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HspB8 Participates in Protein Quality Control by a Non-chaperone-like Mechanism That Requires eIF2α Phosphorylation*[S]

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Aggregation of mutated proteins is a hallmark of many neurodegenerative disorders, including Huntington disease. We previously reported that overexpression of the HspB8·Bag3 chaperone complex suppresses mutated huntingtin aggregation via autophagy. Classically, HspB proteins are thought to act as ATP-independent molecular chaperones that can bind unfolded proteins and facilitate their processing via the help of ATP-dependent chaperones such as the Hsp70 machine, in which Bag3 may act as a molecular link between HspB, Hsp70, and the ubiquitin ligases. However, here we show that HspB8 and Bag3 act in a non-canonical manner unrelated to the classical chaperone model. Rather, HspB8 and Bag3 induce the phosphorylation of the α-subunit of the translation initiator factor eIF2, which in turn causes a translational shut-down and stimulates autophagy. This function of HspB8·Bag3 does not require Hsp70 and also targets fully folded substrates. HspB8·Bag3 activity was independent of the endoplasmic reticulum (ER) stress kinase PERK, demonstrating that its action is unrelated to ER stress and suggesting that it activates stress-mediated translational arrest and autophagy through a novel pathway.

HspB8 (Hsp22/H11/E2IG1) is a member of the human small heat shock protein family (sHsp/HspB; HspB1–11) that shares some of the classical features common to the HspBs, including stress inducibility and *in vitro* chaperone activity (1–4). HspB8 is highly expressed in brain, heart, skeletal, and smooth muscles (5). Interestingly, two missense mutations in HspB8, which result in protein instability and aggregation, have been recently associated with hereditary peripheral neuropathies (6, 7). However, the mechanism responsible for the development of the disease and whether this is due to a toxic gain of function or to a loss of function of the mutated HspB8 is still unknown. We recently found that in mammalian cells endogenous HspB8 forms a stable and stoichiometric complex with the Hsc70/Hsp70 co-chaperone Bag3 (8). Interaction between HspB8 and Bag3 has been further demonstrated using a yeast two-hybrid approach by another independent group (9). Up-regulation of the HspB8·Bag3 complex could decrease the accumulation of mutated huntingtin protein aggregates, whereas its down-regulation enhanced aggregation, suggesting that the complex plays an important physiological role in protein quality control (8).

Expansion of the CAG/polyglutamine repeat in the gene encoding for the huntingtin protein results in Huntington disease, an autosomal dominant neurodegenerative disorder (10) characterized by the presence of polyglutamine aggregates (11, 12). This aggregation is a multistep nucleation-dependent process in which early stage detergent-soluble pre-fibrillar aggregates are gradually converted into detergent-insoluble fibrillar structures that are associated with a rearranged intermediate filament network, named inclusion bodies (13–15). It is thought that a critical concentration of mutated polyglutamine proteins is required to trigger the nucleation process (16). When the cells cannot adequately remove the early toxic microaggregated species either through the proteasome system or the autophagy, oligomerization into detergent-resistant fibrillar structures and inclusion bodies formation will occur. Decreasing the total amount of mutated protein present in the cell by facilitating its degradation seems an early key event to prevent both aggregation and cytotoxicity. Besides degradation via the proteasome, a major role for macroautophagy (here referred to as autophagy) in the clearance of mutant huntingtin is emerging (17–20). Autophagy is a non-selective degradation process that involves the sequestration of portions of the cytoplasm, organelles, and aggregated proteins into double-membrane vesicles, called autophagosomes and delivery of their content to the lysosome for degradation (21, 22). Indeed, loss of autophagy causes neuronal loss and results in accumulation of polyubiquitinated aggregated proteins (23). Consistently, rapamycin, a pharmacological drug that stimulates autophagy, has been shown to protect against mutated polyglutamine-induced neurodegeneration both *in vitro* and *in vivo* (17, 18, 24). Also the action of the HspB8·Bag3 complex on the accumulation of protein aggregates caused by mutated huntingtin seems at least in part linked to a stimulatory effect on its clearance by autophagy (8, 25). In line with its classical role as chaperone, the ATP-independent HspB8 might chaperone misfolded huntingtin,

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keeping it competent for processing by ATP dependent chaperones like Hsp70s. Bag3, with its capacity to also bind Hsp70 via its BAG domain, may form a molecular link between the two chaperones and the degradation machinery. However, such a model yet lacks experimental evidence. Moreover, deletion of the BAG domain that mediates Bag3 interaction with Hsp70 did not affect the activity of the complex (25). Curiously, upon transient overexpression of HspB8 and Bag3, we not only found reduced aggregation of mutant huntingtin, but we also observed a decrease in the total amount of soluble huntingtin, suggesting that the complex may also act on protein synthesis (8, 26). This hypothesis could be in line with recent observations by King *et al.* (24) using rapamycin. Besides its ability to stimulate autophagy, the authors showed that rapamycin treatments simultaneously resulted in inhibition of protein synthesis, which also contributed to decrease mutated huntingtin aggregation (24). This finding suggests that the neuroprotective activity of rapamycin is due to the activation of both translational arrest and autophagy, processes that are directly linked. Such a link is supported by studies on amino acid deprivation and viral infection (27). The mechanism by which this occurs involves the phosphorylation of the translation initiation factor eIF2α (27). Thus, when cells encounter a stressful event (*e.g.* accumulation of misfolded proteins, ER-stress, pathogens infection), they seem to respond by simultaneously inhibiting translation and stimulating autophagy. In the current study, we tested whether our previously observed effects of the HspB8·Bag3 complex on aggregation of mutated huntingtin and autophagy (8) may also occur via eIF2α phosphorylation and also involved translational arrest besides autophagy stimulation.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—pEGFC1 encoding for the GFP[3] protein was from Clontech. pGFP-HDQ74 encoding for the GFP-tagged Huntingtin exon 1 fragment with 74 CAG repeats was from Dr. D. C. Rubinsztein (28). Plasmids encoding human HspB8 and Myc-tagged HspB8 were previously described (26). The plasmid encoding his human Bag3 (pCINHisBag3) and ΔBAG Bag3 (pCINHisΔBAG) were from Dr. E. Kohn (29), whereas the plasmid encoding his human ΔWW Bag3 (pCINHisΔWW) and Δ*PXXP* Bag3 (pCINHisΔ*PXXP*) was previously described (25). The vector encoding for the K113G HspB8 mutant was prepared by mutagenesis reaction using the Myc-tagged HspB8 vector as a template and the primers 5′-GCCAGAGGAGTTGATGGTGGGCACCAAAGATGGATACG-3′ (forward) and 5′-CGTATCCATCTTTGGTGCCCACCATCAACTCCTCTGGC-3′ (reverse). pMyc-LC3 was a kind gift of Dr. T. Yoshimori (Osaka University). pVSV-GADD34 vector encoding for the C-terminal fragment of hamster GADD34 (amino acid 292–590) was a kind gift from Dr. N. Lubsen. Vectors encoding for Myc-tagged wild-type eIF2α, the non-phosphorylatable mutant S51A, wild-type PERK, and the dominant negative mutant PERK K618A were a kind gift from Dr. D. Ron (New York University).

[3] The abbreviations used are: GFP, green fluorescent protein; HA, hemagglutinin; PERK, double stranded RNA-activated eIF2α kinase-like ER kinase; MEF, mouse embryonic fibroblasts; RRL, reticulocyte rabbit lysates; ER, endoplasmic reticulum; mRFP, monomeric red fluorescent proteins.

Antibodies—Anti-HspB8, anti-Bag3, and anti-LC3 are rabbit polyclonal antibodies against human HspB8, Bag3, and LC3, respectively (8, 26). Mouse monoclonal anti-γ-tubulin was from Sigma-Aldrich. Mouse monoclonal anti-total-eIF2α and rabbit polyclonal anti-phospho-eIF2α were from Cell Signaling and Sigma, respectively. Mouse monoclonal anti-GFP was from Clontech.

Cell Culture and Transfection—HEK-293 and HEK-293T (human embryonal kidney) as well as mouse embryonic fibroblasts were grown in Dulbecco's modified Eagle's medium with high glucose (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin. PERK$^{+/+}$, PERK$^{-/-}$, GCN2$^{+/+}$, and GCN2$^{-/-}$ mouse embryonic fibroblasts were kind gifts from Dr. R. Wek (Indiana University). Atg5$^{+/+}$ and Atg5$^{-/-}$ mouse embryonic fibroblasts were kind gifts from Dr. T. Yoshimori (Osaka University). HEK-293 and HEK-293T cells were transfected using Lipofectamine (Invitrogen), whereas mouse embryonic fibroblasts were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfection of siRNA for Bag3 (target sequence, gcaugccagaaaccacuca), HspB8 (target sequence, agagcaguuucaacaacga), and a control sequence (Dharmacon siCONTROL non-targeting siRNA) were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

SDS-soluble and -insoluble Cell Extracts—For PAGE analysis of SDS-soluble and SDS-insoluble proteins, the cells were scraped, homogenized, and heated for 10 min at 100 °C in 2% SDS sample buffer supplemented with 50 mM dithiothreitol. After centrifugation for 20 min at room temperature, two fractions, the supernatants and the SDS-insoluble pellets, were obtained. The supernatants were used as the SDS-soluble fraction. The SDS-insoluble pellets were incubated with 100% formic acid for 30 min at 37 °C, lyophilized overnight, and finally resuspended in 2% SDS sample buffer. Both SDS-soluble and SDS-insoluble fractions were processed for Western blotting.

Recombinant HspB8 Protein Production—Human HspB8 recombinant protein was prepared as previously described (26). The recombinant HspB8 protein concentration was determined using an extinction coefficient of 1.22 for a 1 mg/ml solution at 280 nm. Concentrations were calculated relative to the monomeric protein (HspB8, 22 kDa).

In Vitro Translation Studies—1 μg of pHDQ43-HA encoding a HA-tagged Huntingtin exon 1 fragment with 43 CAG repeats (28) or 1 μg of a vector encoding for luciferase were translated at 30 °C for 2 h in 50 μl of TNT T7 Quick Master Mix (Promega) supplemented with 20 μCi of [^{35}S]methionine alone or with different amounts of recombinant HspB8 or bovine serum albumin (Sigma). When indicated, equal aliquots were removed at different time points (1 and 2 h). 5 μl of each RRL reaction were resolved by SDS-PAGE and visualized by autoradiography. Semiquantitative data on the specific protein levels were obtained by measuring the gray values of the film autoradiograms using the ImageQuant software (Version 1.11).

In Vitro mRNA Decay Assay—Equal amounts of *in vitro* transcribed Huntingtin exon 1 fragment with 43 CAG repeat (Htt43Q) mRNA labeled with [α-^{32}P]CTP were incubated at 30 °C for up to 2 h in 50 μl of TNT T7 Quick Master Mix

(Promega); where indicated, 12 μg of recombinant HspB8 were also added to the reaction mix. Equal aliquots were removed at different time points (1 or 2 h), resolved by SDS-PAGE, and visualized by autoradiography. Semiquantitative data on the specific [α-^{32}P]CTP-labeled mRNA levels were obtained by measuring the gray values of the film autoradiograms using the ImageQuant software (Version 1.11).

RESULTS

Overexpression of HspB8 and Bag3 Results in Induced eIF2α Phosphorylation—We previously showed that upon transient transfection, HspB8 alone and Bag3 alone can induce LC3 lipidation, thus facilitating mutated huntingtin aggregates clearance. Interestingly, stabilization of HspB8 was observed after transient transfection of Bag3, and similar results were also obtained when both HspB8 and Bag3 were simultaneously cotransfected (8). All together these findings suggest that HspB8 and Bag3, which form in cells a stable and stoichiometric complex, likely work through the same pathway. To assess whether HspB8 and Bag3 may exert protein quality control functions that involve the eIF2α pathway, we first tested whether their overexpression affected the phosphorylation levels of endogenous eIF2α. Indeed, an increase in eIF2α phosphorylation was observed upon transient transfection of either HspB8 or Bag3 alone (Fig. 1*A* and supplemental Fig. S1, *A*, *B*, and *D*). Similar effects on eIF2α phosphorylation were observed upon cotransfection of HspB8 and Bag3 (supplemental Fig. S1, *A* and *B*). This effect of Bag3 was independent of Hsc70/Hsp70, as deletion of the BAG domain (ΔBAG), which disrupts Bag3 interaction with Hsc70/Hsp70 (30, 31), did not affect its ability to induce phospho-eIF2α (Fig. 1*A* and supplemental Fig. S1*D*). Similarly, deletion of the WW domain, a module interacting with proline-containing ligands, did not decrease Bag3 ability to modulate eIF2α phosphorylation (Fig. 1*A*). In contrast, overexpression of a deletion mutant of Bag3 lacking the P*XX*P region (ΔP*XX*P) did not result in any significant induction of eIF2α phosphorylation (Fig. 1*A*).

We previously showed that the ΔP*XX*P mutant was incapable of stimulating mutated huntingtin clearance, whereas the ΔBAG mutant was still effective (25). This suggests that the induction of eIF2α phosphorylation mediated by HspB8 and Bag3 might possibly be related to its effect on protein quality control. To directly test this hypothesis within one cell system, we transfected HEK-293 cells with a vector encoding a GFP-tagged Huntingtin exon 1 fragment with 74 CAG repeats (GFP-Htt74Q) alone or together with HspB8, Bag3, or the various Bag3 mutants. 48 h post-transfection we analyzed the accumulation of GFP-Htt74Q in both SDS-soluble and SDS-insoluble fractions (Fig. 1, *B* and *C*). We found that HspB8, Bag3, and the ΔBAG and ΔWW mutants all significantly reduced protein aggregation, whereas the ΔP*XX*P did not (Fig. 1*C*), which perfectly correlated with their effects on endogenous eIF2α phosphorylation. Interestingly, besides reduced aggregation, all proteins that were functional to decrease the accumulation of SDS-insoluble huntingtin also lowered the total amount of SDS-soluble huntingtin (Fig. 1*B*). Correlation between the anti-aggregation activity of HspB8, Bag3, and ΔBAG (supplemental Fig. S1*C*) and the induction of eIF2α phosphorylation (supplemental Fig. S1*D*) was further confirmed using the Filter Trap Assay technique.

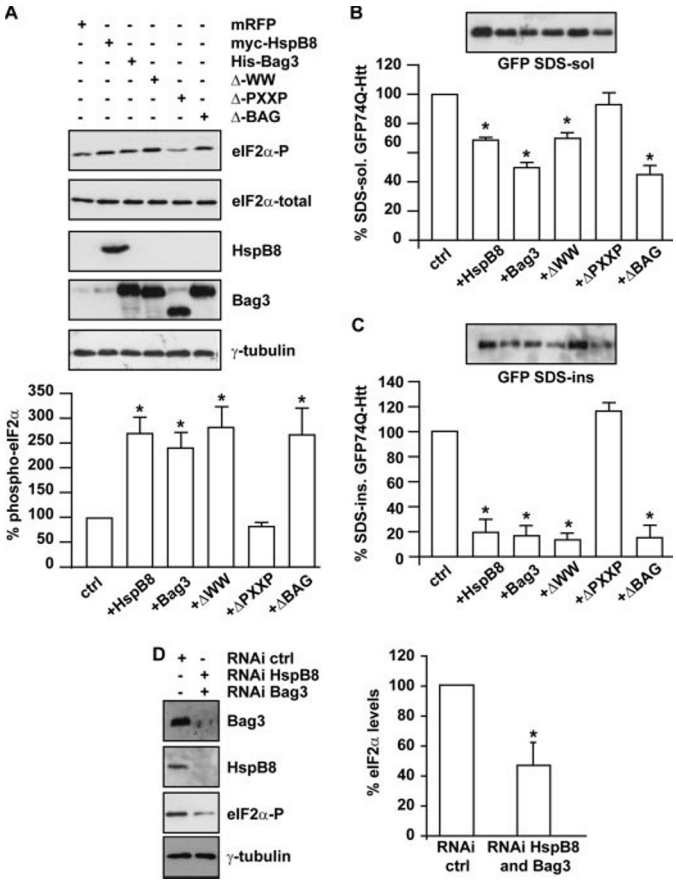

FIGURE 1. **HspB8 and Bag3 induce eIF2α phosphorylation.** *A*, overexpression of HspB8, Bag3, ΔWW, andΔBAG, but not its deletion mutant ΔP*XX*P, significantly induces phospho-eIF2α in HEK-293 cells. Expression levels of the transfected proteins, of total and phospho-eIF2α, and of γ-tubulin (used as a loading control) were analyzed by Western blotting. Quantification of phospho-eIF2α is reported (*, $p < 0.001$; average values ± S.E. of $n > 3$ independent experiments). *B* and *C*, induction of phospho-eIF2α correlates with HspB8·Bag3 anti-aggregation activity. Myc-HspB8, His-Bag3, ΔWW, and ΔBAG, but not ΔP*XX*P, decreased both SDS-soluble and -insoluble levels of GFP-Htt74Q (*, $p < 0.001$; average values ± S.E. of $n = 3$ independent experiments). *D*, knocking down HspB8 and Bag3 significantly decreases phospho-eIF2α levels. HEK-293 cells were transfected with an RNAi control or with RNAi directed against HspB8 and Bag3. 48 h later the cells were transfected with a vector encoding mRFP, a normally folded substrate. Total protein extracts were prepared at 72 h post-transfection. Expression levels of endogenous HspB8, Bag3, phospho-eIF2α, and γ-tubulin (used as a loading control) were analyzed by Western blotting (*, $p < 0.05$; average values ± S.E. of $n = 5$ independent samples).

To assess the importance of translational control in the mechanism of action of HspB8 and Bag3, we next investigated whether knocking down HspB8 and Bag3 could affect the phosphorylation of eIF2α. For this purpose, HEK-293 cells were transfected for 72 h with RNAi targeting HspB8 and Bag3 or with an RNAi control. Knocking down HspB8 and Bag3 caused a significant decrease in phospho-eIF2α levels (Fig. 1*D*), which is in line with our earlier findings that down-regulation of HspB8 and Bag3 enhanced mutant huntingtin aggregation. All together, these results provide further strong evidence that HspB8 and Bag3, which form a stable and stoichiometric complex in cells, indeed work as a functional complex activating protein quality control through the same eIF2α-related pathway.

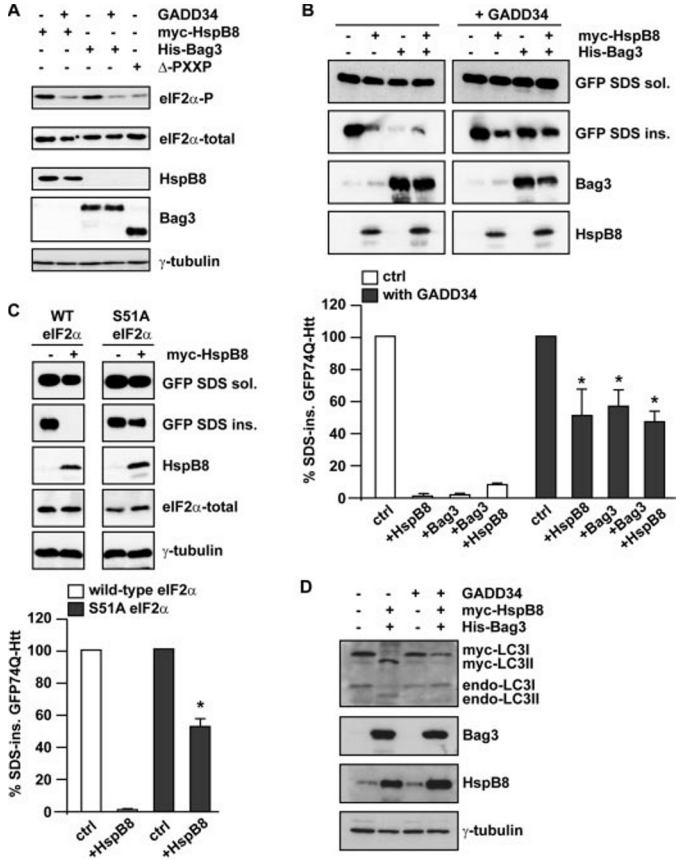

FIGURE 2. **Induction of eIF2α phosphorylation by HspB8·Bag3 is required to suppress huntingtin aggregation and to stimulate autophagy.** *A*, cotransfection with GADD34 blocks HspB8·Bag3-mediated eIF2α phosphorylation. HEK-293 cells were transfected for 24 h with Δ*PXX*P, Myc-HspB8, or His-Bag3. Where indicated (+), GADD34 was also cotransfected. *B*, HEK-293 cells were transfected with vectors encoding for GFP-Htt74Q and either mRFP, His-Bag3, Myc-HspB8, and GADD34. SDS-soluble and SDS-insoluble fractions were prepared 48 h post-transfection, and mutated huntingtin aggregation was analyzed by Western blotting using a GFP antibody. Endogenous levels of γ-tubulin were analyzed as loading control (*ctrl*). Cotransfection of GADD34 significantly decreased HspB8, Bag3, and HspB8·Bag3 ability to decrease mutated huntingtin aggregation (*, $p < 0.001$; average values ± S.E. of $n > 3$ independent experiments). *C*, cotransfection of HspB8 with the eIF2α S51A non-phosphorylatable mutant significantly decreases its effect toward GFP-Htt74Q. HEK-293 cells were transfected with vectors encoding for wild-type (*WT*) eIF2α or the S51A mutant and GFP-Htt74Q plus either mRFP or Myc-HspB8. Samples were prepared and analyzed as described in *B* (*, $p < 0.001$; average values ± S.E. of $n > 3$ independent experiments). *sol.*, soluble; *ins.*, insoluble. *D*, stimulation of autophagy by HspB8·Bag3 requires eIF2α phosphorylation. HEK-293T cells were transfected for 48 h with Myc-LC3 and either an empty vector, HspB8, and His-Bag3; where indicated (+), GADD34 was overexpressed. Levels of both Myc-tagged overexpressed and endogenous LC3 I/II were analyzed by Western blotting using a specific human LC3 antibody.

Blocking eIF2α Phosphorylation Inhibits HspB8·Bag3 Ability to Facilitate Mutated Huntingtin Clearance—To further test the importance of eIF2α phosphorylation for the anti-aggregation effects of HspB8 and Bag3, HEK-293 cells were cotransfected with HspB8 or Bag3 and a vector encoding for the hamster C-terminal domain of the growth arrest and DNA damage-inducible protein, GADD34 (32). GADD34 binds the eukaryotic serine/threonine phosphatase protein phosphatase 1 (PP1) to direct eIF2α dephosphorylation (33). As can be seen in Fig. 2*A*, GADD34 prevented both HspB8 and Bag3 from inducing eIF2α phosphorylation.

We next cotransfected GADD34 and GFP-Htt74Q with either mRFP (used as a control protein), HspB8, and/or Bag3 and analyzed the accumulation of GFP-Htt74Q in both SDS-soluble and SDS-insoluble fractions. GADD34 significantly decreased the protective effect of HspB8, Bag3, and HspB8·Bag3 on huntingtin aggregation (Fig. 2*B*). The implication of eIF2α phosphorylation in the mechanism of action of the HspB8·Bag3 complex was further supported using the S51A non-phosphorylatable mutant of eIF2α (34, 35). Overexpression of this S51A mutant, but not of wild-type eIF2α, together with HspB8 (Fig. 2*C*) and Bag3 (data not shown) significantly impaired their ability to suppress GFP-Htt74Q aggregation. Therefore, eIF2α phosphorylation seems to be required for HspB8 and Bag3 to suppress the accumulation of GFP-Htt74Q aggregates.

The HspB8·Bag3 Chaperone Complex Induces Atg8 (LC3) Lipidation in an eIF2α Phosphorylation-dependent Manner—Induction of eIF2α phosphorylation can result in autophagy stimulation, monitored by the increased Atg12 transcription and the increased conversion of LC3 I into the lipidated form LC3 II (36). We previously showed that overexpression of HspB8·Bag3 also stimulates the turnover of LC3 and postulated this as the mechanism for the clearance of huntingtin aggregates (8). Interestingly, eIF2α phosphorylation and autophagy induction are two mechanistically linked events. Thus, on the basis of our current findings, we next asked whether phosphorylation of eIF2α may represent a key event required for HspB8·Bag3-mediated autophagy stimulation. HEK-293T cells were cotransfected with Myc-LC3, HspB8·Bag3, and either an empty vector or a vector encoding for GADD34. Both levels of transfected Myc-LC3 and endogenous LC3 were analyzed using a specific antibody raised against the human LC3 protein (8). Whereas overexpression of HspB8·Bag3 indeed induced LC3 lipidation (Fig. 2*D*), cotransfection of GADD34 significantly decreased their ability to stimulate LC3 I to II conversion (Fig. 2*D*). This result strongly suggests that the stimulation of autophagy mediated by HspB8·Bag3 requires the induction of eIF2α phosphorylation.

HspB8·Bag3 Inhibits Protein Synthesis of Cotransfected Substrates and Still Decreases Mutated Huntingtin Aggregation in Autophagy-deficient Cells—To further explore the implication of autophagy in the mechanism of action of HspB8·Bag3, we tested their effectivity in autophagy-deficient ($Atg5^{-/-}$) mouse embryonic fibroblasts (MEFs). More aggregated mutated huntingtin was seen in $Atg5^{-/-}$ fibroblasts than in autophagy-proficient, $Atg5^{+/+}$, fibroblasts (Fig. 3*A*, *first through fourth lanes*). This confirms earlier reports that loss of constitutive autophagy activity decreases the cellular ability to cope with mutated huntingtin (17, 18). Intriguingly, overexpression of HspB8·Bag3 could still substantially decrease GFP-Htt74Q aggregation in these $Atg5^{-/-}$ cells. However, the reduction in SDS-soluble levels of GFP-Htt74Q seen after HspB8·Bag3 overexpression in HEK-293 cells (Figs. 1 and 2) or in $Atg5^{+/+}$ cells (Fig. 3*A*, *third lane*) were much less pronounced in $Atg5^{-/-}$ cells (Fig. 3*A*, *sixth lane*; 5–10-fold reduction in clearance efficiency).

In analogy to the effects of HspB8·Bag3, treatment with rapamycin was also effective in both $Atg5^{+/+}$ and $Atg5^{-/-}$ cell lines in decreasing mutated huntingtin aggregation (Fig. 3*A*, *second*

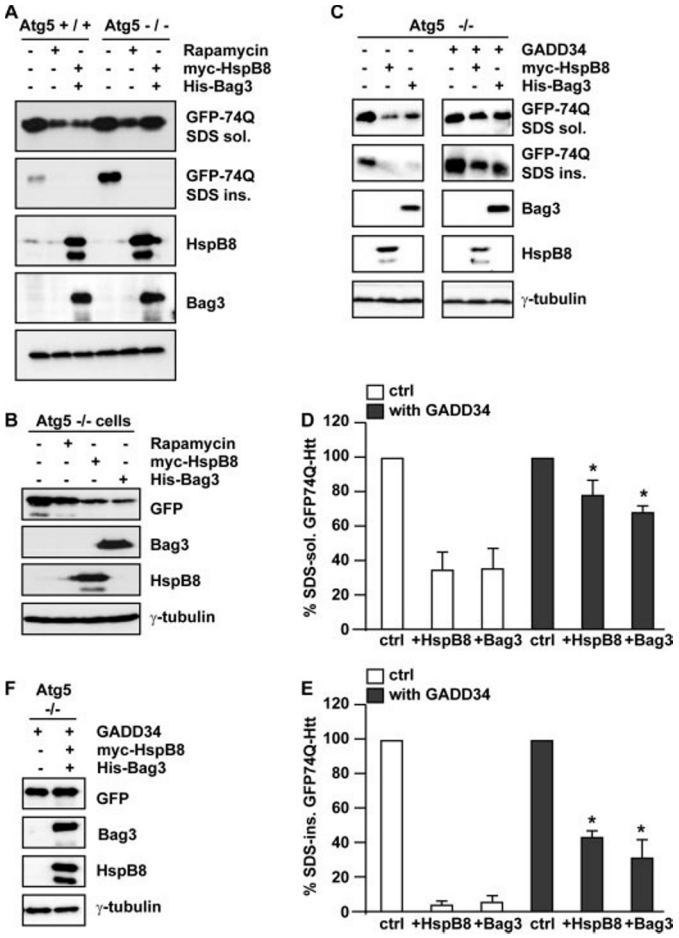

FIGURE 3. **HspB8·Bag3 decreases mutated huntingtin aggregation also in Atg5$^{-/-}$ cells.** *A*, HspB8·Bag3, like rapamycin, are still able to decrease mutated huntingtin aggregation in Atg5$^{-/-}$ cells. Atg5$^{+/+}$ and Atg5$^{-/-}$ mouse embryonic fibroblasts were transfected with vectors encoding for GFP-Htt74Q and either mRFP or Myc-HspB8 and His-Bag3. Where indicated (+), cells transfected with GFP-Htt74Q were treated for 48 h with rapamycin (200 nM). A 5–10-fold increase in GFP74Q-Htt SDS soluble levels was observed in Atg5$^{-/-}$ *versus* Atg5$^{+/+}$ cells cotransfected with HspB8·Bag3. *B*, Atg5$^{-/-}$ cells were transfected and treated as described in Fig. 5*A*, with the exception that GFP, a normally folded substrate, was used instead of GFP-Htt74Q. The effect of HspB8 and Bag3 overexpression and of rapamycin treatment on GFP protein expression was analyzed by Western blotting 48 h later. *C–F*, cotransfection of GADD34 (+) with HspB8·Bag3 in Atg5$^{-/-}$ cells dramatically decrease their effect on GFP-Htt74Q (*C–E*) and on GFP protein synthesis inhibition (*F*). Quantification of GFP-Htt74Q SDS-soluble (*sol., D*) and SDS-insoluble (*ins., E*) levels is reported (*, $p < 0.001$; average values ± S.E. of $n > 3$ independent experiments). *ctrl*, control.

through fourth lanes). This is in line with recent findings showing that, besides autophagy, rapamycin inhibits protein synthesis, which by decreasing the total level of mutated polyglutamine protein also contributes to reduce the formation of aggregates (24). To test whether protein synthesis inhibition may also play a role in the mechanism of action of HspB8·Bag3, we analyzed in Atg5$^{-/-}$ cells their effect on the expression levels of GFP, a normally folded globular protein. Clearly, HspB8 and Bag3 significantly reduced the levels of GFP (Fig. 3*B*, *third* and *fourth lanes*) to an even higher extent than treatment with rapamycin (Fig. 3*B*, *second lane*). This result further suggests that HspB8·Bag3 overexpression may cause translational shutdown besides inducing autophagy. Similar results on GFP expression levels were obtained in HEK-293 cells (supplemental Fig. S2). Furthermore, in Atg5$^{-/-}$ cells cotransfection with GADD34 substantially decreased the ability of HspB8 and Bag3 to prevent aggregation of mutated huntingtin (Fig. 3, *C* and *E*). Moreover, in these GADD34-overexpressing Atg5$^{-/-}$ cells, the soluble levels of GFP-Htt74Q were no longer reduced by HspB8·Bag3 (Fig. 3, *C* and *D*). The implication of eIF2α-mediated protein synthesis inhibition in the mechanism of action of HspB8·Bag3 was further supported by its lack of effect on GFP expression levels in Atg5$^{-/-}$ cells overexpressing GADD34 (Fig. 3*F*). Again, similar data are observed when HspB8 or Bag3 are overexpressed alone or when they are simultaneously coexpressed, further supporting that they work through the same pathway.

To more directly investigate whether HspB8·Bag3 can indeed act on protein synthesis, we used an *in vitro* translation assay. Recombinant HspB8 was added to reticulocyte rabbit lysates (RRL), in which phosphorylation of eIF2α also leads to a shutdown of protein synthesis (37). Indeed, the addition of recombinant HspB8, but not of glutathione *S*-transferase, to the RRL caused a significant increase in the phosphorylation of eIF2α (Fig. 4*A*) without affecting the total levels of eIF2α, which were similar in all conditions (data not shown). The addition of increasing amounts of recombinant HspB8 significantly decreased the translation of both a normally folded protein (Fig. 4*B*, *Luciferase*) and of mutated HA-Htt43Q (Fig. 4*C*). The addition of increasing amounts of recombinant bovine serum albumin (3–18 μg), which was used as negative control, did not significantly affect mutated HA-Htt43Q protein synthesis (data not shown). By an *in vitro* mRNA decay assay it was found that HA-Htt43Q mRNA levels were comparable after 1 or 2 h of incubation with recombinant HspB8 (Fig. 4*D*) at time points at which HA-Htt43Q protein levels were already dramatically decreased (Fig. 4*C*). This excludes the possibility that the reductions in protein levels seen after HspB8 addition were due to effects on mRNA degradation.

All together these results demonstrate that besides inducing autophagy, HspB8 also causes a translation shutdown. Both these processes likely play a role in HspB8 effect on preventing protein aggregation, and both require eIF2α phosphorylation.

Mutation of Lys-113, Which Disrupts HspB8 Kinase Activity, Does Not Affect the HspB8-mediated Induction of eIF2α Phosphorylation—HspB8, also known as H11/Hsp22/E2IG1, shows sequence similarity to the protein kinase coding domain of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) (38). Smith *et al.* (38) showed that HspB8 has a Mn^{2+}-dependent protein kinase activity and identified the lysine 113 as the amino acid residue essential for this activity. The autokinase activity of HspB8 as well as its ability to phosphorylate myelin basic protein were observed by other independent groups (1, 39). Other investigators, however, could not demonstrate that HspB8 is able to phosphorylate substrates like α-casein and histone IIIS (2). Thus, the kinase activity of HspB8 is to date still under debate (40, 41). Notwithstanding the open debate, we decided to mutate the HspB8 lysine residue 113 into a glycine to disrupt its putative kinase activity and tested the ability of both wild-type HspB8 and the presumed kinase-dead K113G mutant to induce eIF2α phosphorylation in HEK-293 cells. Fig. 5 shows that both forms of

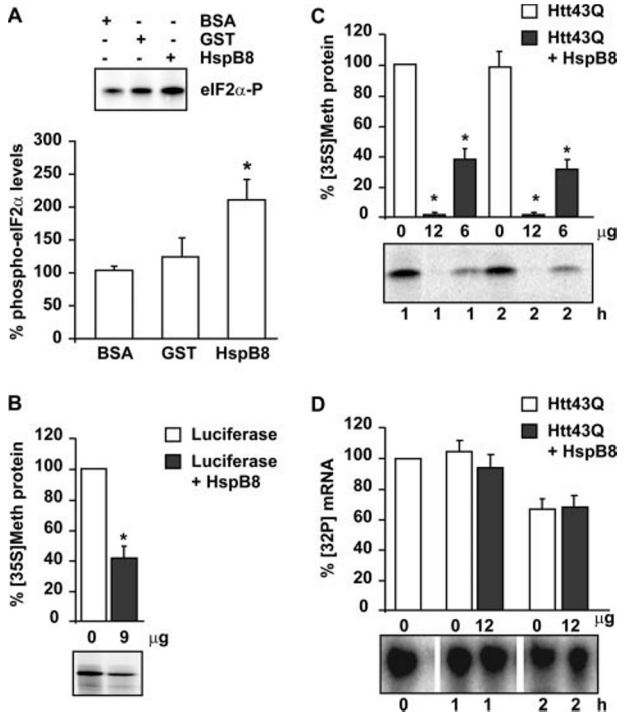

FIGURE 4. **Recombinant HspB8 induces eIF2α phosphorylation and inhibits protein synthesis in reticulocyte rabbit lysates.** *A*, 10 μl of RRL reaction were incubated at 30 °C for 90 min in the presence of either bovine serum albumin (*BSA*), glutathione *S*-transferase (*GST*; used as negative control), or recombinant HspB8. After the addition of 1 volume of 4% SDS buffer, an aliquot of each sample was migrated and probed with a specific phospho-eIF2α antibody. *, $p < 0.05$; average values ± S.E. of $n = 5$ independent samples. *B*, 1 μg of vector encoding for luciferase was incubated at 30 °C for 2 h in 50 μl of RRL reaction without the addition of purified recombinant protein (0 μg, *white columns*) or with the addition of purified recombinant HspB8 (9 μg, *dark gray columns*). *C*, 1 μg of vector encoding for HA-Htt43Q was incubated at 30 °C for 2 h in 50 μl of RRL reaction without the addition of purified recombinant protein (0 μg, *white columns*) or with the addition of purified recombinant HspB8 (6 or 12 μg, *dark gray columns*). RRL reactions were incubated for 1 h or 2 h. [^{35}S]Methionine-labeled translation products were resolved by SDS-PAGE and were visualized by autoradiography. *D*, recombinant HspB8 does not affect mutated huntingtin (Htt43Q) mRNA stability *in vitro*. Equal amounts of *in vitro* transcribed [α-^{32}P]CTP-labeled Htt43Q mRNA were added to 50 μl of RRL reaction and incubated without the addition of any exogenous recombinant protein (*white columns*) or with 12 μg of HspB8 (*dark gray columns*). Equal amounts of the RRL reactions were incubated for 0, 1, or 2 h, resolved by electrophoresis, and visualized by autoradiography. *B–D*, *, $p < 0.001$; average values ± S.E. of $n > 3$ independent experiments).

HspB8 are equally active in inducing phospho-eIF2α. This result suggests that this putative kinase activity of HspB8 is not involved in the modulation of eIF2α phosphorylation.

HspB8·Bag3-mediated Induction of eIF2α Phosphorylation Is PERK-independent—Induction of eIF2α phosphorylation is a crucial response upon endoplasmic reticulum (ER) stress and it is mainly mediated by the ER resident PERK kinase (42, 43). Activation of the PERK/eIF2α signaling pathway occurs after overexpression of mutated huntingtin, and interestingly, it has been recently shown that PERK/eIF2α mediates the stimulation of autophagy (36). To test whether the HspB8·Bag3 complex requires PERK as key molecule to exert its effect on protein synthesis and autophagy, wild-type ($PERK^{+/+}$) and knock-out ($PERK^{-/-}$) MEFs were transfected with a vector encoding GFP-Htt74Q alone or together with HspB8 or Bag3. The total expression level of GFP-Htt74Q was analyzed by SDS-PAGE at

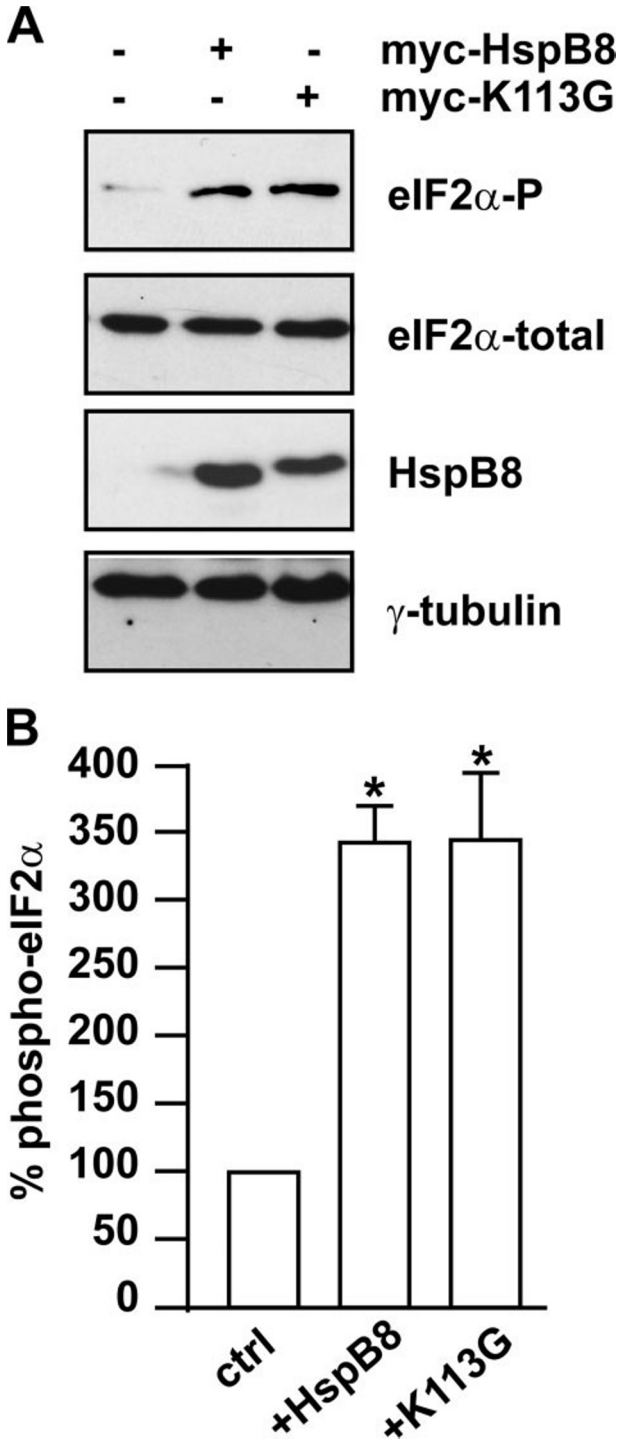

FIGURE 5. **Overexpression of the K113G HspB8 mutant, with disrupted kinase activity, still induces eIF2α phosphorylation.** HEK-293 cells were transfected for 24 h with Myc-HspB8 or Myc-K113G. Expression levels of transfected HspB8 of total and phospho-eIF2α and of γ-tubulin (used as a loading control) were analyzed by Western blotting. Quantification of phospho-eIF2α is reported (*, $p < 0.001$; average values ± S.E. of $n = 3$ independent experiments). *ctrl*, control.

24 h post-transfection (at which time the amount of aggregated GFP-Htt74Q is almost undetectable), whereas the accumulation of both SDS-soluble and -insoluble GFP-Htt74Q was analyzed at 48 h post-transfection. Interestingly, HspB8 and Bag3 were equally efficient in decreasing mutated huntingtin total

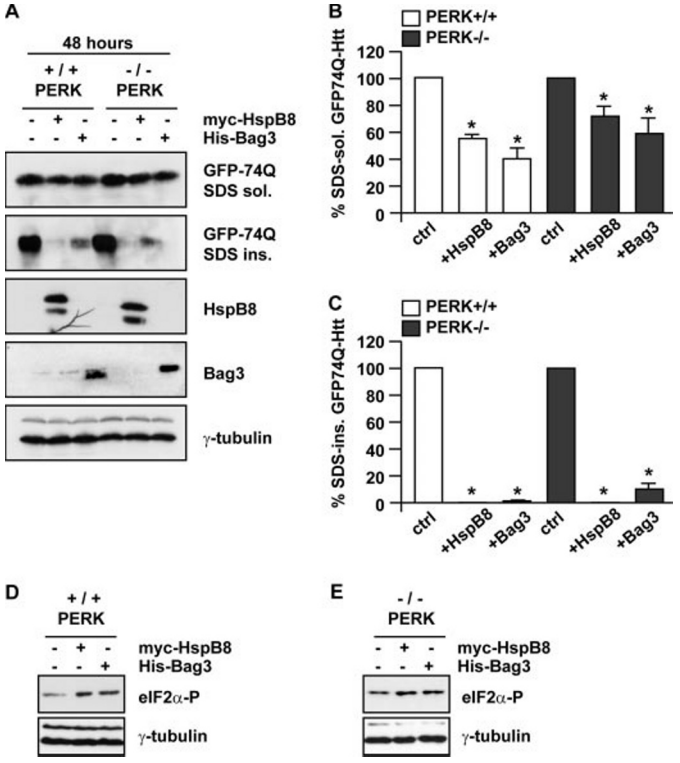

FIGURE 6. **HspB8·Bag3 effect on mutated huntingtin clearance is PERK-independent.** PERK$^{+/+}$ and PERK$^{-/-}$ mouse embryonic fibroblasts were transfected for 48 h with vectors encoding for GFP-Htt74Q and either mRFP, Myc-HspB8, or His-Bag3. HspB8 and Bag3 overexpression significantly decreases GFP-Htt74Q SDS-soluble levels (*A* and *B*) and aggregation (*A* and *C*) in both cell lines (*, $p < 0.001$; average values ± S.E. of $n = 3$ independent experiments). HspB8 and Bag3 induce eIF2α phosphorylation both in PERK$^{+/+}$ (*D*) and PERK$^{-/-}$ (*E*) MEFs. *sol.*, soluble; *ins.*, insoluble.

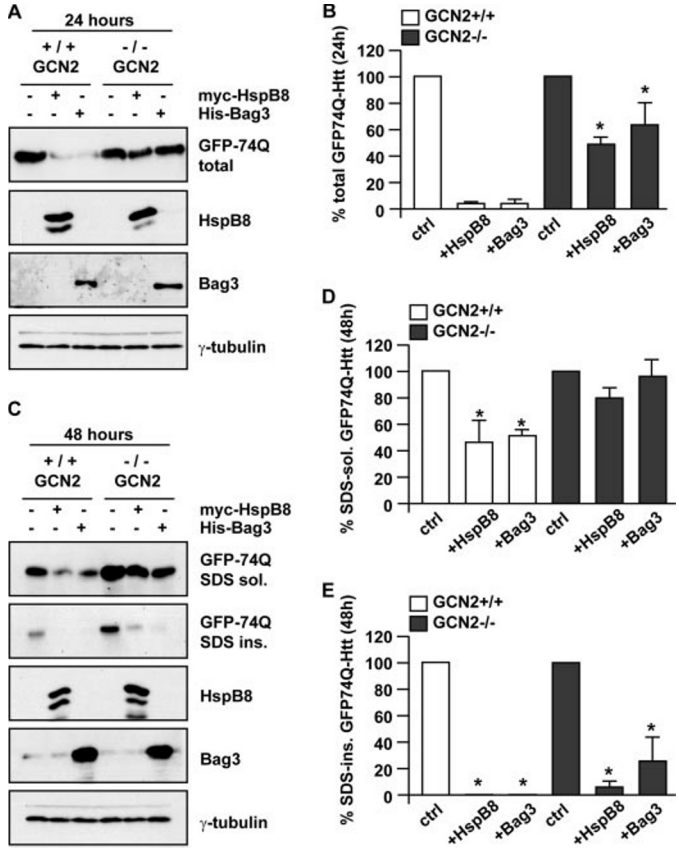

FIGURE 7. **Loss of GCN2 impairs HspB8·Bag3 chaperone-like function.** GCN2$^{+/+}$ and GCN2$^{-/-}$ mouse embryonic fibroblasts were transfected as described in Fig. 6. The effect of HspB8 and Bag3 on total huntingtin levels was analyzed by Western blotting at 24 h post-transfection (*A* and *B*), whereas their effect on GFP74Q-Htt accumulation in both SDS-soluble and SDS-insoluble fractions was analyzed at 48h post-transfection (*C–E*). Quantification of total (*B*), SDS-soluble (*D*), and SDS-insoluble (*E*) GFP74Q-Htt is reported. *A* and *B*, at 24 h post-transfection, in the presence of HspB8 and Bag3, the total levels of GFP74Q-Htt are significantly higher in GCN2$^{-/-}$ than in $^{+/+}$ cells (*, $p < 0.001$; average values ± S.E. of $n > 3$ independent experiments). *ctrl*, control. *sol.*, soluble; *ins.*, insoluble. *D*, 48 h post-transfection overexpression of HspB8 and Bag3 significantly decreases the SDS-soluble levels of GFP74Q-Htt only in GCN2$^{+/+}$ cells (*, $p < 0.001$; average values ± S.E. of $n > 3$ independent experiments). *E*, HspB8 and Bag3 significantly decrease the SDS-insoluble levels of GFP74Q-Htt both in GCN2$^{+/+}$ and GCN2$^{-/-}$ cells (*, $p < 0.001$; average values ± S.E. of $n > 3$ independent experiments).

levels (24 h, data not shown) and SDS-soluble and -insoluble levels (48 h) in PERK$^{+/+}$ and PERK$^{-/-}$ MEFs (Fig. 6, *A–C*). Moreover, transient transfection of HspB8 or Bag3 induced the phosphorylation of eIF2α in both cell lines (Fig. 6, *D* and *E*). Similarly, transfection of HEK-293 cells with the PERK K618A dominant negative mutant, which cannot induce eIF2α phosphorylation, also did not affect HspB8·Bag3 ability to decrease mutated huntingtin aggregation (data not shown). These data strongly suggest that 1) in contrast to mutated polyQ proteins, HspB8·Bag3-mediated eIF2α phosphorylation is PERK-independent, 2) HspB8·Bag3 does not require the ER stress signaling pathway to stimulate autophagy and facilitate mutated huntingtin clearance, and 3) the transfection mediated overexpression of the HspB8·Bag3 complex itself does not cause an ER stress. The latter would have implied that HspB8·Bag3 may eventually also induce cell death, but this was indeed not the case (supplemental Fig. S3).

All together these results strongly suggest that HspB8 is an HspB member that acts in a non-canonical manner together with Bag3 in protein quality control. Increases in HspB8·Bag3 levels lead to inhibition of protein synthesis and simultaneous activation of autophagy in a PERK-independent/eIF2α phosphorylation-dependent manner, and as such, they may protect the cells from the accumulation of misfolded proteins.

Loss of GCN2 Severely Impairs HspB8·Bag3 Function toward Mutated Huntingtin—Another stress kinase that can phosphorylate eIF2α is the general control of nitrogen metabolism kinase (GCN2). GCN2 is activated upon amino acid starvation which, interestingly, robustly stimulates autophagy (44, 45). Because of the ability of HspB8 and Bag3 to modulate autophagy through the induction of eIF2α phosphorylation (in a PERK-independent manner), we next asked whether their function would be impaired by the loss of the GCN2 kinase. We transfected immortalized GCN2$^{+/+}$ and GCN2$^{-/-}$ MEFs with a vector encoding GFP-Htt74Q alone or together with HspB8 or Bag3. Interestingly, HspB8 and Bag3 were significantly less efficient in decreasing GFP74Q-Htt total levels in GCN2$^{+/+}$ *versus* GCN2$^{-/-}$ cells (Fig. 7). In parallel, HspB8 and Bag3 were also significantly less efficient in decreasing SDS-soluble levels of mutated huntingtin in the GCN2$^{-/-}$ cell line (Fig. 7, *C* and *D*), although they could still significantly decrease huntingtin aggregation in GCN2$^{-/-}$ cell line (Fig. 7*E*). These results suggest that HspB8 and Bag3 may act via the GCN2 kinase but also that alternative routes can be used in case cells lack GCN2.

DISCUSSION

HspB8 is a member of the small heat shock protein family that can form both homo-oligomeric and hetero-oligomeric complexes with other HspB members (46, 47). Like most small Hsps isolated from many different species, HspB8 can act *in vitro* as ATP-independent chaperone (1–3) that, in concert with the ATP-dependent chaperones members of the Hsp70 family, can assist in protein quality control (48–52). Our original findings in cells that HspB8 overexpression can suppress aggregation of misfolded proteins like mutated huntingtin (8, 26) would be consistent with its *in vitro* chaperone activity. The finding that it interacts *in vivo* with Bag3, a member of the Bag family of proteins, co-chaperone of Hsc70/Hsp70 (29, 31, 53), further seems to support a classical model of action. In such a model HspB8 complexes would bind denatured proteins, and via Bag3, they would transfer the clients to Hsp70 proteins for further processing, thus avoiding client aggregation. Such a scenario may also be supported by the observation that several Bag proteins act as a link between the ubiquitin/proteasome system and Hsp70, thus modulating substrate processing (54–59).

However, in cells we never found any evidence for stable interaction between HspB8 and any other member of the HspB family (8). Rather, HspB8 was found to almost exclusively co-immunoprecipitate with Bag3 in a stoichiometric 2:1 ratio (8). Also, the HspB8·Bag3 mediated prevention of huntingtin aggregation was independent on the BAG domain of Bag3 (25), suggesting that the complex does not require Hsc70/Hsp70 for its function in protein quality control. Finally, here we show that the HspB8·Bag3 complex also affected the expression levels of the globular and properly folded GFP protein, strongly pointing to a non-classical chaperone-like mode of action.

Rather than via the classical chaperoning model, we found that HspB8·Bag3 acts upstream the α-subunit of the translation initiator factor eIF2. Overexpression of HspB8 alone, Bag3 alone, or simultaneous overexpression of HpsB8 and Bag3 induces eIF2α phosphorylation, which in turn modulates protein synthesis and autophagy. In fact, blocking eIF2α phosphorylation suppresses the effects of HspB8·Bag3 on protein quality control and on LC3 lipidation, indicating that this step is required for these functions of the complex. HspB8·Bag3 are both stress-related proteins whose expression levels are up-regulated by several stress inducers, including heat shock, heavy metal treatment, and viral infection (4, 60–62). Besides the classical chaperones that are up-regulated to refold the misfolded proteins that accumulate after such forms of stress, up-regulation of HspB8·Bag3 may serve as an additional and separate protective response aimed at reducing the total load of proteins for the quality control system. This aim would be achieved by both inducing a general translational arrest (affecting all protein levels) and by facilitating the autophagy-mediated degradation of aggregation-prone substrates. As such, the actions of HspB8 and Bag3 are highly comparable with what is observed after treatment of cells with rapamycin. This drug is mostly known for its stimulatory effect on autophagy and its neuroprotective effect against mutated polyglutamine induced neurodegeneration (18); however, rapamycin also seems to act on eIF2α phosphorylation and protein synthesis inhibition (24, 63, 64). As we report here for the HspB8·Bag3 complex, this protein synthesis inhibition and autophagy stimulation, both, contribute to the protective effect of rapamycin against aggregation of mutated huntingtin (24).

One possible artifact was that overexpression of HspB8·Bag3 by itself could induce a cellular stress, especially knowing that eIF2α phosphorylation response is generally thought to be stress-induced (65). However, eIF2α phosphorylation induced by misfolded or mutated proteins is PERK-mediated (36), whereas we show here that HspB8·Bag3-mediated eIF2α phosphorylation is PERK-independent. This suggests that overexpression of the HspB8·Bag3 complex itself did not induce the unfolded protein response. Moreover, it also did not cause any significant induction of cell death, thus likely excluding that HspB8·Bag3 overexpression may result in a cellular stress. Finally, we previously showed that decreasing the endogenous levels of the HspB8·Bag3 complex by siRNA technique caused a significant increase in mutated huntingtin aggregation and also impaired the basal autophagy activity (8). Therefore, modulating HspB8·Bag3 levels seem to represent a physiological stress-dependent response and point to a new role for HspB8·Bag3 in activating a pro-survival pathway in which eIF2α-mediated translational arrest and autophagy stimulation are key events. This is further suggested by the findings that knocking-down HspB8 and Bag3, which increases mutated protein aggregation and decreases basal autophagy activation (8), also results in less eIF2α phosphorylation.

At this stage the upstream player(s) converting the up-regulation of HspB8·Bag3 into a signal responsible for the phosphorylation of eIF2α is yet to be identified. Part of the effects of HspB8 and Bag3 on protein quality control may take place through the activation of the GCN2 kinase, as suggested by their decreased efficiency in $GCN2^{-/-}$ cells, but alternative routes also may play a role. So far we did not find any evidence that the complex itself can act as a kinase to directly phosphorylate eIF2α. In fact, the HspB8 dead-kinase mutant (K113G) was still able to significantly induce eIF2α phosphorylation. The results obtained with the ΔBAG mutant exclude roles for Hsp70 or Bcl-2 proteins, supporting a non-canonical mechanism of action. Moreover, considering that among the proteins containing a WW domain are ubiquitin ligases (*e.g.* Nedd4), which modulate the ubiquitination and proteasomal degradation of specific substrates (66–68), our result with the ΔWW may likely exclude the implication of the ubiquitin/proteasome system in the mechanism of action of Bag3. However, no protein interacting with the WW domain of Bag3 as well as its function has been yet identified. Finally, our data with the ΔP*XX*P mutant suggest that through this module the HspB8·Bag3 complex interacts with a factor essential in modulating, indirectly, eIF2α phosphorylation. To date, the only protein known to interact with Bag3 P*XX*P domain is phospholipase Cγ1 (69). However, knocking down is phospholipase Cγ-1 by siRNA technique did not affect the HspB8·Bag3 effect toward mutated huntingtin accumulation and aggregation (25) (see also supplemental Fig. S4). Further studies will be focused on the identification of other P*XX*P ligands to gain new insight into HspB8·Bag3 mechanisms of action.

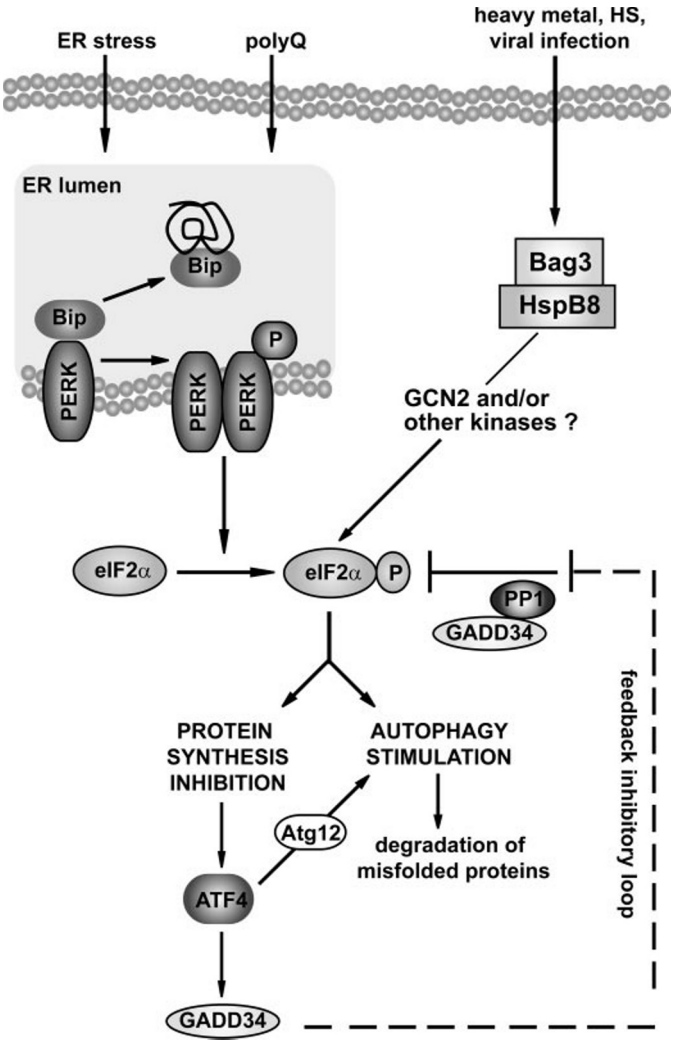

FIGURE 8. **Schematic model showing the putative mechanism of action of HspB8·Bag3.** The accumulation of misfolded proteins like mutated polyglutamine proteins (*polyQ*) provokes the ER stress. As a consequence, Bip dissociates from the PKR-like ER kinase (PERK), causing its dimerization, phosphorylation, and activation. This in turn leads to the phosphorylation of eIF2α, thereby causing a translational shutdown and stimulation of autophagy. Up-regulation of the HspB8·Bag3 complex (which can be achieved by transient transfection or after stress stimuli, including heat shock, heavy metal treatment, or viral infection) induces phospho-eIF2α with a yet unidentified mechanism in which the GCN2 kinase may be involved (GCN2 and/or other kinases?). After eIF2α phosphorylation, the transcription/translation of a specific set of genes is stimulated, among which are the transcription factor ATF4 and, consequently, Atg12 and GADD34. Atg12 participates in stimulating autophagy, whereas GADD34 in turn promotes the dephosphorylation of eIF2α. This feedback inhibitory loop may protect the cells from the deleterious effects of a sustained protein synthesis arrest and hyperactivated autophagy. *HS*, heat shock.

Although the upstream effects remain to be elucidated, the following scenario for the auto-regulatory, stress responsive mechanism of action of the HspB8·Bag3 complex can be hypothesized. Upon stress levels of HspB8·Bag3 rise and participate to increase the levels of phospho-eIF2α (at least in part through the GCN2 kinase). This in turn mediates the up-regulation of the transcription factor ATF4 (the mammalian functional homologue of yeast GCN4, whose expression is up-regulated after the activation of the GCN2 kinase) (36, 65, 70, 71). Indeed a significant increase in both ATF4 mRNA and protein levels has been observed in HEK-293 cells overexpressing HspB8 and Bag3 (supplemental Fig. S5), and conversely, knocking down HspB8 and Bag3 by RNAi treatment decreased the endogenous levels of both phospho-eIF2α and ATF4 (Fig. 1*D* and supplemental Fig. S5). The up-regulation of ATF4 would then 1) induce Atg12 expression, which would stimulate autophagy (36) and 2) induce GADD34 expression (32, 72), which would lead to the dephosphorylation of eIF2α, acting as an inhibitory feedback loop to inhibit the action of HspB8·Bag3 (Fig. 8). This feedback inhibitory mechanism would avoid a sustained arrest of protein synthesis and hyperactivation of autophagy, which may result in cell toxicity and death (73).

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