Chaperones, protein homeostasis & protein aggregation diseases

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CHAPTER 4

Impairment of the proteasomal degradation of ubiquitinated proteins by Bag3 P209L mutant

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Abstract

Recent findings highlighted the central role of BAG3, a co-chaperone of Hsp70s, in protein degradation upon proteasome inhibition. Mutations in BAG3 have been implicated in multiple muscular and cardiac diseases. In particular, a mutation in proline 209 (P209L) leads to a severe and extremely early onset myofibrillar myopathy with associated cardiomyopathy. In this study we characterized how the BAG3-P209L mutation affects BAG3 function. We show that BAG3-P209L induces the sequestration of ubiquitinated clients into cytoplasmic puncta and inhibits their degradation.

Introduction

BAG3 is a member of the human BAG (Bcl-2-associated athanogene) family of molecular co-chaperones. BAG proteins function as nucleotide exchange factor for the HSP70 chaperone family and thereby participate in an ample variety of cellular processes [1,2]. The HSP70 family of molecular chaperones can assist in refolding of client proteins as well as in degradation of client proteins by the autophagy and the ubiquitin proteasomal systems [3–5]. BAG family members have been specifically associated with Hsp70-dependent protein degradation [3,6].

Numerous studies have shown that changes in both the ubiquitin proteasomal system and the autophagy-lysosome system occur with age [7–9]. Protein homeostasis requires an appropriate equilibrium between these protein quality-control components [10]. Using polyglutamine (polyQ) proteins as model for aggregation-prone proteins, it was demonstrated that BAG3, together with its partner HSPB8, facilitates the degradation of misfolded polyQ proteins by stimulating aggregate clearance via autophagy [11,12]. Interestingly, our recent findings so far indicate that BAG3 plays a central role in the mechanism by which eukaryotic cells degrade ubiquitinated proteins when the ubiquitin–proteasome system is impaired. Herein, BAG3 reroutes these ubiquitinated clients to autophagy in a process that is dependent on the interaction of BAG3 with HSP70 and dynein, a process that from now on will be referred to as BAG-Instructed Proteasomal to Autophagosomal Switch & Sorting (BIPASS) [13].

Mutations in BAG3 have been implicated in multiple muscular and cardiac diseases. One mutation, in proline 209 of the IPV (Ile-Pro-Val) motif, is especially detrimental. The BAG3-P209L mutation is a rare mutation that leads to a severe and early onset myofibrillar myopathy with associated cardiomyopathy [14]. Structural studies have shown that the IPV motif is necessary for BAG3 binding to its partner HSPB8, which uses the BAG3 IPV motif to form hetero-oligomers [15]. However, how exactly the P209L mutation affects the (functional) interaction of BAG3 with HSPB8 or any of its other partners of BAG3 has remained unclear. In this study we aimed to characterize how the BAG3-P209L mutation affects BAG3 function. We show that the BAG3-P209L mutant is impaired in its ability to clear protein aggregates. This impairment is related to a decrease in stability of BAG3-P209L and abnormal sequestration of HSPA1A, HSPB8 and ubiquitinated proteins that accumulate in cytoplasmic puncta and become detergent-insoluble.

Results

BAG3 P209L mutation is not affected in its capacity to bind to HSPB8 or Hsp70s.

The P209L mutation within IxI/V sequence of BAG3 causes a dominant form of childhood muscular dystrophy [14]. This motif was shown to be required for binding to its partner HSPB8 [15]. To determine whether the P209L mutation alters the interaction with its chaperone partners HSPB8 a Flag-tagged BAG3 wild-type and P209L mutant were co-expressed with a myc-tagged HSPB8 in HEK293T cells. Unexpectedly, FLAG-BAG3-P209L was found to co-immunoprecipitate equally efficient with HSPB8 as wild-type BAG3 (Fig. 1A). Also interactions with its partners HSPA1A and HSPA8 appeared largely unaffected.

BAG3 P209L mutation is impaired in its ability to clear polyglutamine protein aggregates.

Next, the consequences of the P209L mutation on the previously reported ability of BAG3 to reduce polyQ aggregation [11] was tested. HEK293T cells were co-transfected with a vector encoding a GFP tagged Huntingtin exon 1 fragment with 23 or 74 CAG repeats (Ex1Htt23Q or Ex1Htt74Q) alone, or in combination with either FLAG-tagged BAG3 wild-type or the P209L mutant. As expected, the accumulation of high molecular weight Ex1Htt74Q aggregates retained in the stacking gel was reduced by co-expression of the wildtype BAG3 (Fig. 1B). Interestingly, compared to wild type BAG3, the mutant BAG3 P209L was significantly less able to reduce the formation of high molecular weight Ex1Htt74Q aggregates (Fig. 1B). To obtain a more quantitative measure of anti-aggregation effect, we analyzed the accumulation of Ex1Htt74Q in SDS-insoluble Ex1Htt74Q fractions. Whereas wildtype BAG3 reduced Ex1Htt74Q protein aggregation by more than 60%, the effectiveness of the BAG3-P209L was significantly reduced to less than 30% aggregate reduction (Fig 1C). It is important to note that the P209L mutant still showed residual activity and did not enhance polyQ aggregation as would have been expected if the mutation would have resulted in a gain of toxic function itself. Similar data were obtained using another a vector encoding a YFP tagged ataxin 3 with 64 CAG repeats (YFP-SCA3-64Q), associated with Spinocerebellar Ataxia 3 (data not shown).
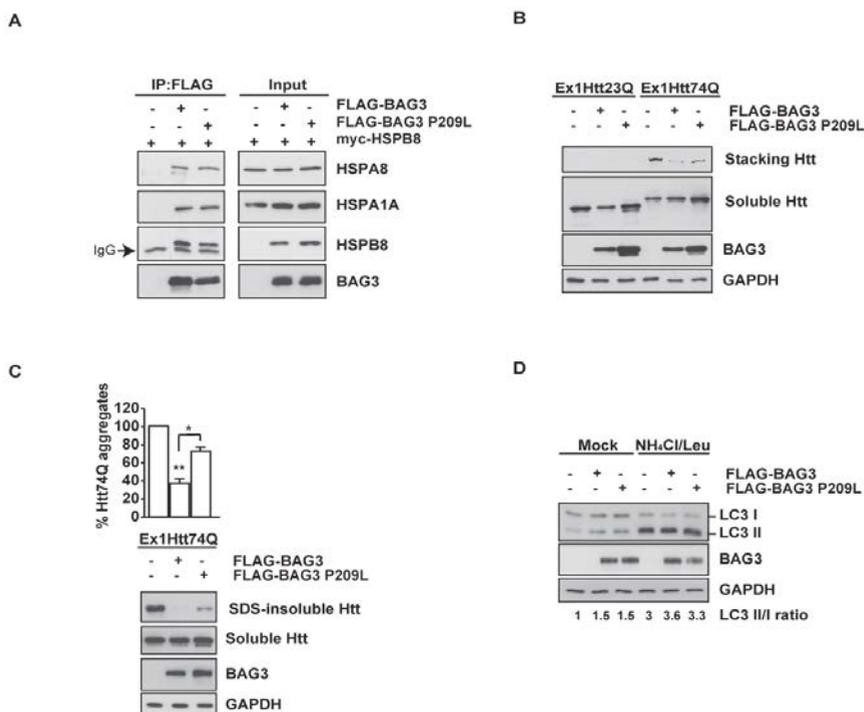
Figure 1. The myofibrillar myopathy associated BAG3 P209L mutation shows a partial impairment in the protective role toward mutated polyQ proteins.

(**A**) HEK293T cells were transfected with an empty vector or vectors encoding for Flag-tagged FL BAG3 or P209L mutant together with Myc-HSPB8. 24 hr post-transfection cells were lysed and subjected to immunoprecipitation with an antibody against Flag-tag. The immunoprecipitated complexes were analyzed by Western blotting (WB) using HSPA1A-, HSPA8 and myc-specific antibodies. (**B**) HEK293T were transfected with plasmids encoding GFP-tagged Ex1Htt23Q or Ex1Htt74Q together with either an empty vector or Flag-tagged FL BAG3 or BAG3 P209L mutant and, total proteins were extracted 48 h post-transfection. The effect of the co-chaperones on the accumulation of the high molecular weight insoluble forms retained in the stacking gel and the soluble monomeric Ex1Htt23Q or Ex1Htt74Q is shown. (**C**) SDS-insoluble levels of GFP- Ex1Htt74Q were quantified (**, $p < 0.001$; *, $p < 0.05$; average values $\pm$ S.E.M (error bars) of $n = 4$ independent samples). (**D**) HEK293T cells were transfected with either an empty vector or vectors encoding for Flag-tagged FL BAG3 or P209L mutant. Prior to extraction of total proteins cells were treated for 2 hr with Leupeptin (200 uM) and ammonium chloride (NH4Cl, 20 mM) to measure autophagy flux. MAP1LC3B-II/I ratio (normalized against GAPDH) was measured.

BAG3 P209L expression leads to an increased autophagic flux similar to wild-type BAG3.

Previous reports showed that increased BAG3 expression stimulates the turnover of autophagic vacuoles [6,11,13]. Here, we confirmed the stimulation of the autophagic flux upon overexpression of wild-type BAG3, which is reflected by increased levels of LC3 II upon inhibition of the final stage of autophagy (fusion of the autophagosomes with lysosomes; Fig 1 D) [11]. We next asked whether the decreased efficiency of P209L BAG3 mutant to inhibit mutated protein aggregation could be ascribed to a change in its ability to induce autophagy. Surprisingly, overexpressing of the P209L mutant revealed that there was no change in its ability to induce an increased turnover of autophagic vacuoles (Fig. 1 D). Altogether, these results show that the P209L mutation leads to a partial loss of BAG3-function, which is reflected in its ability to clear polyglutamine aggregates. However, this partial loss of function is neither due to changes in the mutant ability to bind to its main co-chaperone partners HSPB8 and Hsp70s, nor due to a loss of its ability to induce autophagy.

BAG3 P209L results in an altered intracellular distribution and solubility of itself and its HSP partners.

We recently found that BAG3 is up-regulated under conditions of proteasomal inhibition and subsequently reroutes proteasomal clients to autophagy (BIPASS). In doing so, BAG3 leads to formation of cytoplasmatic puncta that are positive for HSPA1A (Hsp70) and ubiquitinated proteins if the proteasome is inhibited [13]. In non-proteasomally impaired cells, wild type BAG3 showed a diffuse cytoplasmic staining, (Fig 2 A) as before [13]. However, a significant fraction of the mutant BAG3-P209L could be found in cytoplasmic puncta of otherwise normally appearing cells (Fig. 2 A). In line, an increased fraction of BAG-P209L is found in the NP-40 insoluble fraction (Fig. 2 B). Of note, these BAG3 P209L puncta did not overlap with the ER, Golgi, Mitochondria or Lysosomes (Fig. 2 F and data not shown). In parallel with BAG3 P209L insolubilization, we found a significant increase of HSPA8 and HSPB8 and to a lesser extent HSPA1A in the NP-40 insoluble fraction of BAG3-P209L overexpressing cells as compared to BAG3 overexpressing cells. In addition, immunocytochemical analyses revealed that HSPB8 and HSPA1A formed cytoplasmic puncta that co-localize with mutant BAG3 P209L (Fig.2 C and E). Surprisingly, HSPA8 was also found in cytoplasmic puncta, which did not co-localize with BAG3-P209L (Fig.2 D)
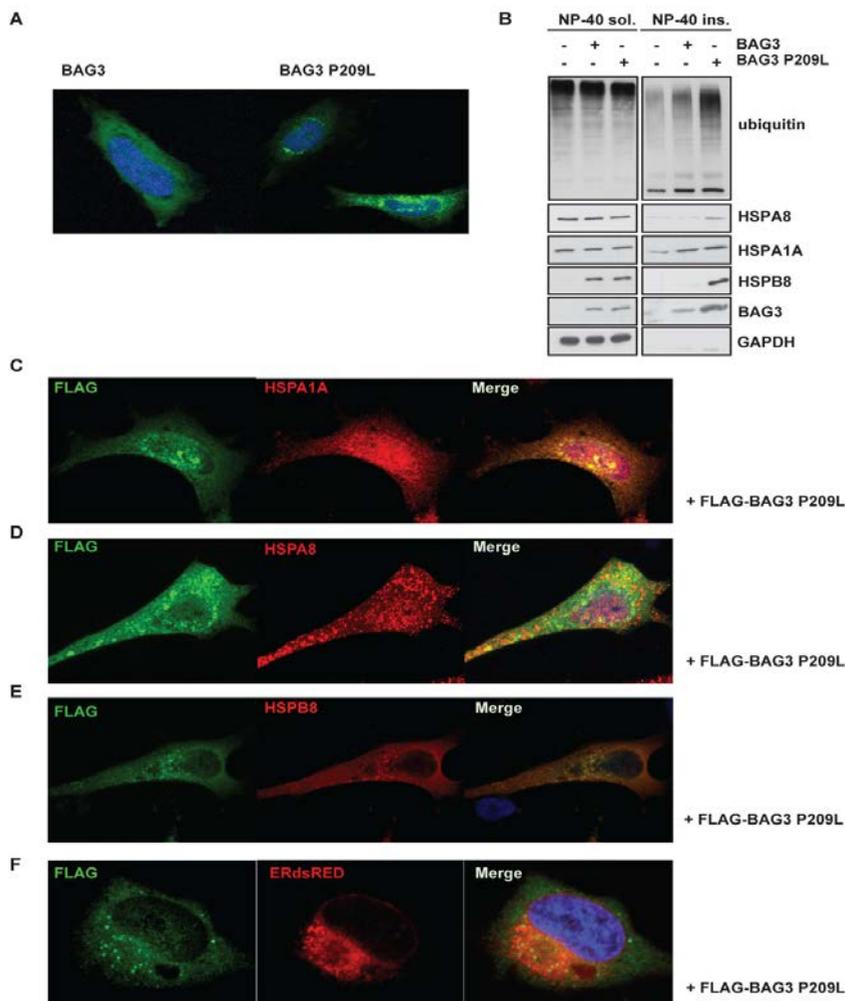
Figure 2. BAG3 P209L is an instable protein that leads to destabilization of its chaperones partners.

(**A**) Hela cells were transfected for 24hr with Flag-tagged BAG3 WT or BAG3 P209L. Subcellular distribution of BAG3 (green) and nuclei (blue) were investigated by immunofluorescence. (**B**) HEK293T cells were transfected as described in Fig. 1 A. 24 hr post-transfection NP-40 soluble and insoluble proteins were fractionated and accumulation of BAG3, HSPA1A, HSPA8, HSPB8 and ubiquitin was analysed in both fractions by Western blotting. (**C-D**) Hela cells were transfected with Flag-tagged BAG3 P209L. Cells were fixed with 2% formaldehyde for 10 min. Subcellular distribution of endogenous BAG3, HSPA1A and HSPA8 was investigated by immunofluorescence using specific antibodies. (**E**) Hela cells were transfected with Flag-tagged BAG3 P209L and Myc-HSPB8. Cells were fixed with 2% formaldehyde for 10 min. Subcellular distribution of endogenous BAG3 and HSPB8 was investigated by immunofluorescence using anti-Flag and anti-Myc antibodies. (**F**) Hela cells were transfected with Flag-tagged BAG3 P209L and ERdsRed. Subcellular distribution of endogenous BAG3 and ER was investigated by immunofluorescence.

Bag3 P209L mutant leads to accumulation of ubiquitinated protein.

As BAG3 was found to redistribute ubiquitinated clients to autophagy for degradation [13], we wondered whether expression of BAG3 P209L may impede on this process during normal growth condition and lead to an altered distribution of ubiquitinated proteins. Similarly to what we previously observed, ectopic overexpression of wild type BAG3 led to the accumulation of ubiquitin in cytoplasmic puncta. A similar effect was observed in non- transfected cells treated with the proteasome inhibitor MG132 (Fig. 3 A), in which endogenous BAG3 was upregulated [13]. Ectopic expression of the BAG3-P209L mutant led to a much larger increase in ubiquitin-positive puncta (Fig. 3 A), a pattern similar to that of the BAG3 DPxxP mutant, which cannot bind to dynein [6]. Furthermore, just as BAG3 P209L, BAG3 DPxxP has been found to be defective in the autophagic clearance of mutated huntingtin (Fig. 1)[16].

To investigate whether the P209L mutant was still able to bind to ubiquitinated proteins, we compared Ni-NTA pull-downs of the His-tagged wild-type BAG3 with the His-tagged P209L BAG3 mutant. We first confirmed previous findings [13,17] that Ni-NTA pull-down of his-tagged BAG3 led to co-precipitation of ubiquitinated proteins. Interestingly, the amount of ubiquitinated proteins pulled-down by BAG3 P209L was larger than for the wild type BAG3 (Fig. 3 B). We next investigated whether the overexpression of BAG3 P209L led to a large accumulation of ubiquitinated proteins in the NP-40-insoluble fraction is a similar manner as was found for the BAG3 DPxxP [13]. In line with our previous findings, overexpression of wild type BAG3 caused some accumulation of ubiquitinated proteins in the NP-40 insoluble fraction (Fig 2 B- 3 D). However, the increase in the amount of ubiquitinated proteins in the NP-40 insoluble fraction was much larger in cells overexpressing BAG3 P209L (Fig 2 B- 3 D). This result implies that the P209L mutant of BAG3 is fully capable of binding and rerouting client to puncta, but deficient in the subsequent stimulation of its degradation (by autophagy).

To further test this hypothesis we used a Hela cell line stably expressing Ub-G76V-GFP, a protein normally degraded by the proteasome [18] but rerouted to autophagic degradation upon BAG3 overexpression [13]. The total protein levels of Ub-G76V-GFP were found to increase upon overexpression of BAG3 P209L but not upon overexpresssion of wild type BAG3 (Fig. 3 C). Similarly, BAG3 P209L, but not wild type BAG3, led to the combined accumulation Ub-G76V-GFP and other ubiquitinated proteins into the NP-40 insoluble fraction (Fig. 3 D). Altogether these results demonstrate that BAG3 P209L, which can still bind HSPA1A (Fig. 1A) and efficiently increases the autophagic flux (Fig. 1 D), leads to the accumulation of aggregate-prone ubiquitinated proteins (Fig. 3). These ubiquitinated proteins seem to be sequestered but not adequately processed by autophagosomes.
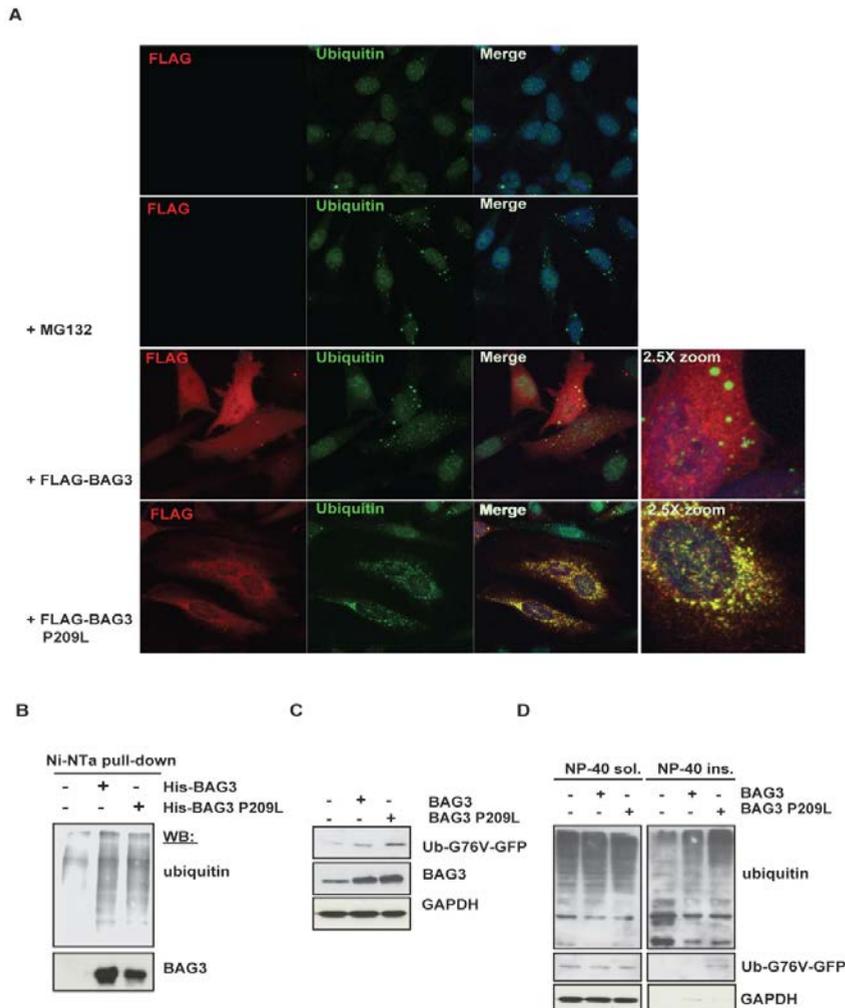
Figure 3. Bag3 P209L mutant impairs proteasomal degradation of ubiquitinated protein.
(**A**) HEK293T cells were transfected with an empty vector or vectors encoding for Flag-tagged
FL BAG3 or P209L mutant. Prior to analysis by confocal microscopy, the cells were treated
for 2 hr with Mg132 20 μM. 48 hr post-transfection cells were fixed with 2% formaldehyde
for 10 min and subjected to immunofluorescence to investigate the subcellular distribution of
ubiquitin. (**B**) HEK293T cells were transfected with an empty vector or vectors encoding for
His-tagged FL BAG3 or P209L mutant. 24 hr post-transfection cells were lysed and subjected
to purification of His-tagged BAG3 with Ni-NTA beads. (C) Hela cells stably expressing
Ub-G76V-GFP cells were transfected with an empty vector or vectors encoding for Flag-
tagged FL BAG3 or P209L mutant. Total proteins were extracted 24 hr post-transfection.
(**D**) Hela cells stably expressing Ub-G76V-GFP were transfected as described in (C).
24 hr post-transfection NP-40 soluble and insoluble proteins were fractionated and
accumulation of BAG3, Ub-G76V-GFP and ubiquitin was analysed in both fractions by
Western blotting.

Discussion

Overexpression of BAG3 was found to promote the autophagy dependent clearance of aggregation-prone protein, such as polyQ proteins and SOD1 [6,16]. In addition, BAG3 can compete with its family member BAG1 for Hsp70-bound clients. Upon BAG3 upregulation routing of polyubiquitinated substrates to proteasomes, which is normally mediated by BAG1 [3], is shifted towards a BAG3-dependent routing of clients to autophagosome (BIPASS)[13]. This BAG3:BAG1 ratio is found to increase during aging, paralleled by a shift from proteasomal to autophagosomal degradation [9]. In this study, we show that a mutation in the proline 209 of BAG3 that causes a dominantly inherited severe and early onset myofibrillar myopathy with associated cardiomyopathy [14], leads to a partial loss of function in the suppression of polyQ aggregates. In addition, we found that overexpression of this mutant leads to an accumulation of endogenous polyubiquitinated proteins. .Interestingly, in all our overexpression experiments, we consistently found higher expression levels of the mutant BAG3 P209L compared to wild type BAG3 (Figure 1), suggesting that increased stability of the mutant has is increased, thereby artificially further raising the BAG3:BAG1 ratios. So, in case of expression of the mutant BAG3 P209L competition for client routing towards the proteasomal routing may be hampered even in cells where the proteasomes are fully functional. Importantly, the P209L mutant is fully capable of binding to its partners HSPB8 and HSPA1/HSPA8, it can sequester clients into puncta and leads to an increased autophagic flux, but it seems to be defective in cargo delivery. In addition, excess of routing to the autophagosomes occurs because of the higher BAG3:BAG1 rations, clients bound to BAG P209L are not delivered to the autophagosomes, resulting in a drastically impaired protein turnover leading to the observed accumulation of ubiquitinated proteins. Note that the magnitude of this effect is comparable to the accumulation of ubiquitinated proteins upon chemical inhibition of the proteasome. In addition, expression of BAG3-P209L leads to an insolubilization of several major chaperones, which may negatively affect protein quality control leading to an even further decline in protein homeostasis.

The combination of biochemical defects we describe here for expression of the BAG3 P209L (increased client routing but defective processing into autophagosomes plus depletion of chaperones) could contribute to the development of severe and early onset myofibrillar myopathy. In fact, myofibrillar myopathy is distinguished by myofibrillar disruption and Z-disc disintegration, associated with protein aggregation and ectopic accumulation of myofibrillar proteins, which reflect imbalances in protein clearance.

Materials and Methods

Plasmids and reagents. FLAG-BAG3 and FLAG-BAG3-P209L plasmids were kindly provided by Shiniji Takayama. pCDNA3-FLAG-C1, used as a control, and ERdsRed were from Invitrogen (Carlsbad, CA, USA). Plasmid encoding human myc-tagged HSPB8 were described previously [11]. MG132 (20 μM for 3 hours) was purchased from Millipore Corporation (Billerica, MA, USA), and Leupeptin (200 uM) and ammonium chloride (NH4Cl, 20 mM) from Sigma-Aldrich (St Louis, MO, USA).

Cell Culture, Transfection, and Immunocytochemistry. HeLa (humancervical cancer), Flp-In T-REx HEK293 and HEK293T (human embryonal kidney) cells were grown in Dulbecco's modified Eagle's medium with high glucose (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin; for Flp-In T-REx HEK293 cells (Invitrogen, Carlsbad, CA, USA), 5 μg/ml Blasticidine (Sigma-Aldrich, St Louis, MO, USA) and 100 μg/ml of Zeocin (Invitrogen, Carlsbad, CA, USA) were used. Hela cell line expressing Ub-G76V-GFP was a kind gift from Dr. N. Dantuma. HEK293 and HEK293T cells were transfected by calcium phosphate precipitation as previously described [11]. Transfection of HeLa cells was performed using Lipofectamine (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. For immunofluorescence, Hela cells were plated on poly-D-Lysine (Sigma-Aldrich, St Louis, MO, USA) coated cover slips. 24 hours after transfection Hela cells were washed twice with PBS (Gibco) and fixed with 2% formaldehyde (Sigma-Aldrich, St Louis, MO, USA) for 10 minutes at room temperature. Cells were permeated using 0.1% triton in PBS for 7 minutes at room temperature and later incubated in PBS+ (0,5% BSA and 0,15% Glycine in PBS). Cells were incubated) primary antibodies overnight at 4°C. Cells were washed four times with PBS+ and incubated with Alexa Fluor 488 Donkey Anti-Rabbit IgG (Invitrogen, Carlsbad, CA, USA), Alexa Fluor 594 Donkey Anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) for 1.5 hours at room temperature. Cells were washed two times and mounted in glycerol (Agar Scientific). Confocal images were obtained using a confocal laser scanning microscope (Leica TCS SP8) with a 63X/1.32 oil objective.

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. Results are expressed as mean ± SEM. Statistical significance was analysed using an independent t-test. P<0.05 was considered statistically significant, * P<0.05, ** P<0.001.

Preparation of protein extracts, co-immunoprecipitation, purification of His-tagged BAG3 with Ni-NTA (Ni2+-nitrilotriacetate) beads and antibodies. For preparation of total protein extracts, cells were scraped and homogenized in 2% SDS lysis buffer as previously described [11]. 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% Nonidet P-40, 3% glycerol, 1 mM DTT, complete EDTA-free (Roche, Penzberg, Upper Bavaria, Germany). The cell lysates were centrifuged and cleared with A/G beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) 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. For preparation of Nonidet P-40 (NP-40) soluble and insoluble fractions, cells were harvested in immunoprecipitation lysis buffer. NP-40 soluble and insoluble fractions were separated by centrifugation at 14000 rpm for 15 min at 4°C. For purification of His-tagged BAG3 with Ni-NTA beads, cells were scraped and homogenized in lysis buffer (20 mM Tris/HCl, pH 7.4, containing 2.5 mM MgCl2, 3% (v/v) glycerol, 0.5% NP40, 150 mM NaCl, 10 mM imidazol and 1× complete protease EDTA-free). His6-tagged FL-Bag3 was purified from NP40-soluble lysates using Ni-NTA agarose beads (Qiagen). After thorough washing, five times with washing buffer 1 (20 mM Tris/HCl, pH 7.4, containing 2.5 mM MgCl2, 3% (v/v) glycerol, 0.5% NP40, 150 mM NaCl, 20 mM imidazole), proteins bound to the beads were recovered by boiling in 2% SDS sample buffer, separated by SDS/PAGE (10% gel) and analysed by Western blotting. The input corresponds to a tenth of the purified fraction. Mouse monoclonal anti-FLAG antibody was from Sigma-Aldrich (St Louis, MO, USA), while mouse monoclonal anti-HSPA1A/Hsp70 and rat polyclonal anti-HSPA8 antibodies were from Stressgen (San Diego, CA, United States). Mouse monoclonal anti-GFP and anti-myc(9E10) antibodies were from Clonetech (Mountain View, CA, USA) and MBL International (Woburn, MA, USA), respectively. Rabbit polyclonal anti-MAP1LC3B and mouse monoclonal anti-ubiquitin (FK2) antibodies were from Novus Biologicals (Littleton, CO, USA) and Enzo Life Science (Zandhoven, Belgio), respectively.

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