity. The data were expressed as the means ± SD of three separate experiments.

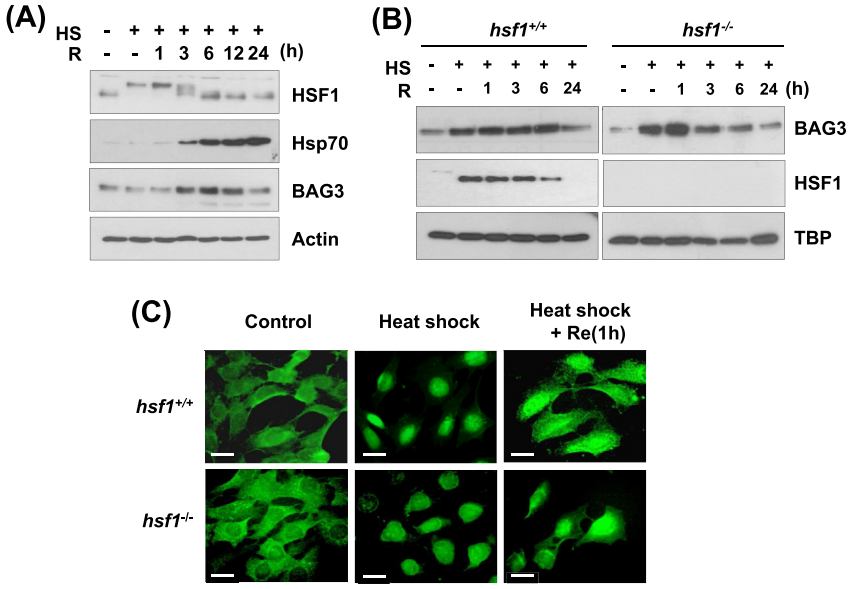

Fig. 4. BAG3 translocates to the nucleus upon heat stress in the *hsf1*$^{-/-}$ MEF cells. (A) MEF (WT) cells were treated with heat shock at 42 °C for 1 h and allowed to recover at 37 °C for the indicated time periods. Total cell lysates were obtained, and the expression levels of HSF1, Hsp70 and BAG3 were detected by Western blot analysis. (B) WT and *hsf1*$^{-/-}$ MEF cells were treated with heat shock at 42 °C for 1 h and then allowed to recover at 37 °C for the indicated time periods. Nuclear proteins were obtained, and the levels of BAG3 and HSF1 were detected by Western blot analysis. (C) WT and *hsf1*$^{-/-}$ MEF cells were treated with heat shock and allowed to recover for 1 h. The cells were immunostained with anti-BAG3 antibodies and then incubated with FITC-conjugated secondary antibodies. The subcellular localization of BAG3 was observed by fluorescence microscopy.

suggesting that BAG3 is the main player for the BAG3-HSF1 nuclear translocation. Considering that HSF1 is a promising target for cancer therapy, it will be of great interest to investigate the molecular mechanism of HSF1 nuclear translocation more thoroughly.

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