CHAPTER 5

Disturbances in Hsp70-Client Processing caused by the Myopathy-Associated BAG3P209L Mutant leads to a Devastating Collapse in Protein Homeostasis

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Abstract

The Hsp70-cycle is a central hub in protein quality control (PQC).\textsuperscript{1} Whereas the chaperones of the DNAJ- and HSPB-families deliver clients to Hsp70s,\textsuperscript{1–4} client-release is mediated by Nucleotide Exchange Factors (NEFs) that reduce Hsp70-client affinity.\textsuperscript{1,5–8} Precise regulation of the combination of co-chaperones in the Hsp70 complex is considered to determine the fate of Hsp70-clients.\textsuperscript{9} BAG3, the only stress-inducible BAG-protein,\textsuperscript{10} is a multi-domain NEF (Fig. 1A) that connects Hsp70 (via the BAG-domain) to proteins such as synaptopodins (WW domain), SH3 domain containing proteins, including dynein motors (PxxP-motif), and small heat shock proteins (IPV motifs).\textsuperscript{11,12} A mutation in the second IPV motif (BAG3\textsuperscript{P209L}) causes a dominant form of myofibrillar myopathy\textsuperscript{13–16} associated with protein aggregation.\textsuperscript{13,17} We found that this aggregation is, unexpectedly, neither related to the loss of HSPB binding, nor to BAG3 misfolding, or the loss of NEF function. Instead, aggregation is mediated through interactions with Hsp70 that lead to subtle defects in Hsp70-mediated client processing, which have devastating dominant negative effects on cellular PQC regulation. Importantly, drug-induced abrogation of BAG3-HSP70 interaction completely rescued these defects.
The BAG-family of NEFs consists of one ER-associated and 5 cytoplasmic BAGs, most of which have been associated with the degradation of HSPA-bound clients via either the ubiquitin-proteasome system (UPS; BAG1, BAG5, BAG6) or autophagy (BAG3).\textsuperscript{6,9,11} Like other BAGs, BAG3 can interact with Hsp70. Unlike other BAGs, BAG3 can bind to the HSPB family members HSPB1, HSPB5, HSPB6, and -with very high affinity- HSPB8 via its IPV-motifs (Fig. S1A).\textsuperscript{12,18} Bag3 binds to HSPBs with a 1:2 stoichiometry in cells\textsuperscript{19} and in vitro (Fig. S1B). The dominantly inherited BAG3\textsuperscript{P209L} mutation has been associated with myofibrillar myopathy (MFM) and associated dilated cardiomyopathy (DCM) with childhood age of onset.\textsuperscript{13–16} BAG3\textsuperscript{P209L} is located in the second of two IPV motifs, which are required for BAG3-binding to HSPBs in vitro.\textsuperscript{17,18} It remains unclear why the BAG3\textsuperscript{P209L} mutation is particularly detrimental. In vitro NMR and SPR data revealed that HSPB-binding to BAG3\textsuperscript{P209L} is reduced (Fig. 1B,C), albeit not completely lost as is the case when both IPV domains are deleted (Fig. 1D). In fact, the P209L-mutation did not affect HSPB1-binding of a BAG3 fragment mainly consisting of both IPV domains (BAG3 56-300) (Fig. S1C). Immunoprecipitation of cell lysates confirmed that the lack of both IPV-motifs (BAG3\textsuperscript{ΔIPV1ΔIPV2}) results in a complete loss of BAG3-HSPB8 binding, whereas BAG3\textsuperscript{P209L}–HSPB8 binding was only mildly reduced (Fig. 1E). Mutating the first IPV motif in the BAG3\textsuperscript{P209L} background (BAG3\textsuperscript{IPV1mut,P209L}) did not affect HSPB-binding, suggesting that the P209L substitution can still interact with HSPBs. Combined, these findings imply that the P209L mutation is not impeding on HSPB-binding per se.

The BAG3\textsuperscript{P209L} mutation has been associated with the aggregation of sarcomeric proteins, including BAG3\textsuperscript{P209L} itself.\textsuperscript{13} Accordingly, a significant portion of ectopically expressed BAG3\textsuperscript{P209L} is detergent insoluble in human cells, whereas BAG3\textsuperscript{WT} is mainly detergent soluble (Fig. S1D). Furthermore, wild type BAG3 was found to be homogenously distributed throughout the cell whereas a significant percentage of cells expressing BAG3\textsuperscript{P209L} showed a decrease in the amount of nuclear BAG3 and a punctae-like distribution pattern (~50%: see also below; Fig. 1F,G; Fig. S1E). These punctae are detergent insoluble (Fig. S1E) and reminiscent of cellular aggregates observed in patients with BAG3\textsuperscript{P209L}-associated MFM/DCM.\textsuperscript{13} Intriguingly, a complete loss in HSPB binding (BAG3\textsuperscript{ΔIPV1ΔIPV2}) did neither result in BAG3 insolubilization, nor in BAG3 punctae formation (Fig. 1F-H; Fig S1F,G), demonstrating that loss of HSPB binding is not sufficient to cause
Figure 1: BAG3P209L aggregation is not caused by a loss of HSPB binding.
BAG3<sup>P209L</sup>: Hsp70 disturbances cause a collapse in PQC

(a) Schematic representation of BAG3 depicting the WW domain, the IPV motifs, the PxxP domain and the BAG domain. The disease-causing mutation P209L resides in the second IPV domain (indicated in light green). (b) Peptides derived from IPV motifs bind Hsp27 crystallin domain. (c) Alanines I and V are necessary for binding. (d) Hsp27 mutant binding to BAG3. (e) Immunoprecipitation using anti-FLAG beads from cells expressing FLAG-BAG3WT or mutant variants using anti-FLAG beads. Western blots for FLAG (BAG3) and MYC (HSPB8) is shown. (f) Immunofluorescence pictures of FLAG-BAG3WT and indicated mutants, using a BAG3 antibody (green). (g) Quantification of the percentage of cells with BAG3 aggregates expressing the indicated variants of BAG3. Data represent the mean and standard deviation of two independent experiments (at least 100 cells were counted per experiment). (h) NP-40 insoluble fraction of cells expressing indicated FLAG-BAG3 variants. Western blot against the indicated antibodies is shown. The soluble fraction can be found in supplemental figure 1G.

the cellular BAG3<sup>P209L</sup> phenotype. In fact, mutating IPV1 in the presence of the P209L mutation (BAG3<sup>ΔIPV1P209L</sup>) reduced BAG3-punctae formation and insolubilization (Fig. 1F-H; Fig S1F,G).

Next, we investigated whether the P209L-mutation initiates self-aggregation of BAG3. This idea is supported by PEPFOLD-predictions that suggest a higher β-sheet propensity for BAG3<sup>P209L</sup> (Fig. 2A). In line with these predictions, purified recombinant BAG3<sup>P209L</sup> but not BAG3<sup>WT</sup> stains positively for thioflavin-T (Fig. 2B). Other hydrophobic mutations at position 209 led to similar results while non-hydrophobic substitutions did not stain for thioflavin-T (Fig. S2A,B), indicating that a hydrophobic amino acid at position 209 is sufficient to drive a higher β-sheet propensity of BAG3. Importantly, CD analysis revealed that recombinant BAG3<sup>P209L</sup> is properly folded (Fig. 2C) and thus does not aggregate in vitro. Yet, in contrast to the largely monomeric BAG3<sup>WT</sup>, BAG3<sup>P209L</sup> displayed an increased propensity to form oligomers (Fig. 2D). The same is true for all hydrophobic mutations at position 209 (Fig. S2C,D) and corresponds to reduced HSPB-binding (Fig. S2E,F). Consistent with these in vitro data, analyses of detergent-soluble cell lysates on sucrose gradients reveal that ectopically expressed BAG3<sup>P209L</sup> sediments at higher densities than BAG3<sup>WT</sup>, corresponding to an oligomeric state of Bag3<sup>P209L</sup> (Fig. 2E).

Subsequently, we asked whether this oligomerization of BAG3<sup>P209L</sup> impaired its NEF function. However, contrary to our expectations, the ability of BAG3<sup>P209L</sup> (Fig. 2F) and all other hydrophobic mutations at 209 (Fig. S2G,H) to bind to Hsp70 was unaffected in vitro. Likewise, the P209L mutation did neither affect BAG3 effects on HSP70-ATPase activity nor on HSP70-client release rates (Fig. 2G-I).
Figure 2: BAG3\textsuperscript{P209L} forms small oligomers, but is not misfolded and not impaired in its function as NEF for Hsp70. (a) PREFOLD \(\beta\)-sheet prediction. (b) ThT fluorescence of BAG3\textsuperscript{WT} and BAG3\textsuperscript{P209L}. (c) CD folding of BAG3 P209L. (d) BAG3\textsuperscript{WT} and BAG3\textsuperscript{P209L} binding to Hsp27C. (e) Fractionation of cells expressing FLAG-BAG3\textsuperscript{wt} or FLAG-BAG3\textsuperscript{P209L} over a sucrose gradient. Western blot against BAG3 and GAPDH is shown. (f) (g) BAG3\textsuperscript{WT} and BAG3\textsuperscript{P209L} ATP-FAM release. (h) ATPase activity of BAG3\textsuperscript{WT} and BAG3\textsuperscript{P209L}. (i) HLA-FAM substrate release activity of BAG3\textsuperscript{WT} and BAG3\textsuperscript{P209L}.
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Since neither loss of HSPB-binding, nor loss of HSP70-binding, or impaired NEF function were associated with BAG3<sup>P209L</sup>-dysfunction, we decided to generated a series of BAG3 single and double mutants by deleting the WW-, PxxP-, or BAG-domain alone or in addition to the P209L mutation (Fig. S3A). Expression of neither single domain deletion (BAG3<sup>ΔWW</sup>, BAG3<sup>ΔPxxP</sup>, or BAG3<sup>ΔBAG</sup>) resulted in punctae-formation or BAG3-insolubilization (Fig. 3A-C; Fig. S3B). However, expression of the BAG3<sup>P209L-ΔWW</sup> and BAG3<sup>P209L-ΔPxxP</sup> double mutants were associated with punctae-formation similar to the effects of the BAG3<sup>P209L</sup> single mutant (Fig. 3A-C; Fig. S3B). These results imply that interactions with BAG3- binding partners, such as synaptopodins (WW domain)<sup>20</sup> and SH3 domain containing proteins (e.g. dynein motors; PxxP-motif)<sup>21</sup>, are not involved in BAG3<sup>P209L</sup>-related aggregation. Surprisingly, however, despite Hsp70-binding and NEF functions being largely unaffected by the BAG3<sup>P209L</sup>-mutation (Fig. 2), expression of the BAG3<sup>P209L-ΔBAG</sup> did not result in P209L-associated punctae-formation and insolubilization (Fig. 3A-C; Fig. S3B). In vitro data confirmed that deletion of the BAG3 domain -but not of the WW or PxxP domains- results in the loss of Hsp70 binding (Fig. S3C,D) while preserving HSPB-binding (Fig. S3E,F). Further, cellular immunoprecipitation experiments confirmed the loss of Hsp70 binding solely upon deletion of the BAG domain (Fig. 3D).

The requirement of HSP70 interaction for BAG3<sup>P209L</sup> aggregation was confirmed by experiments with a single point mutant in the BAG domain (R480A) that abrogates BAG-Hsp70 interaction (Fig. 3D).<sup>22</sup> Indeed, the BAG3<sup>P209L-R480A</sup> double mutant did neither result in insolubilization (Fig. 3E), nor in punctae-formation (Fig. 3A,B). Also experiments with two small molecules, YM01 and JG98, which specifically impair Hsp70-BAG3 interaction (Fig. 3F) by stabilizing the ADP-bound form of Hsp70, confirmed that Hsp70 interaction is required for BAG3<sup>P209L</sup>-insolubilization.<sup>23</sup> Both these compounds reduced the BAG3<sup>P209L</sup>-associated insolubilization in dose dependently (Fig. 3G,H), whilst a non-effective, structural similar drug did not affect BAG3<sup>P209L</sup>-solubility (data not shown). Despite its apparent normal action as a NEF, these data conclusively demonstrate that HSP70 binding is required for BAG3<sup>P209L</sup>-aggregate formation.

To sort out this apparent controversy, we tested the functionality of the Hsp70 machine in the presence of either BAG3<sup>WT</sup> or BAG3<sup>P209L</sup> by measuring Hsp70-dependent refolding of chemically denatured luciferase in vitro. Adding increasing concentrations of BAG3<sup>WT</sup> to the DNAJ/HSP70/luciferase mixture
Figure 3: BAG3<sup>P209L</sup> aggregation requires Hsp70 interaction. (a) Immunofluorescence images of HeLa cells expressing FLAG-BAG3wt and indicated mutants using an antibody recognizing BAG3 (green). (b) Quantification of the fraction of cells with immunofluorescence detectable punctae (2 experiments, at least 100 cells were counted.)
BAG3\textsuperscript{P209L} disturbances cause a collapse in PQC per experiment). (c) Western blot of the NP-40 insoluble fraction of cells expressing BAG3 wt and indicated variants of BAG3. (d) Immunoprecipitation of FLAG-BAG3 variants from cells expressing both Myc-HSPB8 and indicated FLAG-BAG3 variants. (e) Western blot of NP-40 soluble and insoluble fractions of cells expressing indicated BAG3 variants; FLAG (BAG3), Myc (HSPB8) and tubulin antibodies were used. (f) Western blot of immunoprecipitates using anti FLAG beads from HEK293 cells expressing both FLAG-BAG3\textsuperscript{P209L} and Myc-HSPB8 in cells treated with either 0.5 μM JG-98, 5 μM YM-01 or DMSO; FLAG (BAG3), Myc (HSPB8), HSPA1A and actin antibodies were used. (g, h) Western blot of NP-40 soluble and insoluble fractions of cells expressing both FLAG-BAG3\textsuperscript{P209L} and Myc-HSPB8, treated with DMSO or increasing concentration of the drug JG-98 (0.25, 0.5 or 1.0 μM) (g) or YM-01 (1.25, 2.5, 5.0 or 10 μM)(h).

gradually enhanced the yield of luciferase folding (Fig. 4A) comparable to other BAGs.\textsuperscript{9} In contrast, BAG3\textsuperscript{P209L} was unable to stimulate Hsp70-mediated luciferase refolding (Fig. 4A). In living cells, NEF levels are at a near to optimal concentration and further increases in expression-levels through ectopic expression generally reduces the cellular folding capacity.\textsuperscript{24} However, expression of BAG3\textsuperscript{P209L} reduced HSP70-folding capacity even further, whereas the additional mutation in the BAG domain alleviated these effects (BAG3\textsuperscript{P209L-R480A}, Fig. 4B). Albeit not being kinetically affected, the cellular and \textit{in vitro} data conclusively suggest that BAG3\textsuperscript{P209L}-mediated -substrate release is not functionally productive (Fig. 2).

BAG3 has been found to redirect ubiquitinated Hsp70-clients from the proteasome to the autophagy-lysosome system for degradation under conditions of proteasomal stress.\textsuperscript{25} During this process, termed BAG-induced proteasome to autophagy switch and sorting (BIPASS), ubiquitinated Hsp70-clients, BAG3, and Hsp70 temporarily localize to p62/LC3-positive punctae before being degraded by autophagy.\textsuperscript{25} Intriguingly, the punctae formed by BAG3\textsuperscript{P209L} under non-stress conditions stain positive for p62, but not LC3 (Fig. 4C), suggesting that BAG3\textsuperscript{P209L} remains partially functional in initiating BIPASS, but that adequate HSP70/HSPB-client processing is somehow impaired. Both, BAG3\textsuperscript{WT} and BAG3\textsuperscript{P209L} indeed caused an increase in the LC3-II:LC3-I ratio (Fig. 4D,E) as found before for BAG3\textsuperscript{WT}.\textsuperscript{19} However, compared to BAG3\textsuperscript{WT}, the BAG3\textsuperscript{P209L}-ability to support the disposal of protein aggregates initiated by polyglutamine (polyQ) was much reduced (Fig. 4F,G; FigS4A).\textsuperscript{26} This indicates that cargo-delivery to the autophagic system is impaired in BAG3\textsuperscript{P209L}-expressing cells. In line with this hypothesis, BAG3\textsuperscript{P209L} expression led to the accumulation of ubiquitin-positive punctae that co-localized with BAG3\textsuperscript{P209L} (Fig. 4H,I). Moreover, NP40-insoluble
ubiquitinated proteins accumulated upon BAG3\textsuperscript{P209L}-expression (Fig. 4J).

To further investigate whether BAG3\textsuperscript{P209L} affects the disposal of proteasomal clients, we co-expressed the proteasomal reporters Ub-R-GFP (degraded by the proteasome via the N-end-rule pathway) and GFP-ODC (degraded by the proteasome via ubiquitin-independent pathways\textsuperscript{27}) with BAG\textsuperscript{WT} or BAG3\textsuperscript{P209L}.

Expression of BAG3\textsuperscript{WT} led to small increases in both reporters (Fig. 4K), which reflects its role in the re-routing of proteasomal HSP70-clients to autophagosomes.\textsuperscript{25} Mirroring its effects on endogenous ubiquitin, BAG3\textsuperscript{P209L} dramatically increased the levels of both reporters and BAG3\textsuperscript{P209L}-but not BAG3\textsuperscript{WT}- and led to their insolubilization (Fig. 4K). Experiments using a cell line stably expressing yet another reporter, Ub-G76V-YFP (degraded via the UFD-pathway),\textsuperscript{28} showed qualitatively similar results (Fig. 4I). Comparable to BAG3\textsuperscript{P209L}-insolubilization (Fig. 1), the BAG3-IPV mutants BAG3\textsuperscript{ΔIPV1,ΔIPV2} and BAG3\textsuperscript{ΔIPV1P209L} did not lead to the insolubilization of ubiquitinated proteins and their accumulation in BAG3\textsuperscript{P209L} punctae and to relatively less insolubilization and punctae formation of ubiquitinated proteins than BAG3\textsuperscript{P209L}, respectively (Fig. S4C,D). In addition to abrogating punctae formation (Fig. 3), deletion of the BAG domain (but not the WW or PxxP domain) in the background of the P209L mutation negated BAG3\textsuperscript{P209L} effects on insolubilization of ubiquitinated proteins (Fig. S4E) and their accumulation into BAG3\textsuperscript{P209L} punctae (Fig. 4I).

In addition, the BAG3\textsuperscript{P209L-R480A} double mutant neither led to the insolubilization of ubiquitinated proteins (Fig. 4L) nor to the accumulation and precipitation of the UFD reporter (Fig. S4B); the drugs JG98 and YM01 counteracted the effects of BAG3\textsuperscript{P209L} on insolubilization of ubiquitinated proteins (Fig. S4F,G). Withal, these results indicate that BAG3\textsuperscript{P209L} leads to a general impairment of processing ubiquitin-tagged, HSPB- or/and HSP70- bound proteins.

How the accumulation of aggregates in cells can lead to toxic effects has been a matter of dispute for many years and may involve several, perhaps parallel acting, events.\textsuperscript{29} One consequence of aggregation is the sequestration of components of the protein quality control systems, which leads to a vicious and progressive decline in protein homeostasis that subsequently impairs many cellular functions and eventually results in cell death.\textsuperscript{29} Indeed, upon expression of BAG3\textsuperscript{P209L}, we found several HSPs (HSPA1A, HSPA8, HSPB1, HSPB8, DNAJB1, and DNAJB6: Fig 5A), albeit not all HSPs tested (HSPB5 and HSPA6), to be enriched in the same detergent-insoluble fraction as BAG3\textsuperscript{P209L} and ubiquitinated
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Chapter 5

(A) Luciferase refolding

(B) Luciferase refolding

(C) Anti-BAG3 Anti-P62 Merge

(D) Anti-BAG3 Anti-LC3 Merge

(E) LC3 protein levels

(F) HmQ74

(G) SDS-insoluble aggregates (%)
Figure 4: BAG3P209L mutant is partially dysfunctional as Hsp70 co-chaperone. 
(a) NP-40 solubility of BAG3WT and indicated BAG3-mutants. (b) Relative luciferase folding capacity of HEK293 cells expressing HSPB8 and BAG3WT or indicated mutants of BAG3. Luciferase levels were normalized to those in cells expressing BAG3WT. Data represents the mean and standard error of two independent experiments. (c) Immunofluorescence pictures of HeLa cells expressing FLAG-BAG3WT or FLAG-BAG3P209L using antibodies against BAG3 (green: both left and right panel), p62 (red: left panel) or LC3 (red: right panel). (d) Induction of autophagy in cells expressing empty vector, FLAG-BAG3WT or FLAG-BAG3P209L: cells were treated with NH4Cl and Leupeptin or not. Western blot against the indicated antibodies is shown. (e) Quantification of autophagy induction of experiments similar to d. Relative LC3 proteins levels are shown. Data represents the mean and standard deviation of three independent experiments. (f) Suppression of GFP-HttQ74 aggregation of cells expressing a control, FLAG-BAG3WT or FLAG-BAG3P209L. Western blot against indicated antibodies is shown. (g) Quantification of GFP-HttQ74 aggregation of experiments similar to f. Relative percentage of SDS-insoluble protein levels are shown. Data represents the mean and standard deviation of three independent experiments. (h) Immunofluorescence pictures of HeLa cells expressing FLAG-BAG3WT or FLAG-BAG3P209L using antibodies against BAG3 (green) or ubiquitin (red). (i) Quantification of the percentage of cells with BAG3 aggregates (green) and percentage of cells with BAG3 aggregates that co-localize with ubiquitin (red). Cells expressed HSPB8 and BAG3 or the indicated variants of BAG3. Data represents the mean and standard deviation of two independent experiments at least 100 cells were counted per experiment. (j) Fractionation of HEK293 cells expressing a control, FLAG-BAG3WT or FLAG-BAG3P209L. Western blot against FLAG (BAG3), ubiquitin, GAPDH and H2A are shown. (k) Fractionation of HEK293
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cells expressing HSPB8, a control or BAG3<sup>WT</sup> or BAG3<sup>P209L</sup>, together with either Ub-R-GFP or GFP-ODC (ornithine decarboxylase). Western blot against GFP, FLAG (BAG3), Myc (HSPB8) and tubulin are shown. (I) Fractionation of cells expressing HSPB8 and indicated BAG3 variants. Western blot using ubiquitin (FK2) and tubulin antibodies are shown.

proteins (Fig. 5A), co-localizing with the BAG3<sup>P209L</sup>-punctae (Fig. 5B,C). This co-aggregation was exclusively dependend on BAG3<sup>P209L</sup>-HSP70 interaction (Fig. 5A,C; Fig. S5A). Although NEFs and DNAJs are HSP70-co-chaperones, the general idea is that they bind to HSP70 during different phases in the ATP cycle (DNAJs to the ATP-bound state, NEFs to the ADP-bound stage). Indeed, the recruitment of both DNAJB1 and DNAJB6 into the aggregates is independent of the J-domain (Fig. 5D) and thus independent of the DNAJ-HSP70 interaction. These findings imply that these (co)chaperones are most likely recruited to substrates that are trapped in the BAG3<sup>P209L</sup>-punctae after release, when they became insoluble. In line with such a secondary trapping model, only the DNAJ isoform of DNAJB6 (26 KDa) that is cytosolic and nuclear became insoluble in BAG3<sup>P209L</sup>-expressing cells, while the exclusively nuclear isoform (40 KDa) remained soluble (Fig. 5A).

To assess whether trapping of these chaperones indeed exerts negative effects on their cellular functions, we tested the ability of DNAJB6b to suppress polyQ aggregation in the background of BAG3<sup>WT</sup> or BAG3 mutants. Whereas BAG3<sup>WT</sup> can reduce aggregation initiated by short polyQ<sub>43</sub> expansions (Fig. 4E),<sup>26</sup> it cannot reduce aggregation initiated by long polyQ<sub>119</sub> fragments (Fig. 5E). DNAJB6 co-expression alone almost completely suppresses polyQ<sub>119</sub> aggregation (Fig. 5E),<sup>30,31</sup> however, in line with a dominant negative effect on the function of other chaperones, co-expression of BAG3<sup>P209L</sup> -but not BAG3<sup>WT</sup> or BAG3<sup>P209L-R480A</sup>-inhibited DNAJB6-mediated anti-aggregation (Fig.5E).

To address the question if and how endogenous BAG3<sup>P209L</sup> results in similar phenotypes, we performed experiments with fibroblasts derived from a BAG3<sup>P209L</sup> mutation carrier. Endogenous expression of BAG3 is low in most cell types, including fibroblasts, which may be why spontaneous BAG3<sup>P209L</sup>-related punctae were not observed in patient fibroblasts (Fig. 5F). BAG3 is the only stress-inducible BAG-protein<sup>10</sup> and is up-regulated by a variety of stress signaling cascades that overwhelm the capacity of the proteasome, including its chemical inhibition.<sup>25</sup> Correspondingly, short bortezomib treatment of BAG3<sup>P209L</sup> mutation carrier-derived fibroblasts -but not of fibroblasts derived from donors without the mutation- led to the formation of persistent BAG3 punctae (Fig. 5F,G).
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Figure 5. BAG3$^{P209L}$ aggregates sequester chaperones in an HSP70-binding dependent manner. (a) Fractionation of HEK293 cells expressing Myc-HSPB8 and BAG3 variants. Western blots using the indicated HSP antibodies is shown. (b) Immunofluorescence pictures of HeLa cells expressing FLAG-BAG3$^{P209L}$. Cells were stained with the indicated antibodies for staining endogenous chaperones except HSPB8. (c) Quantification of the percentage of cells with aggregates and cells with aggregates that colocalize with the indicated chaperones. Data represents the mean and standard deviation of two independent experiments (at least 100 cells were counted per experiment). (d) Immunofluorescence pictures of HeLa cells expressing BAG3 WT or BAG3$^{P209L}$ (upper row in green) with V5-DNAJB6bWT or V5-DNAJB6bH32Q (middle row in red). (e) Filter trap assay of cells expressing HttQ119-YFP together with Tetracyclin-inducible DNAJB6b and the indicated FLAG-BAG3 variants. Western blot using a GFP antibody is shown. (f) Immunofluorescence picture of primary control or BAG3$^{P209L}$ patient fibroblasts. Cells were either untreated, treated for 6 hours with bortezomib followed by a 20 hour recovery or treated for 6 hours with bortezomib followed by a 20 hour recovery in the presence of 0.05 μM JG98. Arrows indicate BAG3-punctae.
Co-treatment of BAG3\textsuperscript{P209L} fibroblasts with bortezomib and YM01/JG98 reduced the frequency of persistent punctae (Fig. 5F,G).

Withal, our data have shown that minimal disturbances in the functionality of the Hsp70 machine can have devastating effects on cellular functioning. The BAG3\textsuperscript{P209L} mutant, which causes a childhood onset fulminant form of MFM and DCM with childhood age of onset,\textsuperscript{13-16} is properly folded, fully capable of binding HSP70 and of functioning as NEF for HSP70, but slightly impaired in the proper processing of HSP70-bound clients. This subtle primary defect is associated with BAG3\textsuperscript{P209L}-oligomer formation and leads to a dramatic secondary disruption of the normal protein homeostasis by trapping crucial chaperones via the interaction with Hsp70 through the BAG-domain. Remarkably, these (secondary) trapped chaperones become partly non-functional, which is exemplified by the BAG3\textsuperscript{P209L}-effects on DNAJB6 function. Intriguingly, just like BAG3 mutations, mutations in DNAJB6 have been linked to myopathies.\textsuperscript{32,33}

The selective sensitivity of skeletal and cardiac muscle cells to BAG3 mutations may be linked to the fact that BAG3 is constitutively expressed at low levels in most tissues. Stress situations that overload the proteasome specifically increase BAG3 levels -but not other BAGs\textsuperscript{10}, thereby enabling cells to re-route the proteasomal clients to autophagosomes.\textsuperscript{25} Muscle cells are repeatedly exposed to cellular stress (e.g. mechanic stress from exercise) that may cause BAG3 levels to rise to high enough levels that the primary defects of mutant like BAG3\textsuperscript{P209L} may start to have an impact. Amongst the BAGs, BAG3 has the highest binding-affinity for Hsp70s,\textsuperscript{9} which implies that small increases in BAG3 may suffice to initiate the negative cascade described here; the data on patient-derived fibroblasts provides a proof-of-concept for this idea. Most importantly, we show that drugs specifically interfering with BAG3-Hsp70 binding can not only attenuate this cascade but also minimize its toxic downstream effects. These drugs have been tested in the concept of cancer and were shown to have minimal negative effects on otherwise healthy cells\textsuperscript{9} and thus have the potential to serve as therapeutic treatment in BAG3-related myopathies.
Materials and Methods

Recombinant proteins and constructs
Plasmids encoding myc-tagged human HSPB8 and peGFP-HDQ74, which was kindly provided by Dr. D.C. Rubinsztein were described before. FLAG-BAG3 WT and P209L constructs were kindly provided by Dr. S. Takayama. The primers and plasmids used in this study are listed in Supplementary Table S1 and Supplementary Table S2. To generate Flag tagged human BAG3 (pcDNA3 FLAG-BAG3), BAG3 delta BAG (pcDNA3 FLAG-BAG3 ΔBAG), and BAG3 delta PxxP (pcDNA3 FLAG-BAG3 ΔPxxP), the BAG3 encoding sequences of the following constructs were amplified by polymerase chain reaction (PCR) with Phusion polymerase, purified by agarose gel electrophoresis and ligated to EcoRI and XhoI digested pcDNA FLAG-Parkin: BAG3 (pCN His-BAG3), BAG3 ΔBAG (pCN His-BAG3 ΔBAG) and BAG3 ΔPxxP (pCN His-BAG3 ΔPxxP). Using specific primers, the P209L mutation was introduced into Flag-tagged BAG3 constructs with the Pfu turbo DNA polymerase site directed mutagenesis kit to generate the following constructs: BAG3P209L (pcDNA3 FLAG-BAG3P209L), BAG3 delta BAG P209L (pcDNA3 FLAG-BAG3 ΔBAG P209L), BAG3 delta PxxP P209L (pcDNA3 FLAG-BAG3 ΔPxxP P209L), BAG3 delta WW P209L (pcDNA3 FLAG-BAG3 ΔWW P209L), and BAG3 IPV1 IPV2 (pcDNA3 FLAG-BAG3 IPV1 AAA IPV2 AAA) were constructed in a similar fashion; all constructs were sequence verified.

Antibodies and reagents
Antibodies (dilutions are indicated in brackets for western blot (WB), immunofluorescence (IF) or immunoprecipitation (IP)) against FLAG (Sigma, clone M2; Sigma, M, Wb 1:1000, IF 1:200), ubiquityl-histone H2A (Millipore, clone E6C5), ubiquitin (Norvus Biologicals, FK2, M, WB 1:1000, IF 1:1000; Dako WB), K48-linkage specific polyubiquitin (Enzo lifesciences, WB 1:1000), K63-linkage specific polyubiquitin (Cell Signalling, clone D7A11, 1:1000), myc (MBL, clone PL14, WB 1:3000, IF 1:100), HSC70 (Stressgen, WB 1:5000, IF 1:100), HSP70 (Stressgen, SP-A810, WB 1:1000, IF 1:50), HSPA1A (Enzo life sciences, clone XXX, Rb, dilution?), HSPB1 (Stressmarq biosciences), GAPDH (Fitzgerald, clone 6C5, WB 1:50000), histone H2A (Abcam, WB 1:5000), MYC (Clonetech, Mountain View, CA, USA), DNAJB1/Hsp40 (Stressgen, San Diego, CA, USA, Rb, dil) were used.

MG132 (20uM for 3-6 hours), rapamycin, Pepstatin A (10ug/ml), E64d (10ug/ml), 3-Methyladenine (3-MA, 10mM) ammonium chloride (NH4Cl, 20mM) were from sigma.
Cell culture and transfection

Standard cell culture techniques were used. HeLa (human cervical cancer), HEK293, and HEK293T (human embryonal kidney) cells were grown at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium with high glucose supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Gibco). Cell lines stably expressing GFP-ubiquitin (Dantuma) were generated by transfecting GFP-ubiquitin into HEK293-cells with lipofectamine using standard procedures. HEK293T and HeLa-cells were transfected with calcium phosphate precipitation, as previously described; for higher efficiency in immunofluorescence experiments, HEK293 and HeLa cells were transfected with lipofectamine (Invitrogen) or polyethylenimine (PEI) (Sigma-Aldrich, St Louis, MO, USA) according to manufacturer’s protocols.

Protein-pull down

For immunoprecipitation, cells were lysed on ice in IP lysis buffer 24 hours post transcriptional activation (buffer: 60/80mM KCl, 50mM HEPES pH 7.5, 1.5 mM MgCl₂, 0.4% Nonidet P-40, 10/3% glycerol, 0.5mM DTT, complete EDTA-free (Roche Applied Sciences) and 10mM NEM). Cell lysates were homogenized by passing lysates 5/6 times through a 26G needle and the total or ‘whole cell lysate’ was collected. Lysates were centrifuged at maximum speed for 15 minutes at 4°C in order to separate the supernatant (input or soluble fraction) from the pellet fraction (insoluble fraction). In the meanwhile magnetic beads complexed with FLAG-antibodies (14ul beads/sample; Sigma, FLAG M2, clone) were washed with IP lysis buffer. Pellet fractions were resolubilized with 2%SDS buffer containing betamercapthoethanol, boiled and stored for future use. Co-immunoprecipitation was performed by adding the input fraction onto the washed magnetic beads and incubating them at slow rotation (5RPM) for at least 2 hours at 4°C. The first wash was performed using the IP-volume in lysis buffer and a magnetic stand. In between washes, beads were incubated on the rotator for 5 minutes at slow rotation at 4°C. Further 3 washes were performed in a similar way using 1ml of IP lysis buffer. During the last wash, beads were transferred into new cups and washed with IP lysis buffer without detergent. Proteins were eluted using ½ volume 2%SDS buffer and ½ volume 4x2%SDS buffer and 10% betamercaptoethanol. Samples were kept on ice until they were boiled for 5 minutes. Co-immunoprecipitated proteins and input fractions were resolved on SDS-PAGE the same day as the IP was performed.

Fractionations

Protein measurement was performed using the nanophotometer (company), protein content was equalized and equal amounts of volume and protein were used for subsequent fractionation. SDS fractionation was performed as previously described. For NP40 fractionations, cell-pellets were resuspended in NP40 fractionation buffer (Fractionation
**Western Blotting**

Following the preparation of protein samples, proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and processed for Western blotting. Primary antibodies (at concentrations mentioned above) were prepared in 3% BSA/PBSTween, secondary antibodies (Invitrogen, horse peroxidase conjugated IGG or IGM) in 5% milk/PBSTween. For visualization membranes were incubated with ECL western blotting substrate (Pierce, cat. No. 32106) for 2 minutes and developed (Sigma, cat. No. P7042-1GA) and fixed (Sigma, cat. No. P7167-1GA) using luminescent films (Amersham hyperfilm, GE-healthcare, cat. No. 28906837).

**Immunofluorescence**

HeLa cells were seeded on poly-D-lysine coated glass slides and fixed 48 hrs post-transfection. HeLa cells were washed once with PBS (Gibco) and fixed with 2% formaldehyde (Sigma-Aldrich, St Louis, MO, USA) for 15 minutes at room temperature with 2% paraformaldehyde for 15 minutes. Fluorescent stainings were performed as previously described. Primary antibody incubation was performed overnight at 4°C in a humid chamber and Alexa-conjugated dyes (Invitrogen, Carlsbad, CA, USA; Alexa Fluor 488 goat-anti-Rabbit, Alexa Fluor 488 donkey-anti-Rabbit, Alexa Fluor 594 goat-anti-Rabbit, Alexa Fluor 594 donkey-anti-Rabbit, Alexa Fluor 488 goat-anti-mouse, Alexa Fluor 594 goat-anti-mouse, Alexa Fluor 594 chicken-anti-rat) were applied for 1.5 hrs at room temperature to visualize primary antibodies. Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) or Hoechst 33258 for 5 minutes, and samples were and embedded in glycerol (CitiFluor, Agar Scientific).

**Imaging**

Immunofluorescence (IF) images were captured using confocal laser scanning microscope (Leica TCS SP8) with a 63X/1.40 objective lens. Z-stack images were obtained to check for the aggregates in different Z-planes. Quantification of the aggregates in various mutants was carried out manually using Leica DM6000 microscope. Imaris, photoshop, and Image J software was used for image processing.
## Supplementary materials

Supplementary Table S1: Primers used for cloning *E.coli* strains in this study

<table>
<thead>
<tr>
<th>Primer</th>
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<td>Bag3 EcoRI F</td>
<td>TACTACGAATTCTATGAGCGGCGCCACCCAC TGG</td>
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<td>bag3_revers e_Xhol</td>
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<tr>
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**BAG3^{P209L}:** Hsp70 disturbances cause a collapse in PQC

**Supplementary Table S2: Plasmids used in this study**

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<td>pcDNA containing FLAG tagged human Parkin, Amp&lt;sup&gt;β&lt;/sup&gt;</td>
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<td>pCINeo containing His tagged human BAG3, Amp&lt;sup&gt;β&lt;/sup&gt;</td>
<td>Serenn a carra</td>
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<tr>
<td>pCIN His-BAG3&lt;sub&gt;ΔBAG&lt;/sub&gt;</td>
<td>pCINeo containing His tagged human BAG3 with BAG domain deletion, Amp&lt;sup&gt;β&lt;/sup&gt;</td>
<td>Serenn a carra</td>
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Figure S1. (a) BAG3-binding of Hsp27, Hsp27-mutants, αβ-crystallin, Hsp20, and Hsp22. (b) Molecular weight of Hsp27c, BAG3, and Hsp27. (c) BAG3-binding of indicated BAG3 mutants. (d) NP40-solubility of BAG3WT and BAG3P209L. (e) Cellular BAG3WT and BAG3P209L distribution of detergent-resistant BAG3. (f) Schematic overview of experimental BAG3-mutants. (g) NP40-soluble fraction of BAG3WT and indicated experimental BAG3-mutant.
Figure S2. (a) BAG3-binding of Hsp27, Hsp27-mutants, αβ-crystallin, Hsp20, and Hsp22. (b) Molecular weight of Hsp27c, BAG3, and Hsp27. (c) BAG3-binding of indicated BAG3 mutants. (d) NP40-solubility of BAG3WT and BAG3P209L. (e) Cellular BAG3WT and BAG3P209L distribution of detergent-resistant BAG3. (f) Schematic overview of experimental BAG3-mutants. (g) NP40-soluble fraction of BAG3WT and indicated experimental BAG3-mutant.
Figure S3. (a) Schematic representation of BAG3WT and experimental BAG3-domain replacements. (b) Cellular BAG3-distribution in mammalian cells transfected with BAG3WT and indicated BAG3-domain replacements. (c) BAG3-binding of BAG3WT and indicated BAG3-domain replacements. (d) ITC stoichiometry of BAG3WT and indicated BAG3-domain replacements to BAG3. (e) Hsp27-binding of BAG3WT and indicated BAG3-domain replacements. (f) ITC stoichiometry of BAG3WT and indicated BAG3-domain replacements to Hsp27.
BAG3_P209L: Hsp70 disturbances cause a collapse in PQC

Chapter 5

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**S4A**

GF-HiQ23  | GFP-HiQ74  
---|---
Ct. | BAG3

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**S4B**

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Flag-BAG3  | Flag-BAG3_pIM  | Flag-BAG3_pIM  |
---|---|---|
Flag-BAG3_pIM | Flag-BAG3_pIM  | Flag-BAG3_pIM  |
Flag-BAG3_pIM | Flag-BAG3_pIM  | Flag-BAG3_pIM  |
Flag-BAG3_pIM | Flag-BAG3_pIM  | Flag-BAG3_pIM  |
Flag-BAG3_pIM | Flag-BAG3_pIM  | Flag-BAG3_pIM  |
Ub_pIM_GFP  | MG132 (5 µM) |

**S4C**

NP-40 Sol.  | NP-40 Insol. |
---|---|
| + + + + + + + + | + + + + + + + + |
| + + + + + + + + | + + + + + + + + |
| + + + + + + + + | + + + + + + + + |
| + + + + + + + + | + + + + + + + + |
| + + + + + + + + | + + + + + + + + |

Flag-BAG3  | Flag-BAG3_pIM  |
---|---|
Flag-BAG3_pIM | Flag-BAG3_pIM  |
Flag-BAG3_pIM | Flag-BAG3_pIM  |
Flag-BAG3_pIM | Flag-BAG3_pIM  |
Flag-BAG3_pIM | Flag-BAG3_pIM  |
Flag-BAG3_pIM | Flag-BAG3_pIM  |
Myc-HspB8 |

**S4D**

% of cells with aggregates correlating with Ub
Figure S4. (a) GFP-HttQ23- and GFP-HttQ74-accumulation following BAG3WT and BAG3 P209L–transfection. (b) NP40-solubility of BAG3WT and BAG3 P209L with and without R480-mutation following. MG132 was used as a control. (c) NP40-solubility of ubiquitin following cellular transfection with BAG3WT and BAG3-domain replacements. (d) Percentage of cells with aggregates co-localizing with ubiquitin following the transfection of BAG3-domain replacements. (e) NP40-solubility of ubiquitin following transfection with BAG3 and BAG3-domain replacements. (f) NP40-solubility of ubiquitin following BAG3 P209L-transfection in combination with JG98-treatment. DMSO was used as control treatment. (g) NP40-solubility of ubiquitin following BAG3 P209L-transfection in combination with YM01-treatment. DMSO was used as control treatment.
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Figure S5. (a) NP40-insoluble fraction of cells transfected with BAG3WT or BAG3-domain-replacements. (b) Co-aggregation of BAG-domain replacements with HSPB5 and HSPA6. (c) Cellular localization of ubiquitin and BAG3 in BAG3P209L and control fibroblasts.
Conflict of interest

HHK was involved in a regional initiative (SNN project Transitie II & Pieken) called ChaperoneAge, a consortium with commercial partners Syncom, ABL, Axon MedChem, Nyken, Brains-on-line, Angita Pharma and the RuG/UMCG. HHK and SC received research grants from Prinses Beatrix Spierfonds; HHK received grants from the Hersenstichting, the High-Q foundation, the Ministry of Economic Affaires (senternoven.nl), and the National Ataxia Foundation. SB received grants from the Hersenstichting and NWO-ALW. MMB was a graduate student at the University Medical Center Groningen at the time the study was conducted and is currently employed by PAREXEL International.
BAG3^P209L: Hsp70 disturbances cause a collapse in PQC

References


22. Gentilella, A. & Khalili, K. BAG3 Expression in Glioblastoma Cells Promotes...