BAG3 induces the sequestration of proteasomal clients into cytoplasmic puncta: implication for a proteasome-to-autophagy switch

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Abstract

Eukaryotic cells use autophagy and the ubiquitin–proteasome system as their major protein degradation pathways. Upon proteasomal impairment, cells switch to autophagy to ensure proper clearance of clients (the proteasome-to-autophagy switch). The HSPA8/HSPA1A co-chaperone BAG3 has been suggested to be involved in this switch. However, at present it is still unknown whether and to which extent BAG3 can indeed re-route proteasomal clients to the autophagosomal pathway. Here, we show that BAG3 induces the sequestration of ubiquitinated clients into cytoplasmic puncta co-labelled with canonical autophagy linkers/markers. Following proteasome inhibition, BAG3 upregulation significantly contributes to the compensatory activation of autophagy and to the degradation of the (poly) ubiquitinated proteins. BAG3 binding to the ubiquitinated clients occurs through the BAG domain, in competition with BAG1, another BAG family member, that normally directs ubiquitinated clients to the proteasome. Therefore, we propose that following proteasome impairment, increasing the BAG3/BAG1 ratio ensures the “BAG-Instructed Proteasomal to Autophagosomal Switch & Sorting” (BIPASS).
Introduction

The aggregation of misfolded, mutated proteins is a common basis for many adult onset neurodegenerative diseases (e.g. Parkinson disease, Huntington disease, Spinocerebellar Ataxias, Spinal and Bulbar Muscular Atrophy, Amyotrophic Lateral Sclerosis). Cells have evolved an elaborate protein quality control system, which acts to facilitate the (re)folding of un- or misfolded protein species by molecular chaperones. When folding is unsuccessful, chaperones can also target the misfolded proteins for degradation, thereby preventing protein aggregation. Intracellular degradation is primarily mediated by two proteolytic systems: the ubiquitin proteasome system and autophagy. The proteasome generally recognizes (poly)ubiquitinated substrates, which are primarily short-lived proteins. The autophagic-lysosomal pathway is responsible mainly for the degradation of long-lived proteins, protein aggregates and entire (damaged) organelles. Sequestration of cytosolic regions by macroautophagy was initially perceived as an “in bulk” process. Nevertheless, there is growing evidence supporting the existence of different forms of selective macroautophagy that include the degradation of misfolded, aggregated and/or (poly)ubiquitinated proteins. These selective forms of macroautophagy are complementary to the ubiquitin-proteasome system and compensatory for proteasomal degradation, when the latter is impaired. Numerous studies have shown that changes in both the ubiquitin proteasome system and the autophagy-lysosome system occur with age. This suggests that a proper balance between these protein quality control systems is required for protein homeostasis, also referred to as proteostasis, and that its alteration may contribute to ageing and disease. However, little is known about mechanisms controlling autophagic degradation of ubiquitinated and/or damaged substrates and, in particular, their re-routing from proteasomal to autophagosomal degradation (here referred to as the proteasome-to-autophagy switch).

Recent studies implicated several members of the BAG (Bcl-2-associated athanogene) protein family in cellular protein quality control. BAG1 has been suggested to bridge HSPA1A-bound clients to the proteasome through its ubiquitin-like domain. BAG3 was found to stimulate the selective degradation of several disease-associated proteins such as polyQ huntingtin and superoxide dismutase-1 (SOD1), associated with Huntington disease and Amyotrophic Lateral Sclerosis, respectively, by the autophagic machinery. To do so, BAG3 cooperates with HSPA8/Hsc70, HSPA1A/Hsp70, and/or HSPB8, a member of the family of small heat shock proteins and with the macroautophagy receptor protein SQSTM1/p62.39

Interestingly, during ageing, a change in the expression of BAG1 and BAG3 seems to occur: whilst BAG1 is expressed at relatively higher levels in young tissue, BAG3 is expressed at relatively higher levels in aged tissue and this has been correlated with a higher proteasomal activity in young tissues and a more intensive use of the
autophagic system in aged tissue. These correlative data suggested that the BAG3/BAG1 expression ratio may play a crucial role in the balance between proteasomal and autophagosomal degradation. However, direct evidence for this hypothesis is yet lacking.

Here, we show that BAG3 interacts with HSPA1A-bound ubiquitinated proteasomal clients and induces their sequestration into cytoplasmic puncta that also contain SQSTM1 and that are next labeled with autophagic markers for degradation. Moreover, in the recovery phase after proteasome inhibition, BAG3 not only is required for the efficient compensatory activation of autophagy, but also for the autophagy-mediated clearance of the accumulating (poly)ubiquitinated clients.

Another issue that has remained unclear to date is which signalling pathway regulates this proteasome-to-autophagy switch. Likely, the accumulation of misfolded and/or (poly)ubiquitinated proteins due to proteasomal failure or saturation may be the trigger for such a switch, implying that either the cytosolic Heat Shock Response (HSR), the Nuclear Factor Kappa B (NFkB) pathway and/or the Unfolded Protein Response in the ER (UPR) might play a role. Interestingly, several studies showed a link between the HSR, the NFkB pathway, as well as the UPR and autophagy activation. However, whether these pathways do so through similar or distinct routes has remained unclear. Here we show that modulation of all these three pathways leads to BAG3 upregulation, further pointing to BAG3 as a key player in the execution of (poly)ubiquitinated client disposal. In summary, our data demonstrate that upon proteotoxic stress conditions, BAG3 plays a key role in re-routing (poly)ubiquitinated proteins to autophagy thus restoring protein homeostasis.

Results

BAG3 binds to ubiquitinated proteins and re-routes them into cytoplasmic puncta in an HSPA1A-dependent manner.

Upon treatment with proteasome inhibitors (poly)ubiquitinated proteins accumulate and tend to aggregate. In attempt to clear these accumulating (poly)ubiquitinated proteins and to restore protein homeostasis, cells induce autophagy as an alternative degradative pathway (proteasome-to-autophagy switch). Interestingly, treatment of HEK293T cells with the proteasomal inhibitors Bortezomib or MG132 leads, in parallel to an accumulation of ubiquitinated proteins, to a significant upregulation of BAG3 protein levels (Fig. 1A and B), similarly to what has been observed in other cell lines. Furthermore the protein levels of HSPB8, which forms a stoichiometric complex with BAG3, increase upon proteasome inhibition (Fig. S1A and B).
The increase in the levels in both proteins is likely due to a transcriptional activation since both BAG3 and HSPB8 mRNA levels are increased (Fig. 1C, Fig. S1C).

Since accumulation of ubiquitinated proteins is correlated with an up-regulation of BAG3 and since BAG3 has been implicated in autophagy,\textsuperscript{11, 17, 20, 21, 28, 38} we wondered whether and to which extent BAG3 participates in the re-routing of (poly)ubiquitinated proteins to autophagy for degradation upon proteasome inhibition. First, we investigated the effect of proteasomal inhibition on the subcellular localisation of BAG3. Strikingly, BAG3 colocalization with ubiquitin-positive cytoplasmic puncta strongly increased following inhibition of the proteasome (Fig. S1D). After proteasomal inhibition, BAG3 was found to colocalize both with ubiquitin- and HSPA1A- positive puncta (Fig. 1D and F) and both BAG3 and HSPA1A puncta contained SQSTM1 (Fig. 1E and G). SQSTM1 puncta are also decorated with ubiquitin (Fig. 1H), consistent with its function as a ubiquitin-binding scaffold protein that binds both to (poly)ubiquitinated substrates and the autophagosome marker MAP1LC3B/LC3 to assist in their delivery to the autophagosomes for degradation.\textsuperscript{39}

We next confirmed findings of others\textsuperscript{36, 39} that Ni-NTA pull-down of his-tagged BAG3 leads to co-precipitation of (poly)ubiquitinated proteins (Fig. 2B). To address how BAG3 binds to (poly)ubiquitinated proteins, we generated a series of BAG3 deletion mutants either lacking the fragment containing the two IPV sequences (BAG3-ΔHSPB8), the BAG domain (BAG3-ΔBAG), the PxxP region (BAG3-ΔPxxP), the WW domain (BAG3-ΔWW) or the C-terminal region (BAG3-ΔC) (Fig. 2A). In line with previous data,\textsuperscript{36, 40} binding to the (poly)ubiquitinated proteins was only lost upon deletion of the BAG domain, but not upon deletion of any of the other domains (Fig. 2B).

The BAG domain in all BAG proteins is required for its interaction with the ATPase domain of HSPA family.\textsuperscript{41} Given that the BAG domain is required for the interaction of BAG3 with (poly)ubiquitinated proteins and since the HSPA system is not only involved in protein refolding but also in the proteasomal degradation of ubiquitinated misfolded proteins,\textsuperscript{42} we next tested the role of HSPA1A in the BAG3 interaction with ubiquitinated proteins. In BAG3 expressing cells, HSPA1A was knocked-down to below 10% by siRNA (Fig. 2C). In these HSPA1A depleted cells the amount of ubiquitinated protein pulled-down with BAG3 was drastically reduced (Fig. 2C). To exclude that the ubiquitinated pattern was from ubiquitinated BAG3, we compared Ni-NTA pull-downs of the different His-tagged proteins under native conditions and denaturing conditions (Fig. 2D). Clearly, the smear of ubiquitinated bands in pull-downs in BAG3 expressing cells detected under native conditions is lost under denaturing conditions. Again, in BAG3-ΔBAG expressing cells no ubiquitinated proteins were pulled-down. Yet, pull-down under both native and denatured conditions suggested a potential (mono)ubiquitination of BAG3 for both wildtype and BAG3-ΔBAG mutant (Fig. 2D, arrow). These data imply that although
BAG3 itself can undergo (mono)ubiquitination, the major fraction of ubiquitinated proteins seen upon Ni-NTA pull-down corresponds to HSPA1A-bound, (poly) ubiquitinated clients. Interestingly, in cells expressing his-tagged HSPA1A, the Ni-NTA pull-downs reveal that besides HSPA1A clients, also HSPA1A itself is ubiquitininated (Fig. 2D).43

Figure 1. BAG3 is induced upon proteasome inhibition and forms cytoplasmic puncta. (A-C) HEK293T cells were either left untreated or treated with 20 μM MG132 for 5 h or 100 nM Bortezomib overnight. Total proteins (A, B) or mRNA (C) were extracted and BAG3 or BAG1 protein or BAG3 mRNA levels were measured (* = p < 0.05 compared to control; n > 3 independent samples +/- sem). (D-H) HeLa cells were treated for 3 h with 20 μM MG132 and cells were fixed with formaldehyde followed by acetone treatment. Subcellular distribution of endogenous BAG3, HSPA1A, SQSTM1 and ubiquitin was investigated by immunofluorescence using specific antibodies.
Figure 2. BAG3 binds to (poly)ubiquitinated proteins in an HSPA8/HSPA1A-dependent manner.

(A) Schematic representation of the binding domains and partners of BAG3.

(B) HEK293T cells were transfected with an empty vector or vectors encoding for His-tagged FL BAG3 or deletion mutants each lacking one specific binding domain (ΔWW, ΔB8, ΔPxxP, ΔBAG or ΔC). 24 h post-transfection cells were lysed and subjected to purification of His-tagged BAG3 with Ni-NTA beads. (C) HEK293T cells were transfected with a control siRNA (-) or a siRNA directed against HSPA1A (+). 48 h post-transfection, cells were transfected with an empty vector or a vector encoding for His-tagged BAG3. Cells were subjected to Ni-NTA pull-down as described above. (D) HEK293T cells transfected with an empty vector or vectors encoding for His-tagged FL BAG3, BAG3-DBAG or HSPA1A were subjected to Ni-NTA pull-down under native or denatured conditions. Levels of (poly)ubiquitinated proteins, BAG3 and HSPA1A were analyzed in the pulled-down and/or input fractions. Concerning ubiquitin, while the FK2 ubiquitin antibody was used in panel B-D, an antibody specific for K48 ubiquitin was used in panel E.
Most proteasomal clients are poly-ubiquitinated through lysine-48 (K48) chain linkage. To test whether the BAG3-HSPA1A substrates that are bound under conditions of proteasomal inhibition are indeed proteasomal clients, we treated cells for 3 h with MG132 and stained BAG3 pull-down material with a K48-linkage specific (poly)ubiquitin antibody (Fig. 2E). The data clearly show that upon MG132 treatment, an increased amount of K-48 (poly)ubiquitinated proteasomal clients become associated with BAG3-HSPA1A.

To further investigate the involvement of BAG3 in the sequestration of (poly)ubiquitinated clients, we studied the subcellular distribution of ubiquitin in cells overexpressing either BAG3 wildtype or deletion mutants in HeLa cells. Interestingly, overexpression of wildtype BAG3, but not of BAG3-ΔBAG, lead to the accumulation of ubiquitin in cytoplasmic puncta (Fig. 3A). The BAG3-ΔPxxP mutant also increased the appearance of ubiquitin-positive puncta (Fig. 3A). Similar results were obtained also in HEK293T cells (Fig. S2). Together with the pull-down data (Fig. 2), these results suggest that BAG3 binds via HSPA1A to (poly)ubiquitinated proteins and promotes their sequestration in cytoplasmic puncta.

To exclude the possibility that BAG3 up-regulation might directly inhibit the proteasome, thus inducing the formation of these ubiquitin-containing cytoplasmic puncta, enzymatic (caspase, trypsin and chymo-trypsin) activities of the proteasome were analysed in cell lysate of BAG3 overexpressing cells. All three enzymatic activities of the proteasome were unaffected by the increased BAG3, whilst epoxymicin addition (as positive control) severely inhibited all activities (Fig. 3B). Together, these data point to a specific role of BAG3, independent from proteasomal inhibition, in binding to and sequestering ubiquitinated HSPA1A-clients to cytoplasmic puncta.

Next, we investigated biochemically the fate of ubiquitinated proteins and to what extent they might be re-routed to autophagy by BAG3. In cells overexpressing the BAG3-ΔBAG, the levels of ubiquitinated proteins did not profoundly change in both NP-40 soluble and insoluble fractions (Fig. 4A). However, in cells overexpressing the wildtype BAG3 protein, we found significant accumulation of ubiquitinated proteins in the NP-40 insoluble fraction (Fig. 4A). This increase in NP-40 insoluble ubiquitinated proteins was further enhanced if lysosomal degradation was blocked with ammonium chloride (Fig. 4B), suggesting that BAG3-mediated puncta formation precedes autophagic degradation. Interestingly, this increase in the amount of ubiquitinated proteins in the NP-40 insoluble fraction was also much larger for the BAG3-ΔPxxP mutant (Fig. 4A). BAG3-ΔPxxP is unable to interact with dynein, a key motor protein involved in the retrograde transport of cargo towards the perinuclear region of the cells (Microtubule-Organising Center). The Microtubule-Organising Center is enriched in autophagosomes and lysosomes.
and it has been suggested that cargo is transported to these sites (often referred to as aggresomes) for autophagosomal-lysosomal degradation.\textsuperscript{45} Thus, the accumulation of ubiquitinated proteins in the NP-40 insoluble fraction in BAG3-ΔPxxP expressing cells could be related to the inability of BAG3-ΔPxxP to transport these bound clients towards the perinuclear region (defective protein re-routing) and/or to the inability of BAG3-ΔPxxP to efficiently induce autophagy. The latter is unlikely, since the BAG3-ΔPxxP mutant still leads to increased MAP1LC3B conversion similar as wildtype

![Figure 3. BAG3 induces the accumulation of ubiquitin in cytoplasmic puncta.](image)

(A) HeLa cells were transfected with GFP and an empty vector or his-BAG3, his-BAG3-ΔBAG or his-BAG3-ΔPxxP encoding vectors. 48 h post-transfection cells were fixed with Methanol 100% for 10 min at -20 °C and subjected to immunofluorescence to investigate the subcellular distribution of ubiquitin. The percentage of cells containing ubiquitin positive cytoplasmic puncta is indicated.

(B) The caspase, trypsin and chymo-trypsin activities of the proteasome were measured in cell lysates from BAG3 overexpressing cells (green line) versus control cells (blue line) and in cells treated with the proteasome inhibitor epoxomycin (red line) for 30 min. Degradation of the fluorogenic peptide substrates was not impaired in BAG3 overexpressing cells.
Figure 4. BAG3 is required to induce autophagy and clear aggregate-prone (poly) ubiquitinated proteins following proteasome inhibition.

(A) BAG3-ΔPxxP leads to accumulation of insoluble ubiquitinated proteins. HEK293T cells were transfected with either an empty vector or vectors encoding for his-BAG3, his-BAG3-ΔBAG or his-BAG3-ΔPxxP. 48 h post-transfection NP-40 soluble and insoluble proteins were fractionated. Ubiquitin protein levels were measured in both fractions (* = p < 0.05 and ** = p < 0.001 compared to empty vector; n = 6 independent samples +/- sem). (B) HEK293T cells were transfected with either an empty vector or a vector encoding for His-tagged BAG3. 20 h post-transfection, cells were treated with NH4Cl 20 mM for 6 h and subsequently let recover overnight. The day after, NP-40 soluble and insoluble proteins were fractionated. Ubiquitin protein levels were measured in both fractions (** = p < 0.001 compared to empty vector;
n = 3 independent samples +/- sem). (C) BAG3-ΔPxxP is defective in client re-routing rather than in autophagy induction. HEK293T cells were transfected with either an empty vector or vectors encoding for His-tagged FL BAG3 or ΔPxxP. Prior to extraction of total proteins cells were treated for 4 h with 10 μg/ml E64d and 10 μg/ml Pepstatin A (+E64d+Peps.A) to measure autophagy flux. MAP1LC3B-II/I ratio (normalized against TUBA4A) was measured. (D) HeLa cells were transfected with mRFP-GFP-MAP1LC3B and either an empty vector, His-tagged FL BAG3 or ΔPxxP. Prior to analysis by confocal microscopy, the cells were treated for 2 h with 20 mM NH4Cl and 0.2 mM Leupeptin (+NH4Cl/Leu) to block the autophagy flux. Colocalization efficiency of mRFP with GFP signals was measured using ImageJ software and is shown as the percentage of the total number of mRFP puncta that colocalize with GFP puncta (yellow bars). The number of mRFP puncta (that do not colocalize with GFP) in all conditions is also shown as percentage compared to control cells (red bars). The value indicates average and sem from at least six images (** = p < 0.01). (E) Knocking-down BAG3 impairs the compensatory activation of autophagy occurring following proteasome inhibition. HEK293T cells were transfected with either a control siRNA or a specific siRNA for BAG3. 48 h post-transfection, cells were treated with 10 μM MG132 for 5 h and 30 min and let recover in drug-free medium for 24 h (* = p < 0.05 compared to untreated cells; n = 4 independent samples +/- sem). (F) BAG3 deficient cells accumulate more insoluble ubiquitinated proteins following proteasome inhibition. HEK293T were treated as described in C, but recovery was prolonged up to 40 h prior to extraction and fractionation of NP-40 soluble and insoluble proteins. (* = p < 0.05 compared to untreated cells; n = 4 independent samples +/- sem).

BAG3 (Fig. 4C), suggesting that the observed accumulation and insolubilization of ubiquitinated proteins in BAG3-ΔPxxP expressing cells indeed relates to inefficient client routing to the perinuclear region following puncta formation (Fig. 3A ). Moreover, the findings that the BAG3-ΔPxxP mutant cannot facilitate the clearance of polyQ containing huntingtin or mutated SOD1 aggregates would imply that the accumulation of ubiquitin-positive puncta per se is not sufficient to stimulate the autophagic flux and to ensure proper client disposal. We next monitored autophagy using the tandem mRFP-GFP- MAP1LC3B reporter in live imaging experiments. HeLa cells were transfected with mRFP-GFP- MAP1LC3B alone or in combination with either wildtype BAG3 or BAG3-ΔPxxP. The GFP signal is sensitive to the acidic lysosomal pH, whereas mRFP is more stable. Thus, colocalization of GFP and mRFP fluorescence indicates phagophores or autophagosomes that have not fused with a lysosome. Instead, the mRFP signal without GFP corresponds to amphisomes or autophagolysosomes. As expected, co-treatment with leupeptin and ammonium chloride, the latter raising lysosomal pH, allowed to detect both GFP and mRFP fluorescence and lead to increased colocalization of mRFP with GFP signals as compared to untreated cells (Fig. 4D, yellow bars). Instead, both BAG3 and BAG3-ΔPxxP lead to a decrease in the colocalization of mRFP with GFP signals, further confirming that both wildtype BAG3 and BAG3-ΔPxxP induce the autophagic flux (Fig. 4D, yellow bars), while increasing the percentage of mRFP puncta as compared to control cells (Fig. 4D, red bars).
If BAG3 can indeed re-route (poly)ubiquitinated clients to autophagy, it is expected that upon proteasome inhibition, siRNA-mediated BAG3 depletion would increase the accumulation of aggregate-prone (poly)ubiquitinated clients. Indeed, we found that BAG3 is significantly induced in the recovery phase after exposure to the proteasome inhibitor MG132 (24 h after recovery; Fig. 4E). This induction correlates with the accumulation of ubiquitinated proteins and the induction of autophagy (increased MAP1LC3B-II/I ratio; Fig. 4E and F). Interestingly, blocking the MG132-mediated induction of BAG3 by treatment with siRNA abrogated the compensatory activation of autophagy occurring during the recovery phase after proteasome inhibition (Fig. 4E) and lead to an increased accumulation of aggregate-prone (poly)ubiquitinated proteins (Fig. 4F; 24 h after recovery), again pointing to a key role for BAG3 in the proteasome-to–autophagy switch. These data corroborate a recent report by Rapino et al. where they show that cancerous cells deficient in BAG3 do accumulate a significantly higher amount of ubiquitinated proteins in the detergent soluble and insoluble fractions upon proteasomal inhibition.

Further evidence for such re-routing was next obtained by investigating the effect of BAG3 on Ub-R-GFP, a typical proteasomal activity reporter. Without BAG3 overexpression, the fraction of non-degraded Ub-R-GFP is mainly diffusely distributed throughout the cytoplasm and nucleus of HeLa cells (Fig. 5A and B). In cells overexpressing BAG3, however, a significant fraction of the Ub-R-GFP was found in cytoplasmic puncta and in aggresome-like structures in the perinuclear region (Fig. 5A and B). Overexpression of the BAG3-ΔBAG mutant only marginally affected the subcellular distribution of Ub-R-GFP (Fig. 5A and B), again pointing to requirement of HSPA1A as the chaperone required for the recognition and binding of the proteasomal clients. In line, BAG3, but not BAG3-ΔBAG, leads to the accumulation of Ub-R-GFP into the NP-40 insoluble fraction (Fig. 5C). Similar results were obtained also in HEK293T cells (Fig. S3A and B). In cells overexpressing the BAG3-ΔPxxP mutant, Ub-R-GFP accumulated also in puncta, supporting that (only) binding via HSPA1A is a pre-requisite to BAG3-driven puncta formation (Fig. 5A-C) and consistent with our findings that the PxxP domain is not involved in binding to proteasomal clients (Fig. 2). Note, however, that in cells expressing the BAG3-ΔPxxP mutant the accumulation of Ub-R-GFP in perinuclear structure is impaired, consistent with earlier observations by Gamerdinger et al.

The Ub-R-GFP cytoplasmic puncta accumulating in cells overexpressing BAG3 were next found to colocalise with the autophagy markers MAP1LC3B (Fig. 6A), WIPI1 (Fig. 6B) and the autophagy linker SQSTM1 (Fig. 6C). Similar data were obtained in HEK293T cells (Fig. S3C-E). Also in case of overexpression of the BAG3-ΔPxxP mutant, the Ub-R-GFP cytoplasmic puncta significantly overlapped with these three autophagy markers (Fig. 6), in agreement with the fact that it is only
Figure 5. BAG3 sequesters the proteasomal reporter Ub-R-GFP into cytoplasmic insoluble puncta.

(A) HeLa cells were transfected with Ub-R-GFP and either an empty vector, His-BAG3, His-BAG3-ΔBAG or His-BAG3-ΔPxxP. Cells were fixed with formaldehyde/acetone 48 h post-transfection. (B) The percentage of cells containing Ub-R-GFP positive cytoplasmic puncta is depicted (n = 3 independent samples +/- sem). (C) HEK293T cells were transfected as described in A. 48 h post-transfection NP-40 soluble and insoluble proteins were fractionated and accumulation of Ub-R-GFP in both fractions was analysed by Western blotting.
Figure 6. The Ub-R-GFP containing puncta induced by BAG3 colocalize with SQSTM1 and canonical autophagy markers

(A-C) HeLa cells were transfected with Ub-R-GFP and his-BAG3, his-BAG3-ΔBAG or his-BAG3-ΔPxxP and subjected, 48 h post-transfection, to immunofluorescence to investigate Ub-R-GFP colocalization with MAP1LC3B (A), WIPI1 (B) and SQSTM1 (C).
defective in transport towards the perinuclear region, downstream foci formation. Taken together, our data strongly suggest that, in the presence of BAG3, model proteasomal reporters as well as endogenous ubiquitinated proteins are sequestered into cytosolic puncta that now can be degraded through autophagy.

Next, we followed the fate of a specific endogenous proteasomal client upon BAG3 overexpression. Based on a Mass Spectrometry study on BAG3 co-IP proteins showing that BAG3 interacts with the E3 ligase Anaphase-Promoting Complex subunit 1 (to be published elsewhere), we selected PTTG1/securin since it is targeted for proteasome-mediated degradation via the E3 ligase Anaphase-Promoting Complex subunit 1. Indeed, in control cells, PTTG1 levels significantly increased after proteasomal inhibition with Bortezomib, while autophagy inhibition with 3-methyladenine (3-MA) and wortmannin showed no effect (Fig. 7A). However, after overnight overexpression of BAG3, the endogenous PTTG1 levels were significantly decreased (Fig. 7B). Interestingly, no significant effect on PTTG1 levels was observed upon overexpression of the BAG3-ΔBAG mutant, again supporting the key role of HSPA1A in recognizing and binding (at least some of) the clients (Fig. 7B). Moreover, BAG3 could still partly decrease PTTG1 levels in cells treated with the proteasome inhibitor Bortezomib, but not in cells treated with the autophagy inhibitors wortmannin and 3-MA (Fig. 7C). Consistently with our biochemical data, PTTG1 was found to colocalize with MAP1LC3B-positive puncta (Fig. 7D) and (partially) with the lysosomal marker LAMP2 (Fig. 7E) in cells overexpressing BAG3; this suggests that upon overexpression BAG3 can re-route PTTG1 towards autophagy degradation. No significant colocalization of PTTG1 with MAP1LC3B puncta was observed in control cells or cells expressing BAG3-DBAG (Fig. 7E).

Intriguingly, another BAG family member, BAG1 has been shown to be engaged in HSPA1A dependent routing of proteins to the proteasome. We therefore investigated the fate of PTTG1 in BAG3 or BAG1 depleted cells under otherwise normal growth conditions. While knock-down of BAG1 lead to PTTG1 accumulation, knock-down of BAG3 had no effect (Fig. 7F). This suggests that in resting cells BAG1 participates in the proteasomal degradation of PTTG1, but that under conditions of proteasomal impairment BAG3 is up-regulated and re-routes clients like PTTG1 towards degradation by autophagy.
Figure 7. BAG3 re-routes PTTG1 to autophagy for degradation.

(A) HEK293T cells were either left untreated or treated with 10 mM 3-methyladenine and 200 nM wortmannin (3-MA+wort.) or 100 nM Bortezomib overnight. (B) HEK293T cells were transfected with an empty vector, his-BAG3 or his-BAG3-ΔBAG encoding vectors. (A and B) Expression levels of PTTG1 were measured using a specific antibody. Average levels of PTTG1 are reported (** = p < 0.001 compared to untreated cells; n = 4 independent samples +/- sem and * = p < 0.01 compared to empty vector; n = 3 independent samples +/- sem). (C) HEK293T cells overexpressing empty vector or His-BAG3 were either left untreated or treated overnight with 200 nM wortmannin and 10 mM 3-methyladenine (Wort.+3-MA) or with 100 nM Bortezomib (Bort.) prior to extraction of total proteins. Average levels of PTTG1 are reported (** = p < 0.001 compared to empty vector; n = 5 independent samples +/- sem).
(D, E) PTTG1 partially colocalizes with myc-MAP1LC3B and LAMP2 in cells overexpressing BAG3. (D) HEK293T cells were transfected with a myc-MAP1LC3B encoding vector and either an empty vector or his-BAG3. 24 h post-transfection cells were treated with Bafilomycin A (100 nM) for 4 h and fixed with formaldehyde; subcellular distribution of myc-MAP1LC3B and endogenous PTTG1 were investigated by immunofluorescence. (E) HEK293T cells were transfected with His-tagged FL or ΔBAG BAG3 encoding vectors. 24 h post-transfection cells were treated with Bafilomycin A for 4 h and fixed with formaldehyde; subcellular distribution of endogenous LAMP2 and PTTG1 were investigated by immunofluorescence. (F) HEK293T cells were transfected with a scrambled siRNA or with a BAG3 and BAG1 specific siRNA. PTTG1 expression levels were measured 72 h post-transfection. Average levels of PTTG1 are reported (** = p < 0.001 compared to scrambled siRNA; n = 3 independent samples +/- sem).

Figure 8. BAG3 and BAG1 compete for binding to ubiquitinated proteins.

(A) The active binding to the proteasome of a fluorescent activity probe was measured in lysates from cells expressing either BAG3 alone or together with increasing amount of BAG1. Average levels of fluorescent activity probe are reported (** = p < 0.01 and * = p < 0.05 compared to cells transfected with BAG3 alone; n = 4-5 independent samples +/- sem). (B) HEK293T cells were transfected with either an empty vector (-) or with vectors encoding for HA-BAG1 and/or His-BAG3 alone or together (+). 48 h post-transfection His-BAG3 was pulled-down using the Ni-NTA beads and the amount of (poly)ubiquitinated proteins and HSPA1A bound to His-BAG3 was analyzed in the pulled-down fraction. (C) The amount of BAG3 binding to HSPA1A increased with time after MG132 treatment. Immunoprecipitation with a specific HSPA1A antibody was carried out using HEK293T extracts from control cells and cells treated with 20 μM MG132 for 5 h. Interaction of endogenous HSPA1A with BAG3 was investigated by Western blotting using specific antibodies.
Client destination is determined by the BAG3/BAG1 ratio.

The PTTG1 data suggested that BAG3 and BAG1 may compete for the binding of clients and thereby potentially influencing their destination. Intriguingly, during ageing, a condition characterized by a reduction in the proteasome function and a concomitant increase in autophagy activation, a relative change in the expression levels of BAG3 and BAG1 was found. We therefore tested the hypothesis that BAG3 re-routes clients to cytoplasmic puncta and autophagosomes by competing with BAG1 for the HSPA1A-bound (poly)ubiquitinated substrates. We measured the activity of the proteasome activity-based probes that fluorescently label proteasomal active sites in lysates from cells expressing either BAG3 alone or together with increasing amount of BAG1. Increasing the levels of BAG1 at a fixed BAG3 concentration gradually increased the fluorescent signal of the activity probe, indicating that proteasome activity was increased (Fig. 8A). Moreover, BAG1 overexpression decreased the total amount of HSPA1A and (poly)ubiquitinated proteins pulled-down by BAG3 (Fig. 8B). In addition, the amount of BAG3 binding to HSPA1A increased after MG132 treatment (Fig. 8C). Intriguingly, recent findings demonstrate not only that BAG3 and BAG1 compete for binding to HSPA1A but that there is a hierarchical affinity of BAG3 to BAG1, further strengthening our data.

The Heat Shock Response, the NFkB signalling pathway and the Unfolded Protein Response all participate to ensure BAG3 upregulation upon proteasome inhibition.

The above described assumption that BAG3:BAG1 ratios are key to client routing towards either the proteasome or autophagosome is strongly supported by findings that BAG3 expression is significantly induced upon proteasome inhibition (Fig. 1) Since various stress conditions are known to lead to proteasomal impairment or overload, we investigated which of the main stress pathways activated upon proteotoxic stress (the Heat Shock Response (HSR), the NFkB signalling pathway and the Unfolded Protein Response (UPR)) could modulate BAG3 expression. We show here, in line with previous reports, that BAG3 expression is induced by Heat Shock (Fig. 9A). Upregulation of HSPA6, which is only expressed after Heat Shock was included as positive control (Fig. 9A). Moreover, activation of the NFkB pathway by treatment with TNF alpha (Fig. 9D), as well as activation of the UPR (here illustrated by the overexpression of the ERN1/IRE-1 alpha activated transcription factor X-box binding protein 1, XBP1) (Fig. 9G), both also lead to upregulation of BAG3. These results in HEK293 cells are in line with previous data in other cell lines that identified BAG3 as a target for NFkB and XBP1, respectively. Interestingly, we observed that impairment of each of these three pathways by overexpression of either a dominant negative form of HSF1 (DN-HSF1; Fig. 9B), a superdominant active
form of IkB alpha (IkB alpha SD), which acts as NFkB/RELA repressor (Fig. 9E),\textsuperscript{54} or of a dominant negative form of ERN1 (DN-ERN1; Fig. 9H),\textsuperscript{55} also lead to a significant upregulation of BAG3. Furthermore, cells lacking respectively HSF-1 (Fig. 9C) or XBP1 (Fig. 9I) show higher basal BAG3 expression levels than their wildtype counterpart cell lines. This suggests the existence of cross-talk between these pathways and that under fragile conditions of impaired stress responses that may impede on proteosomal client degradation, cells can still maintain protein homeostasis by elevating BAG3 expression ensuring BAG3-directed autophagic client clearance.

Finally, we tested which of these pathways is essential for BAG3 induction upon chemical inhibition of the proteasome. Although in resting conditions the expression levels of BAG3 were higher in XBP1\textsuperscript{-/-} cells (Fig. 9I) as compared to their wildtype counterpart, treatment with Bortezomib led to further increase in BAG3 expression in RELA\textsuperscript{-/-} (Fig. 9F) and XBP1\textsuperscript{-/-} cells (Fig. 9I). However, such further increase in BAG3 expression after proteasome inhibition was absent in HSF1\textsuperscript{-/-} cells (Fig. 9C), implying the HSR as the main regulator for BAG3 up-regulation upon treatment of cells with chemicals that impair proteosomal activity.

**Discussion**

Upon a variety of stress treatments, proteins can become damaged (oxidized), unfolded, misfolded and/or may aggregate. Cells have a number of stress response pathways (HSR, UPR, NFkB) that activate protein quality control systems to either refold or dispose the damaged proteins. Depending on the acuteness, type and severity of the stress, chaperone-assisted refolding or proteasomal degradation may be sufficient to deal with the damage. However, when these systems are overloaded or when the type of damage has become merely non-resolvable, cells can still switch to autophagy as a “non-specific” route to degrade (poly)ubiquitinated or/and aggregated proteins. How this proteasome-to-autophagy switch is regulated is still largely unknown. Yet, several data already had suggested involvement of BAG3.\textsuperscript{11,47} Here we show that BAG3 is not only upregulated upon chemical inhibition of the proteasome, but indeed re-routes (poly)ubiquitinated clients to autophagy for degradation, thus ensuring their proper clearance and avoiding their accumulation/aggregation (Fig. 4). Re-routing of clients is not a (passive) consequence of proteasome impairment and increased autophagy per se. In fact, the delta PxxP mutant of BAG3, which can still bind (via HSPA1A) to the ubiquitinated clients (Fig. 2) and efficiently increases the autophagic flux (Fig. 4), lead to the accumulation of aggregate-prone ubiquitinated proteins (Fig. 4), due to deficient cargo dynein-mediated transport towards autophagosomes.\textsuperscript{21} Rather, BAG3 actively recruits HSPA1A-bound (poly)
Figure 9. The Heat Shock Response, the NFκB signalling pathway and the Unfolded Protein Response all participate to BAG3 upregulation upon proteasome inhibition. (A) HEK293 cells were subjected to a heat shock (HS) for 30 min at 42 °C. Total proteins were extracted either immediately after heat shock or 16 h after recovery. Expression levels of HSPA6, which is only expressed after heat shock, were analysed as a positive control. (B) HEK293 cells stably expressing a tetracycline-inducible DN-HSF1 were either left untreated (-) or treated with tetracycline (+) for 48 h. Expression levels of BAG3, HSPA6 and TUBA4A were analyzed. (C) Wild-type (+/+) and knockout (-/-) HSF1 mouse embryonic fibroblasts were either left untreated (-) or treated with 20 μM MG132 for 5 h or 100 nM Bortezomib overnight. Expression levels of BAG3 and TUBA4Awere analyzed. (A-C) Average levels of BAG3 are reported (** = p < 0.01 and * = p < 0.05 compared to control; n = 3 independent samples +/- sem). (D) HEK293T cells were treated with TNF alpha for 15 min followed by a recovery
for 90 min. Expression levels of tumor necrosis factor alpha-induced protein 3 (TNFAIP3), whose expression is rapidly induced by the tumor necrosis factor, were analysed as positive control. (E) HEK293 cells were transfected for 24 h with either an empty vector (-) or a vector encoding for IKBα SD (+). (F) Wild-type (+/+), and knockout (-/-) RELA mouse embryonic fibroblasts were either left untreated (-) or treated with 20 μM MG132 for 5 h or 100 nM Bortezomib overnight. Expression levels of BAG3 and TUBA4A were analyzed. (E-F) Average levels of BAG3 are reported (** = p < 0.01 and * = p < 0.05 compared to control unless indicated; n = 3 independent samples +/- sem). (G) HEK293 cells stably expressing XBP1 in a tetracycline-inducible manner were either left untreated (-) or treated with tetracycline (+) for 48 h. Expression levels of BAG3, HSPA5/GRP78, used as positive controls, HSPB8 and TUBA4A were assessed using a specific antibody. (H) HEK293 cells stably expressing a tetracycline-inducible DN-ERN1 were either left untreated (-) or treated with tetracycline (+) for 48 h. Expression levels of BAG3 and TUBA4A were analyzed. Splicing of XBP1 was investigated by PCR. (I) Wild-type (+/+) and knockout (-/-) XBP1 mouse embryonic fibroblasts were either left untreated (-) or treated with 20 μM MG132 for 5 h or 100 nM Bortezomib overnight. Expression levels of BAG3 and TUBA4A were analyzed. (G-I) Average levels of BAG3 are reported (** = p < 0.01 and * = p < 0.05 compared to control unless indicated; n = 5 independent samples +/- sem).

ubiquitinated clients via its BAG domain and next re-routes them via the PxxP domain for autophagic clearance (Fig. 10). Once bound to BAG3, the (poly)ubiquitinated clients are at first sequestered into cytoplasmic puncta that contains SQSTM1. SQSTM1 is a molecule able to bind both to ubiquitin and the autophagosome marker MAP1LC3B, which has been shown to facilitate the degradation by autophagy of aggregated (poly) ubiquitinated proteins.39, 55 BAG3 not only colocalizes with HSPA1A, ubiquitin and SQSTM1 into cytoplasmic puncta, but its overexpression induces their formation in an HSPA1A-dependent manner. The ubiquitin-containing cytoplasmic puncta induced by BAG3, but not by BAG3-ΔBAG, also colocalize with the autophagosome markers MAP1LC3B and WIPI1, strongly suggesting their labelling is required for re-routing towards autophagy (Fig. 3 and 5). That this indeed results in autophagic degradation of clients and to what extent it is dependent on BAG3 was evidenced by the accumulation of aggregate-prone (poly) ubiquitinated proteins in cells where the upregulation of BAG3 following proteasome inhibition was impaired by BAG3 knockdown (Fig. 4).47 The ability of BAG3 to influence the fate of typical proteasome clients was further supported by the accumulation of the proteasomal activity reporter Ub-R-GFP into cytoplasmic puncta co-labelled with autophagy linkers/markers (Fig. 5) and of the endogenous protein PTTG1 of which the degradation upon overexpression of BAG3 coincided with its appearance in MAP1LC3B/LAMP2-positive puncta (Fig. 6). While our data suggest a key role for BAG3 upon proteasomal inhibition in the proper clearance of accumulating (poly)ubiquitinated clients, it is still unknown to what extent BAG3 decides on the fate of typical proteasomal clients in resting cells.

Considering that the ubiquitinated proteins pulled-down by BAG3 are not directly bound to BAG3, but via HSPA1A, that only BAG3 levels significantly
Figure 10. Working model: BIPASS: BAG-Instructed Proteasomal to Autophagosomal Switch & Sorting.

When BAG1:BAG3 protein ratio is in favour of BAG1 (1), HSPA1A-bound clients (70) are targeted by BAG1 to the proteasome for degradation (2). Under conditions of proteasomal overload, BAG3 expression is upregulated (3), thus changing the ratio in favour of BAG3. HSPA1A-bound clients are now mainly taken care by BAG3 (4). BAG3 promotes the sequestration of these clients into cytoplasmic puncta that are co-labelled with the autophagy adapter SQSTM1 (p62) and the autophagy marker MAP1LC3B (LC3) (5), to be next re-routed to autophagosomes for degradation (6).

raise upon proteasome inhibition, and that BAG3 competes for binding to HSPA1A with a higher affinity as compared to BAG1,51 overall our data suggest that the BAG1:BAG3 expression ratio is key to the fate of ubiquitinated clients to be degraded through either the proteasome or autophagosomes (BAG-Instructed Proteasomal to Autophagosomal Switch & Sorting; BIPASS; Fig. 10).

Amongst the stress pathways that respond to protein damage are the HSR, the NFkB pathway and the ERN1 branch of the UPR (Fig. 9). Upon chemical proteasome
inhibition, HSF1 seems the key-regulator of BAG3 induction. However, we found that genetic deletion of HSF1 and XBP1, as well as impairment of the HSR, the NFkB pathway and ERN1 branch of the UPR (by overexpression of dominant negative forms of these transcription factors or chemical inhibition) upregulated BAG3 expression, as compared to wildtype or resting cells. Together, these data suggest that a tight and parallel regulation of the protein homeostasis network by multiple pathways exists and underscores the central role that BAG3 plays in protein homeostasis.

While our data demonstrate a direct implication of HSPA1A in the recognition and binding to ubiquitinated proteins, we cannot at present exclude that BAG3 also modulates the fate of clients bound e.g. by its partner HSPB8, which also can pull-down ubiquitinated proteins (data not shown). However, surprisingly, deletion of the IPV-containing HSPB8 binding domain did not affect BAG3-mediated client sequestration and re-routing to autophagy (Fig. 2). This domain is involved in binding to the small HSP proteins HSPB8 and to a lesser extent also alpha B crystallin/HSPB5 and heat shock protein, alpha-crystallin-related, B6/HSPB6. Like BAG3, HSPB8 was also upregulated following proteasome impairment (Fig. S1). Interestingly, we observed that upon proteasomal inhibition HSPB8 also localised to the SQSTM1 puncta but only upon later time points. Since HSPB8 itself can bind to (poly)ubiquitinated proteins (data not shown), it thus might also assist BAG3 in the autophagy-mediated cargo disposal during persisting stress.

Concerning the pathophysiological significance of BAG3 (and HSPB8) upregulation, they were found to be upregulated in the post-mortem brain tissue of patients suffering of several protein-aggregate diseases and in aged tissue that are all characterized by the accumulation of aggregated and oxidized proteins that cannot be handled by the proteasome. In addition, genetic manipulation of BAG3/HSPB8 levels in cell and fly models can attenuate the accumulation of aggregates of disease-causing poly-Q proteins and of SOD1 and TAR DNA binding protein/TDP-43 mutants that cause Amyotrophic Lateral Sclerosis. Therefore, this complex is not only important for and used by cells to alleviate damage caused by acute forms of stress, but its boosting may also serve to clear aggregates in chronic neurodegenerative diseases.

Materials and Methods

**Plasmids and reagents.** Plasmids encoding human non tagged and myc-tagged HSPB8 as well as plasmids encoding for HA-tagged BAG1, His-tagged human HSPA1A (pcDNA5.1-FRT/TO-His-HSPA1A), BAG3 FL (pCINHisBAG3) or the delta WW, delta B8, delta BAG and delta PxxP were described previously. The plasmids encoding for His-tagged human Bag3 delta C, lacking the C-terminal tail was created...
by PCR using specific primers and pCINHisBag3 as template. The plasmids encoding for IκB alpha SD was a kind gift from Dr. A.J.A. van de Sluis.\textsuperscript{54} The plasmid expressing Ub-R-GFP was provided by Dr. N.P. Dantuma.\textsuperscript{48} The plasmid encoding for Myc-MAP1LC3B and for mRFP/GFP-MAP1LC3B were a kind gift of Dr. T. Yoshimori. The following reagents were used: Bortezomib (100 nM overnight; Selleck Chemicals, S1013), MG132 (20 μM for 3-6 hours; Calbiochem, 474790). 3-Methyladenine (3-MA, 10 mM, M9281), wortmannin (200 nM, W1628), Bafilomycin A (100 nM, B1793), Pepstatin A (10 μg/ml, P5318), E64d (10 μg/ml, E8640), N-ethylmaleimide (NEM, 20 mM, E3876), Leupeptin (200 nM, L5793) and ammonium chloride (NH4Cl, 20 mM, A9434) were from Sigma. Human TNF alpha was provided by the Endothelial Biomedicine Group of the UMCG (The Netherlands).

Cell Culture, Transfection, and Immunocytochemistry. HeLa (humancervical cancer), HEK293, Flp-In T-REx HEK293 and HEK293T (human embryonal kidney) cells were grown in Dulbecco’s modified Eagle’s medium with high glucose (Invitrogen, 41966-052) supplemented with 10% fetal bovine serum (Greiner Bio-One, 758093) and penicillin/streptomycin (Invotrogen, 15140-163); for Flp-In T-REx HEK293 cells (Invitrogen, K6010-01), 5 μg/ml Blasticidin (Invitrogen, A11139-03) and 100 μg/ml of Zeocin (Invitrogen, R250-01) were used. Flp-In T-REx-HEK293 cell cell lines expressing either a tetracycline-inducible dominant negative form of HSF1 (HSF1 DN) or ERN1 (ERN1-DN) or an active form of XBP1 were a kind gift from Dr. N.H. Lubsen.\textsuperscript{61} Cells were transfected by calcium phosphate precipitation as previously described.\textsuperscript{59} Transfection of siRNA for BAG3 (target sequence: gcaugccagaaaccacuca), siRNA for BAG1 (target sequence: aggaagagguugaacuaauu), siRNA for HSPA1A (Dharmacon’s SMARTpool siRNA) and a control sequence (Dharmacon’s siCONTROL non-targeting siRNA) were performed using Lipofectamine 2000 (Invitrogen, 11668019), according to the manufacturer’s instructions. Immunocytochemistry was performed as previously described,\textsuperscript{59} except that the cells were either fixed with 4% formaldehyde buffer for 9 min at room temperature or with methanol at -20 °C. Wildtype (+/+) and knockout (-/-) HSF1 mouse embryonic fibroblasts were kindly provided by Dr. Ivor J. Benjamin; wildtype (+/+) and knockout (-/-) XBP1 mouse embryonic fibroblasts were kindly provided by Dr. L. Hendershot; knockout (-/-) RELA mouse embryonic fibroblasts were kindly provided by Dr. L. Schmitz.

Preparation of protein extracts, Purification of His-tagged BAG3 with Ni-NTA (Ni2+-nitrilotriacetate) beads, co-immunoprecipitation and antibodies. For preparation of total protein extract, cells were scraped and homogenized in 2% SDS lysis buffer as previously described.\textsuperscript{59} For preparation of NP-40 (IGEPAL CA-630, Sigma, I3021) soluble and insoluble fractions, cells were harvested in lysis buffer containing
20 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2, 100 mM KCl, 0.5% NP-40, 3% glycerol, 1 mM dithiothreitol (DTT), complete EDTA-free (Roche, 11873580001). NP-40 soluble and insoluble fractions were separated by centrifugation at 14000 rpm for 15 min at 4°C. Purification of His-tagged BAG3 with Ni-NTA (Ni2+-nitrilotriacetate) beads was previously described.\footnote{17, 56} For Ni-NTA pull-down under denaturing conditions, cells were washed twice in PBS with NEM (20 mM) and were lysed in 6 M guanidinium-HCl, 0.01 M NaH2PO4, 0.05% Tween 20, 0.1 M Tris (pH 8.0) plus 20 mM imidazole. Lysates were sonicated for 30 s at setting 4 to reduce viscosity. Lysates were centrifuged at 14000 rpm for 20 min. Supernatant was mixed on a rotator with Ni-NTA-agarose beads (Qiagen, 30210) overnight at 4 °C. Ni-NTA-agarose beads were successively washed twice with 1 ml of 6 M guanidinium-HCl, 0.01 M NaH2PO4, 0.05% Tween 20, 0.1 M Tris/HCl and 20 mM imidazole (pH 8.0), followed by three washes with the buffer A containing 8 M urea, 0.1 M NaH2PO4, 0.05% Tween 20 and 0.01 M Tris/HCl (pH 8.0). The bound material was eluted by adding 2% SDS, 10% β-mercaptoethanol and incubating at 99 °C for 5 min. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and then processed for western blotting. Membranes were subsequently incubated with HRP-conjugated secondary antibodies (Amersham, anti-rabbit, GEHNA9340; anti-mouse, GEHNXA931) at 1:7000 dilution. Visualization was performed with enhanced chemiluminescence (ECL) and Hyperfilm (Amersham, PR32106 and GEH28906837, respectively).

For immunoprecipitation from transfected cells, 24 h post-transfection, cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2, 100 mM KCl, 0.5% IGEPAL CA-630, 3% glycerol, 1 mM DTT, complete EDTA-free. The cell lysates were centrifuged and cleared with A/G beads (Santa Cruz Biotechnology, sc-2003) at 4 °C for 1 h. A/G beads complexed with specific antibodies (anti-HSPA1A antibody) were added to the precleared lysates. After incubation for 2 h at 4 °C, the immune complexes were centrifuged. Beads were washed four times with the lysis buffer; both co-immunoprecipitated proteins and input fractions were resolved on SDS-PAGE. Anti-BAG3 and anti-HSPB8 are rabbit polyclonal antibodies against human BAG3 and HSPB8, respectively.\footnote{17} Mouse monoclonal anti-myc (9E10) was a kind gift of Dr. R. Tanguay. The following commercial antibodies were also used in this study: mouse monoclonal anti-TUBA4A/α-tubulin (Sigma, T6074), anti-HA (Sigma, H3663), anti-HSPA1A/Hsp70 (Stressgen, SPA-810), anti-GFP (Clonotech, 632381), anti-IkBα (Cell Signaling, 9242), anti-BAG1 (Cell Signaling, 3920S), anti-TNFAIP3 (Cell Signaling, 5630S), anti-ubiquitin FK2 (Enzo Life Sciences, BML-PW8810), anti-LAMP2 (Santa Cruz, sc-18822), mouse polyclonal anti-HSPA6 (Stressgen, SPA-754-F), rabbit monoclonal anti-ubiquitin K48 (Cell Signalling, 8081S), rabbit polyclonal anti-HSF1 (Stressgen, SPA-901), anti-SQSTM1/p62 (Enzo Life Sciences, BML-PW9860), anti-ubiquitin (DAKO, Z0458), anti-PTTG1 (Abcam, Mab79546), anti-HSPA5/
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GRP78 (Stressmarq, SPC-180), anti-MAP1LC3B (Novus Biologicals, NB100-2220), goat polyclonal anti LMNB1/Lamin B (Santa Cruz, sc-6217). For statistical analysis, at least three independent samples were analyzed using analysis of variance (one-way ANOVA) and Student’s t-test.

Quantitative PCR. Total RNA was extracted from HEK293T cells using the Absolutely RNA kit (Stratagene, 400800). 1 μg of total RNA was transcribed in first strand cDNA using M-MLV reverse transcriptase (Invitrogen, 28025-013). The cDNA synthesis was performed with oligo (dT) 12-18 (Invitrogen, 18418-012). Relative changes in transcript levels were determined on the Icycler (Bio-Rad) using SYBR green supermix (Bio-Rad, 170-8885RK). Calculations were done using the comparative CT method according to User Bulletin 2 (Applied Biosystems). For each set of primers, the PCR efficiency was determined. Primer sequences used in this study were as follows: GAPDH-for: 5’-tgccaccaccaacctgcttagc-3’, GAPDH-rev: 5’-ggcatggactgtgtgcatgag-3’; BAG3-for: 5’-tcctggacacatcccaattc-3’; BAG3-rev: 5’-tctctttctgttagccacactc-3’; HSPB8-for: 5’-gacgacttgacagcctcttg -3’, HSPB8-rev: 5’-gacacctccagcttcttg -3’.

In vitro analysis of the caspase, trypsin and chymo-trypsin activities of the proteasome. 48 h post-transfection HEK293 cells were harvested in TSDG-buffer (10 mM TRIS pH7.5, 25 mM KCl, 10 mM Nacl, 1.1 mM MgCl2, 0.1 mM EDTA, 10% glycerol) and lysed by three freeze-thaw cycles. Lysates were cleared by a 15 min cold centrifugation at 14000 rpm and protein concentrations were determined by a Bradford assay. Non-transfected cell lysates were than incubated with 0.5 μM epoxomycin for 30 min prior to the assay. Then 20 μg of the lysates were incubated in a final volume of 100 ml with fluorogenic peptide substrates (final concentration 10 mM), Suc-LLVY-AMC for Chymotryptic activity, Ac-RLR-AMC for tryptic activity and Ac-GPLD-AMC for Caspase activity (Enzo life sciences, BML-P802, BML-AW9785, BML-AW9560, respectively) and fluorescent read-out was done for 10 h at 37 °C in a Fluostar plate reader (BMG).

In vitro analysis of the proteasome activity. HEK293 cells were resuspended in cold TSDG-buffer and lysed by three freeze-thaw cycles. Lysates were cleared by 15 min cold centrifugation at 14000 rpm and protein concentrations were determined by a Bradford assay. 20 μg of protein lysate were incubated with 0.5 μM of the activity-based proteasome probe Bodipy-epoxomycin (kind gift of Dr. H. S. Overkleeft) for 1 h at 37 °C. Native SB (50 mM Tris pH 6.8, 50% glycerol, 0.1% BPB) was added and the samples were loaded on a 4-12% Bis-tris gel (Biorad). Wet gel slabs were than imaged on a Typhoon scanner (GE healthcare) with Cy3/Tamra settings (lex= nm, lem= nm).
**XBP1 splicing assay.** XBP1 splicing was measured by PCR (32 cycles: 95 °C for 30 s; 58 °C for 30 s; and 72 °C for 2 min and 4 min in the final cycle), using specific primers flanking the splicing site (XBP1 PCR up, 5’-ctggaacagcaagtggtaga-3’ and low, 5’-actgggtccttgggtaga-3’) producing the following PCR product sizes: 398 and 424 bp fragments representing spliced (XBP1s) and unspliced (XBP1u) XBP1, plus a hybrid (XBP1h) migrating as a fragment of approximately 450 bp. Products were resolved on 3% agarose gels. XBP1h represents a mixture of two hybrid structures. Each structure contains one strand from XBP1s and one strand from XBP1u and is formed in the final annealing PCR step.\(^6^1\)

**Tandem mRFP/mCherry-GFP fluorescence microscopy.** 24 h post-transfection cells were directly examined without fixation under the confocal microscope at 37 °C. Images were collected using Zeiss LSM780 Confocal Laser Scan Microscope, 63x/1.3Imm, equipped with incubation chamber with CO\(_2\) and temperature control. Images were processed using ImageJ software (http://rsb.info.nih.gov/ij/). As minor manipulation, background correction was applied to all parts of the image. Colocalization efficiency of mRFP with GFP signals was measured using Image J Co-localization Coloc 2. mRFP puncta that do not colocalize with GFP were then calculated by subtracting the colocalized fraction.

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Figure S1. HSPB8 is induced upon proteasome inhibition.

(A-C) HEK293T cells were either left untreated or treated with 20 μM MG132 for 5h or 100 nM Bortezomib overnight. Total proteins (A, B) or mRNA (C) were extracted and HSPB8 protein or mRNA levels were measured (* = p < 0.05 compared to control; n > 3 independent samples +/- sem). (D) HeLa cells were treated for 5 h with 20 μM MG132 and cells were fixed with methanol. Subcellular distribution of endogenous BAG3 and ubiquitin was investigated by immunofluorescence using specific antibodies.
Figure S2. BAG3 induces sequestration of ubiquitin into cytoplasmic puncta.

HEK293T cells were transfected with GFP, GFP-BAG3, GFP-ΔBAG or GFP-ΔPxxP encoding vectors. 48 h post-transfection cells were fixed with Methanol 100% for 10 min at -20 °C and subjected to immunofluorescence to investigate ubiquitin subcellular distribution. Immunofluorescence pictures show accumulation of ubiquitin in cytoplasmic puncta in cells expressing GFP-BAG3 and GFP-ΔPxxP. The percentage of cells containing ubiquitin positive cytoplasmic puncta is depicted (** = p < 0.001 compared to GFP; n = 3-4 independent samples +/- sem).
Figure S3. BAG3 sequesters the proteasomal reporter Ub-R-GFP into cytoplasmic insoluble puncta that colocalize with SQSTM1 and canonical autophagy markers. (A) HEK293T cells were transfected with a Ub-R-GFP encoding vector and either an empty vector or his-tagged FLBAG3 or ΔBAG encoding vectors. Cells were fixed 24 h post-transfection. The percentage of cells containing Ub-R-GFP positive cytoplasmic puncta is depicted (** = p < 0.001 compared to empty vector; n = 3-4 independent samples +/- sem). (B) NP-40 soluble and insoluble proteins were fractionated and accumulation of Ub-R-GFP in both fractions was analysed by Western blotting. (C-E) HEK293T cells were transfected with Ub-R-GFP and BAG3 encoding vectors and subjected, 24 h post-transfection, to immunofluorescence to investigate Ub-R-GFP colocalization with MAP1LC3B (C), SQSTM1 (D) and WPII1 (E).