Overexpression of human BAG3\textsuperscript{P209L} in mice causes restrictive cardiomyopathy

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An amino acid exchange (P209L) in the HSPB8 binding site of the human co-chaperone BAG3 gives rise to severe childhood cardiomyopathy. To phenocopy the disease in mice and gain insight into its mechanisms, we generated humanized transgenic mouse models. Expression of human BAG3\textsuperscript{P209L}-eGFP in mice caused Z-disc disintegration and formation of protein aggregates. This was accompanied by massive fibrosis resulting in early-onset restrictive cardiomyopathy with increased mortality as observed in patients. RNA-Seq and proteomics revealed changes in the protein quality control system and increased autophagy in hearts from hBAG3\textsuperscript{P209L}-eGFP mice. The mutation renders hBAG3\textsuperscript{P209L} less soluble in vivo and induces protein aggregation, but does not abrogate hBAG3 binding properties. In conclusion, we report a mouse model mimicking the human disease. Our data suggest that the disease mechanism is due to accumulation of hBAG3\textsuperscript{P209L} and mouse Bag3, causing sequestering of components of the protein quality control system and autophagy machinery leading to sarcomere disruption.
Bcl2-associated athanogene 3 (BAG3) is a co-chaperone and proteostasis factor, which mediates the degradation of unfolded proteins under stress, such as mechanical strain. In humans, a single base mutation in BAG3 leads to an amino acid exchange (P209L) causing severe childhood restrictive cardiomyopathy, muscular dystrophy, respiratory insufficiency, and peripheral polyneuropathy resulting in increased mortality by the second decade (OMIM MFM6 #612954).

The BAG3 protein is strongly expressed in striated muscle and localizes to thin filament-anchoring Z-discs. A critical target protein of BAG3 is the actin-crosslinking protein filament C (FLNC). BAG3 mediates the degradation of mechanically unfolded and damaged forms of FLNC through chaperone-assisted selective autophagy (CASA). Thus, the co-chaperone exerts key proteostasis functions under mechanical strain. This is supported by the analysis of BAG3+/− mice, which exhibit disrupted Z-discs in striated muscle cells resulting in growth retardation and fulminant myopathy after birth in response to muscle usage. Interestingly, in humans with the BAG3 (P209L) mutation, Z-disc disruption and formation of protein aggregates have been reported pointing to a possible disease mechanism in agreement with recent in vitro work. However, this aspect needs to be explored in vivo but this has not been possible because of the lack of a mouse model displaying the typical features of the human disease. For instance, insertion of the homologous point mutation into the mouse BAG3 gene did not produce any obvious phenotype.

Likewise, cardiomyocyte (CM)-specific overexpression of human BAG3 harboring the P209L point mutation (hBAG3P209L) in mice caused mild dilated cardiomyopathy with late-onset but no aggregate formation, sarcomere disintegration, or the prototypic restrictive cardiomyopathy. The reasons underlying the lack of a typical phenotype is unclear, but the degree of BAG3P209L transgene expression was not determined in this study. Thus, the need for an animal model that mimics the human pathology is critical because the pathophysiology of this severe, life-threatening cardiac disease cannot be studied in humans at the cellular and molecular level due to the general lack of heart biopsies from patients.

Herein we report the establishment of several transgenic mouse lines expressing hBAG3P209L in striated muscle cells. We find that these humanized mouse models mimic key pathophysiological features of patients enabling us to explore the underlying molecular disease mechanisms and its rescue via an AAV-mediated gene therapy approach.

**Results**

**Generation and analysis of αMHC-BAG3P209L mice.** To gain insights into the pathomechanisms underlying BAG3P209L-induced cardiomyopathy, we generated transgenic mouse lines with CM-specific overexpression of either hBAG3WT-eGFP (αMHC-BAG3) or hBAG3P209L-eGFP (αMHC-BAG3P209L) (Supplementary Fig. 1a). This strategy was chosen to investigate and to rule out potential adverse effects of hBAG3WT-expression. Mice of both transgenic lines were viable, had normal litter sizes, and did not display any obvious phenotype. Macroscopically, the hearts did not display any differences at 3- and 10-weeks of age and cardiac dry weights to tibia lengths did not differ between αMHC-BAG3WT, αMHC-BAG3P209L, and control (WT) mice (Supplementary Fig. 1b). Both transgenic mouse lines displayed eGFP expression in their hearts with postnatal onset, but the expression pattern in αMHC-BAG3P209L–transgenic hearts was more heterogeneous and patchy (Fig. 1a). Quantification of eGFP expression in sections from αMHC-BAG3WT and αMHC-BAG3P209L hearts revealed that 83.13 ± 2.58% of CMs (n = 3) expressed BAG3WT, while expression of BAG3P209L was found in only 36.28 ± 5.56% of CMs (n = 3). The observed difference in transgene penetration between the two mouse lines is most likely due to positional effects of the random integration of the transgene into the genome (18 copies of the transgene for αMHC-BAG3WT and 32 for αMHC-BAG3P209L) and/or silencing of the promoter by epigenetic effects. Therefore, we isolated and sorted eGFP+ CMs from αMHC-BAG3WT and αMHC-BAG3P209L mice and determined by immunoblotting the degree of overexpression of hBAG3P209L in comparison to endogenous mouse Bag3. This ratio was found to be 1.27 ± 0.66 (n = 3) for αMHC-BAG3WT, and 1.04 ± 0.26 (n = 3) for αMHC-BAG3P209L (Supplementary Fig. 1c, d).

**Aggregate formation and disruption of sarcomers in αMHC-BAG3P209L CMs.** Microscopic analysis of isolated single CMs from adult αMHC-BAG3P209L mice demonstrated that BAG3P209L–eGFP expression led to almost complete Z-disc disintegration, accompanied by aggregation of α-actinin and BAG3P209L (Fig. 1b). Quantification of isolated CMs showing either formation of BAG3-aggregates or aggregates and disturbances of α-actinin cross striation at 10 weeks of age revealed that the majority (64.66 ± 1.57%) of eGFP+ CMs in BAG3P209L-eGFP expressing hearts were affected. In contrast, only 10.67 ± 1.15% and 3.33 ± 0.58% of CMs from αMHC-BAG3WT and WT hearts, respectively, displayed some structural abnormalities (Supplementary Fig. 1e).

Next, we extended this analysis to BAG3-interacting proteins and cytoskeletal components. The distribution of both the BAG3 client FLNC and the BAG3 partner protein SYNPO2 was severely altered in BAG3P209L–eGFP+ CMs, as both proteins displayed loss of their typical cross-striated pattern and formation of small aggregates (Fig. 1c). Also, HSPA8 and HSPB8, chaperone partners of BAG3, formed aggregates upon BAG3P209L expression (Fig. 1d). In contrast, BAG3P209L expression in CMs did not affect the sarcomeric protein titin, which is not under control of BAG3-mediated protein quality control (Supplementary Fig. 1f). Indeed, regions with large BAG3P209L aggregates were devoid of titin (Supplementary Fig. 1f, arrow), whereas the intermediate filament protein desmin displayed structural alterations (Supplementary Fig. 1f) due to hBAG3P209L expression. These findings illustrate (i) that there is a profound dominant-negative impact of BAG3P209L on sarcomeric architecture and (ii) that different Z-disc components may depend on different protein quality control (PQC) systems or they are less affected by the aggregation cascade initiated by BAG3P209L.

Ultrastructural analysis revealed severe sarcomeric disruption upon BAG3P209L expression in adult CMs, with sarcomeric lysis and readily detectable formation of electron-dense aggregates (Fig. 1e). Also, mitochondria in CMs were disordered to some extent compared to WT controls (Fig. 1e). These striking structural disarrangements were exclusively found in hBAG3P209L–eGFP+ CMs, but not in hBAG3P209L–eGFP− neighboring CMs of the same hearts, as demonstrated by immunogold staining for eGFP (Fig. 1f). Staining against eGFP labeled structures and aggregates identical to those labeled by the BAG3 antibody that recognizes both hBAG3 and endogenous mouse Bag3 (Fig. 1g, Supplementary Fig. 2a). This suggested that the aggregates were composed of both transgenic BAG3P209L and endogenous Bag3. Aggregate formation was often detected adjacent to either intact (I), or partly (P) or severely (S) disintegrated sarcomeres (Fig. 1g), thereby strongly resembling the phenotype in human patients. We also analyzed the composition of the aggregates by immunogold EM and found that they contained Bag3, but only negligible amounts of α-actinin (Fig. 1g), fully in line with our immunofluorescence analysis (Fig. 1b). Additional staining against titin, FLNC, desmin, and β-actin revealed that in BAG3P209L CMs, titin was exclusively found in sarcomeres, whereas FLNC and desmin were also detected in...
electron-dense aggregates (Supplementary Fig. 2b). In contrast, β-actin localized solely to Z-discs and intercalated discs, but not to aggregates (Supplementary Fig. 2c). Although hBAG3P209L-eGFP+ CMs displayed pathological features resembling those observed in patients, the corresponding mice neither presented signs of cardiomyopathy nor strongly compromised heart function and early mortality. This was most likely due to the low degree of penetrance (36.28 ± 5.56% of CMs) of the transgene.

**Overexpression of human BAG3P209L causes restrictive cardiomyopathy.** Because of the obvious limitations of our aMHC-
BAG3P209L mouse model described above, we subsequently generated a transgenic mouse line enabling the conditional expression of hBAG3P209L-eGFP under the control of the CAG-promoter (Supplementary Fig. 3a). This promoter was chosen because of its known strong expression in muscle tissue10. The construct was inserted into the Rosa26 locus of mouse G4-embryonic stem cells (ESCs) by using zinc-finger nucleases and a transgenic mouse line named Tg(CAG-flox-hBAG3P209L-eGFP) was derived from a positive ESC-clone. This line was bred to homozygosity with PGK-Cre transgenic mice (Supplementary Fig. 3a), because these displayed a more consistent phenotype, whereas heterozygous mice had a quite variable phenotype.

Our initial characterization of this humanized mouse model revealed that only 50% of the expected number of homozygous PGK-Cre/CAG-flox-hBAG3P209L-eGFP mice (referred to as CAG-BAG3P209L) from heterozygous matings were born, which points to an increased embryonic lethality. After birth, we found that CAG-BAG3P209L mice were substantially smaller than their littermates (Fig. 2a), displaying growth retardation. The body-weight was significantly reduced by 2 weeks of age and amounted to 12.6 ± 4.1 g by 5 weeks of age (n = 6; controls: 23.4 ± 1.8 g, n = 14, Fig. 2b). Because of the reduced body weight, the animals had to be euthanized at the age of 5 weeks; 12% of mice died between 2 and 5 weeks of age (Supplementary Fig. 3b). We found no difference in the heart-weight to tibia length ratio, whereas the heart weight to bodyweight ratio was increased in CAG-BAG3P209L mice (Supplementary Fig. 3c). Reminiscent of the human pathology and starting at around 2 weeks of age, the mice displayed prominent signs of skeletal muscle weakness with some variations in onset and severity of the overall phenotype.

To better understand the striking phenotype of the mice, we performed macroscopic and microscopic analysis of eGFP-fluorescence of 1- to 5-week-old CAG-BAG3P209L mouse hearts. The transgene was found to be expressed in 75.3 ± 14.1% of ventricular CMs at 2 weeks of age, 76.7 ± 14.5% at 3 weeks, and 74.9 ± 8.9% at 5 weeks of age (n = 3–4 mice; Fig. 2c, Supplementary Fig. 3d, e). Transgene expression in hearts was detected exclusively in CMs, as demonstrated by immunofluorescence staining (Supplementary Fig. 3f). Moreover, transgene expression proved to be muscle-specific, as BAG3P209L-eGFP expression was predominantly visible in striated muscle (Supplementary Fig. 4a).

To determine if the ratio of transgenic hBAG3 to endogenous mouse Bag3 protein in hearts from 5-week-old CAG-BAG3P209L mice was comparable to the estimated 1:1 ratio of BAG3P209L to BAG3 in patients with the P209L mutation, we performed immunoblotting, yielding a ratio of 1.05 ± 0.08 (Supplementary Fig. 4b). Next, we performed histological analysis of 1-, 3-, and 5-week-old hearts from CAG-BAG3P209L and control mice. Left and right ventricles displayed similar dimensions (Fig. 2d, Supplementary Fig. 4c, d), but we found that the number of enlarged CMs (Fig. 2e, arrows) increased with age. In 5-week-old mice large, swollen nuclei and the formation of vacuoles in CMs became evident (Fig. 2e, arrowheads, and insets). Quantification yielded a significant increase in CM size starting at 1 week and progressing further at 3–5 weeks of age (Fig. 2f). This was accompanied by disruptions of the regular structure of cardiac muscle fibers seen in controls (Fig. 2e), possibly indicating fibrotic alterations. To test this, we determined the extent of fibrosis in CAG-BAG3P209L mice by Sirius-red staining. Indeed, enhanced cardiac fibrosis was already detected at 2 weeks of age and it further increased significantly in 3- and 5-week-old CAG-BAG3P209L mice, when compared to controls (Fig. 3b). At 5 weeks of age, fibrosis amounted to more than 40% (Fig. 3a, b). As cardiac fibrosis can be caused by collagen deposits replacing apoptotic cells, we stained cardiac sections from 2- and 5-week-old CAG-BAG3P209L and control mice for the apoptosis marker activated caspase 3 (Supplementary Fig. 4e, f) and counted the number of positive CMs. There was a gradual increase of CM apoptosis from 2 to 5 weeks of age (Supplementary Fig. 4f), which is concordant with the observed increase in fibrosis (Fig. 3b). At 2 and 5 weeks of age, the number of apoptotic CMs was significantly higher in CAG-BAG3P209L mice compared to controls (Supplementary Fig. 4f), suggesting partial replacement fibrosis.

Immunofluorescence staining against BAG3 revealed that transgene-expressing CMs from 3- to 5-week-old CAG-BAG3P209L mice displayed BAG3P209L-eGFP containing aggregates (Fig. 3c–e, Supplementary Fig. 5a–g) and that these also contained endogenous mouse Bag3 (Supplementary Fig. 5b). This was accompanied by structural changes of the microfilament system and the intermediate filaments in CMs (Fig. 3c, d), which progressed with age (Supplementary Fig. 5b, c). Similar to our findings in CMs from αMHC-BAG3P209L transgenic mice, immunostainings revealed that the CASA client FLNC and the Bag3 partner protein SYNPO2 were found in aggregates in CAG-BAG3P209L mice (Fig. 3e, f), while proteins not associated with CASA, such as titin, were not detected in the aggregates (Supplementary Fig. 5d). Remarkably, the intermediate filament (IF) protein vimentin, which was not expressed in control CMs, progressively accumulated in aggregates in CMs from CAG-BAG3P209L mice with increasing age (Fig. 3g, Supplementary Fig. 5e), possibly indicating a switch to a fetal gene program. We also found prominent infiltration of CD45+ immune cells into the myocardium (Fig. 3h, Supplementary Fig. 5f); at least in part, these were CD68+ macrophages phagocytosing the apoptotic CMs (Supplementary Fig. 5g).

Next, we performed ultrastructural analysis of hearts from 4-week-old CAG-BAG3P209L mice and found severe sarcomere disruption and lysis in CMs with formation of electron-dense aggregates (Fig. 4a). Mitochondria in CMs were disordered compared to controls, possibly due to the severe Z-disc alterations (Fig. 4a). Immunogold staining for eGFP revealed BAG3P209L.
eGFP localization to electron-dense aggregates between partially or completely disintegrated sarcomeres (Fig. 4b). Thus, the ultrastructural data corroborate our immunohistochemical findings and reveal that overexpression of BAG3P209L in CMs leads to aggregate formation and disintegration of sarcomeres.

Given the prominent alterations of CM morphology and the strong cardiac fibrosis, we next investigated heart function using echocardiography in 21–25-day-old CAG-BAG3P209L mice, because at this age, the mice could still endure this procedure. We found typical features of restrictive cardiomyopathy: The left ventricular inner diameter was smaller in CAG-BAG3P209L mice compared to WT littermates yielding an increase in relative wall thickness, reduced stroke volume, and cardiac output despite preserved ejection fraction (Fig. 4c–e, Supplementary Fig. 6a–b).
Supplementary Table 1). Reduced relaxation of the left ventricle during diastole caused impaired diastolic filling (LVPWd/LVIdD, Fig. 4c, d, Supplementary Fig. 6a, Supplementary videos 1–2). Doppler measurements revealed a restrictive mitral inflow (E/A ratio > 2.5) in 4/8 CAG-BAG3P209L– transgenic versus 0/6 control mice (Fig. 4f, g). Pulmonary artery pressure, as derived from pulmonary artery measurements, was elevated in CAG-BAG3P209L mice, and accordingly also right ventricular function was impaired (Fig. 4h, i, Supplementary Table 1). At 5 weeks of age, CAG-BAG3P209L mice either died or suffered from heart failure (cardiac output severely reduced, Supplementary Fig. 6a) and had to be euthanized, as explained above.

Changes in the POC system and increased autophagy in CAG-BAG3P209L mice. To gain deeper molecular insights into the pathophysiological mechanisms occurring in hearts from CAG-BAG3P209L mice, we performed single-cell and tissue transcriptomic analysis, as well as high-resolution proteomic analysis. Since the expression level of hBAG3 of the CAG-BAG3P209L eGFP is heterogeneous across the myocardium and the observed pathologic phenotypes (and presumably the changes in gene expression) are only evident in eGFP+ CMs, we performed single-cell RNA-Seq analysis of eGFP+ CMs in control and CAG-BAG3P209L mice at the earliest stage of disease onset (2 weeks of age). These experiments showed that the ratio of hBAG3 to mouse Bag3 was in the range of 0.5- to 2-fold, in accordance with both the measured eGFP fluorescence intensity (dim and bright) of individual CMs (Supplementary Fig. 7a) and the protein levels determined by immunoblotting (Supplementary Fig. 4b). To expand our single eGFP+ CM-expression data, we also performed differential expression analysis for our scRNA-seq data from clusters of 33 control (n = 2 mice) and 80 bright (n = 2 mice) individual CMs to determine changes in transcription by KEGG pathway analysis. We found 35 significantly downregulated genes (Supplementary Data 1); some of these pointed to an impairment of energy metabolism pathways, such as the TCA cycle, oxidative phosphorylation pathways, as well as genes involved in fatty acid elongation, were affected (Supplementary Fig. 7b, Supplementary Data 2). Other pathways, such as the fetal gene program, indicative of heart failure, were not activated at this stage of disease onset. To further assess the global transcriptional derangement in failing CAG-BAG3P209L hearts, we performed tissue RNA-Seq analysis from hearts of 5-week-old CAG-BAG3P209L and control mice, since single-cell RNA-Seq was not feasible due to the prominent cardiac fibrosis. This analysis revealed that a total of 3804 genes were differentially expressed, of which 1950 were upregulated and 1854 downregulated in homozygous CAG-BAG3P209L mouse hearts compared to controls (Fig. 5a, Supplementary Data 3). Among the most strongly upregulated genes were components of the protein quality control system (e.g. *Hspa1a, Hspa8, Cryab*), fibrotic pathways (e.g. *CTGF, Galectin-3, Endothelin-1*) and of the fetal CM gene program (e.g. *Nppa, Myh7*). Following GO-analysis for KEGG pathway terms with either the set of upregulated or downregulated genes, the most affected genes were revealed in energy metabolism pathways such as the TCA cycle and oxidative phosphorylation (Supplementary Fig. 5b). Also, genes involved in cardiac muscle contraction were downregulated, as would be expected considering the loss of sarcomeres. Upregulated genes were related to the KEGG terms lysosome, phagosome, and endocytosis, indicating accumulation of many proteins involved in these protein quality control pathways (Fig. 5b, Supplementary Data 4).

Given that BAG3 is involved in the post-translational control of protein homeostasis, we next analyzed the heart proteome of CAG-BAG3P209L and control mice at the age of 2 and 5 weeks using an unbiased label-free shotgun proteomics approach. This identified 2351 proteins in the hearts of 2-week-old mice, but only a single protein, Myeloid leukemia factor 1, showed significant accumulation according to our stringent criteria for determining significant changes in abundance (two-sample t-test, Benjamini–Hochberg adjusted FDR < 0.05) in CAG-BAG3P209L mice compared to controls (Supplementary Data 5). In contrast, 5-week-old mice revealed massive alterations of the heart proteome: Out of 1347 identified proteins, 351 proteins were either significantly up (217 proteins) or downregulated (134 proteins) (Fig. 5c, Supplementary Data 6). GO enrichment analysis of the proteins with significantly lower abundance in 5-week-old CAG-BAG3P209L mice showed that most belonged to major metabolic pathways of the mitochondria, including oxidative phosphorylation and TCA cycle (Supplementary Fig. 8a). In addition, also proteins required for cardiac muscle contraction, including (e.g., TNNT2 and TPM1) were depleted. In contrast, protein subunits of the proteasome, focal adhesion, and tight junctions, as well as heat shock proteins (CRYAB, HSPBP7) and proteins involved in autophagy/phagosome formation, were accumulating in the hearts of 5-week-old CAG-BAG3P209L mice (Fig. 5d). Consistent with the physiological phenotype and transcript abundance data, also proteins involved in fibrotic pathways (Galectin-3) and associated with cardiomyopathies, the fetal cardiac gene program (LMNA, MYH7, NPPA) and vasopressin-regulated water reabsorption, as a sign of heart failure, were accumulating. Overall, the changes in protein abundance in 5-week-old mice in comparison to transcript abundance showed a significant correlation with the RNA data (Pearson correlation coefficient 0.68, −lg10(pval) > 15.65), suggesting that most protein changes were the result of changes in transcription (Supplementary Fig. 8b). Importantly, we noticed that proteins listed as BAG3-interactors in Biogrid13 displayed on average a significantly higher accumulation than all other proteins (Mann–Whitney- U test p-val > 0.001), consistent with a BAG3-driven protein accumulation process (Fig. 5e).
**Fig. 3 Cardiac fibrosis and formation of aggregates in CMs from CAG-BAG3P209L mice.**

**a** Analysis of cardiac fibrosis by Sirius red and fast green stainings: red collagen staining indicates massive fibrosis in hearts from CAG-BAG3P209L mice. **b** Quantification of the fibrotic area in CAG-BAG3P209L mice and CTRL. Mean ± SD. *N* (1 week) = 3 CTRL and 3 CAG-BAG3P209L mice; *n* (2 weeks) = 3 CTRL and 6 CAG-BAG3P209L mice; *n* (3 weeks) = 10 CTRL and 13 CAG-BAG3P209L mice; *n* (5 weeks) = 4 CTRL and 6 CAG-BAG3P209L mice. One-way ANOVA. **c–h** Sections of hearts from 5-week-old CAG-BAG3P209L and CTRL mice were stained for α-actinin (**c**), desmin (**d**), FLNC (**e**), SYNPO2 (**f**), vimentin (**g**), and CD45 (**h**). In BAG3P209L-eGFP (green) CMs the formation of large aggregates was observed. Scale bars: 50 μm. **c–h** The experiments were repeated three times from three independent biological replicates with similar results. CTRL, control mice (siblings of CAG-BAG3P209L mice, which are either WT, PGK-Cre, or CAG-flox-hBAG3P209L).
As our proteome analysis suggested accumulation of small heat shock proteins and increased autophagic processes, we next validated these observations by immunoblot analysis of total heart protein from 5-week-old CAG-BAG3P209L and control mice. In accordance with the increased number of autophagic vesicles detected in the EM analysis (Figs. 1e, 4a, b), we found strong induction of SQSTM1 and LC3B in CAG-BAG3P209L mice (Fig. 5f). This suggested a strong impact on autophagic flux, leading to the formation of more autophagosomes, as has also been described in patient samples14. In addition, endogenous mouse Bag3, as well as BAG3-associated proteins, such as CRYAB, were found to accumulate compared to controls (Fig. 5f), underscoring our immunofluorescence results. Likewise, we confirmed accumulation of the small heat shock proteins HSPB6 and HSPB7 (Fig. 5f).

Thus, RNA-Seq and proteomics data demonstrated accumulation of components of the protein quality control system and the autophagy machinery in hearts from CAG-BAG3P209L mice.

**BAG3P209L has altered protein solubility, and decreased mobility.** We next investigated the biochemical properties of the BAG3P209L protein to better understand the disease mechanisms. First, we analyzed whether its interaction with partner proteins

**Fig. 4 Disintegration of sarcomeres and development of restrictive cardiomyopathy in CAG-BAG3P209L mice.** a, b Ultrastructural analysis of hearts from 4-week-old control and CAG-BAG3P209L mice (a) and (b) immunogold staining for eGFP, revealing disintegration of sarcomeres and formation of aggregates in CMs from CAG-BAG3P209L mice (arrows). MYOF = myofibril. Scale bars: 1000 nm. The experiments were repeated three times from three independent biological replicates with similar results. c–i Echocardiography of 4-week-old CAG-BAG3P209L and CTRL mice: c–i Increased relative wall thickness (c, d) and reduced stroke volume (c, e) were found in CAG-BAG3P209L mice; data were obtained from parasternal M-Mode of the left ventricle. Tendency of increased mitral valve E/A ratio (f, g) and increased pulmonary artery acceleration/ejection time (PAT/PET; h, i) were found in CAG-BAG3P209L mice; data were derived from Doppler flow measurements. LVPWd left ventricular posterior wall during diastole, LVIDd left ventricular inner diameter during diastole. Mean ± SEM. (d, e, g, i) n = 6 CTRL and 8 CAG-BAG3P209L mice. Two-sided Student’s T-test. CTRL, control mice (siblings of CAG-BAG3P209L mice, which are either WT, PGK-Cre, or CAG-flox-hBAG3P209L).
was impaired by performing in vitro binding studies with purified components. We focused on the binding of BAG3 to HSPB8, which has been reported to be mediated by two conserved IPV (Ile-Pro-Val) motifs\(^1\), of which the second one is affected by the P209L mutation (Supplementary Fig. 9a). Surprisingly, BAG3\(^{P209L}\) was found to associate with HSPA8, FLNC, and HSPB8 similar to BAG3\(^{WT}\) (Supplementary Fig. 9b–d). Since binding was unaltered, conformational changes might occur in BAG3, potentially explaining its tendency to aggregate. Therefore, purified hBAG3\(^{WT}\) and hBAG3\(^{P209L}\) were subjected to limited proteolysis by trypsin treatment (Supplementary Fig. 9e). We found that the mutation caused several changes in the fragment pattern following treatment (Supplementary Fig. 9e, open arrowheads), pointing to an altered tertiary/quaternary structure.
of hBAG3P209L. We also investigated the solubility and heat sensitivity of purified hBAG3WT and hBAG3P209L (Supplementary Fig. 9f). These experiments revealed that the amount of insoluble hBAG3P209L significantly increased following heat treatment, pointing to a more aggregation-prone conformation of the mutant cochaperone. Thus, the P209L mutation does not per se alter the binding properties of the protein, but rather its susceptibility to aggregate formation.

To corroborate the increased tendency of BAG3P209L to form aggregates in vivo, we performed differential centrifugation of cardiac protein extracts (Fig. 6a) from adult CAG-BAG3P209L and homozygous PGK-Cre/CAG-flox-hBAG3WT-eGFP mice (referred to as CAG-BAG3WT, Supplementary Fig. 10a–h). Strikingly, hBAG3P209L–eGFP was mainly found in the insoluble pellet fraction, underscoring the strong predisposition of hBAG3P209L to form aggregates in vivo. Besides, in the presence of hBAG3P209L endogenous mouse Bag3 was driven into the insoluble fraction, indicating that the mutant protein also sequesters mouse Bag3 into aggregates (Fig. 6b). Similar results were obtained for CMs isolated from MHC-BAG3WT or MHC-BAG3P209L transgenic mice (Supplementary Fig. 10i).

As hBAG3P209L was preferentially detected in the insoluble fraction, we next tested its mobility by performing fluorescence recovery after photobleaching (FRAP) experiments with hBAG3P209L and hBAG3WT control CMs. After bleaching of BAG3WT–eGFP (Fig. 6c), a fast recovery rate with a half-life of 21 ± 5 s and a mobile fraction of 73 ± 8% was measured, illustrating that the protein undergoes highly dynamic interactions at the Z-disc (Fig. 6d, e). In strong contrast, exchange rates for hBAG3P209L were significantly reduced, in terms of both the fluorescence half-life, which could not even be calculated within the time of the experiment, and the mobile fraction (13 ± 3%; Fig. 6d, e).

AAV-mediated knockdown of hBAG3 mitigates the disease phenotype. Given the prominent cardiac phenotype of our mouse model and its resemblance to the human disease, we wondered, whether we could halt or even reverse its key features using gene therapy. CAG-BAG3P209L mice were treated at P15 with a single intrajugular injection of AAV/rh10, harboring a cassette for the expression of either a shRNA targeting hBAG3P209L or a randomly scrambled shRNA (Supplementary Fig. 11a). AAV/rh10 was chosen because of its strong tropism and expression in striated muscle16. We analyzed the mice at P37 and found that the body weight in the hBAG3-shRNA group was significantly higher compared to the mice treated with scrambled shRNA (Fig. 7a), indicating the effectiveness of our therapy (Fig. 2b). Next, the transduction rate of AAV/rh10 in CMs was determined, and mCherry expression was detected in 69.8 ± 10.3% of CMs after treatment with AAV hBAG3-shRNA and in 63.5 ± 11.6% after treatment with AAV scrambled shRNA (Supplementary Fig. 11b), whereas non-CMs were found to be mCherry-negative. EGFP-fluorescence was analyzed as a readout for the pathological protein expression, and we detected in most animals of the hBAG3-shRNA group a striking decrease of hBAG3P209L–eGFP fluorescence, whereas mCherry fluorescence intensity was comparable in both treatment and control groups (Supplementary Fig. 11b, c). This was further corroborated by immunoblot analysis of hearts from hBAG3-shRNA and scrambled shRNA-treated mice, which revealed a prominent reduction of hBAG3P209L protein (Fig. 7b). Moreover, the fluorescence intensities of mCherry and hBAG3P209L–eGFP were found to be inversely correlated (Supplementary Fig. 11b, c). In parallel with the reduction of the hBAG3P209L–eGFP fluorescence, we also noticed a significant reduction of the number of aggregates in CMs of the hBAG3-shRNA treated group. Furthermore, even regular cross-striation could be detected in the vast majority of CMs after staining for α-actinin, desmin, or FLNC (Fig. 7c–e), and CM swelling and immune cell infiltration were strongly reduced (Fig. 7f) in the hBAG3-shRNA treated group. As a further proof of the rescue effect, the prominent cardiac fibrosis seen in CAG-BAG3P209L mice was significantly decreased upon knockdown of hBAG3P209L (Fig. 7g, h).

Thus, AAV-shRNA-based reduced hBAG3P209L–eGFP expression results in mitigation of the cardiac phenotype.

Discussion
Herein, we demonstrate that our CAG-BAG3P209L mouse model phenocopies the devastating human cardiac disease. In fact, we detected a progressive early-onset restrictive cardiomyopathy, as reported for the patients suffering from BAG3P209L myofibrillar myopathy1,9,17. The mice developed increasingly severe symptoms of heart failure accompanied by growth retardation starting shortly after birth and, therefore, requiring their euthanization at 5 weeks of age. In patients with the BAG3P209L mutation, the restrictive cardiomyopathy is due to extensive fibrosis caused by stiffening of the myocardium18 and an impairment of diastolic filling of the ventricles. Congruent with the human pathology we detected in CAG-BAG3P209L mice cardiac fibrosis starting at 3 weeks and becoming very extensive by 5 weeks of age. It manifested as a diffuse interstitial and replacement fibrosis triggered by the loss of CMs by apoptosis. Mouse models for restrictive cardiomyopathy are rare and mainly based on mutations in proteins determining the passive properties of CMs, such
as troponin I or troponin T. This is different in our mouse model, where dysfunction of an important component of the protein quality control system causes CM apoptosis, activation of a fetal gene program and pronounced replacement fibrosis. Interestingly, another mouse line with CM-specific overexpression of hBAG3P209L was reported to lack an overt cardiac phenotype or typical changes of CMs except for an accumulation of pre-amyloid oligomers at the adult stage. One reason potentially underlying this difference could be the ratio of hBAG3P209L to mouse Bag3, which in our mouse models appears comparable to that in human patients, who are heterozygous for the dominant-negative P209L mutation. Similarly, a mouse model harboring the homologous P209L point mutation in mouse Bag3 protein (P215L) did not present an obvious cardiac phenotype.
when bred to homozygosity, indicating subtle differences between human and mouse Bag3 proteins.

We next explored the disease mechanisms and focused first on the CMs, which are also severely affected in the human pathology. In both of our mouse models, CAG-BAG3P209L-GFP and αMHC-BAG3P209L-GFP, we observed the second hallmark of the human disease, namely aggregation of proteins in cytoplasmic inclusions that correlated directly with the Bag3P209L protein expression and sarcomere disintegration. The aggregates consisted mainly of Bag3, α-crystallin, desmin, and vimentin, whereas FLNC and α-actinin formed separate aggregate entities with a morphology distinct from those containing hBag3P209L (Fig. 3c, e). Our data imply that hBag3P209L expression directly causes formation of aggregates leading to a collapse of the Bag3-associated proteostasis network over time, resulting in sarcomere dissolution as a consequence of muscle usage. At 2 weeks of age, a strong hBag3P209L-eGFP expression was evident in a minority of CMs, which were swollen and in which aggregates were already formed. At this stage, the disease phenotype was still relatively mild, which was supported by the RNA-Seq and proteomics data. However, for the next three weeks, more CMs displayed strong Bag3P209L-eGFP expression, a massive formation of aggregates, and cell death resulting in the striking cardiac phenotype. We also noticed an impairment of metabolic pathways of the mitochondria, such as oxidative phosphorylation and TCA cycle. These changes could be partly due to a loss of CMs and future experiments need to explore, whether they are primary caused by hBag3P209L expression or secondary due to CM loss.

We also explored the impact of the altered biochemical properties of the hBag3P209L protein and its potential effects on the endogenous Bag3 protein. Our data argue against altered binding properties of hBag3P209L as the molecular mechanism responsible for the observed phenotype, because hBag3P209L retained its ability to bind to HSPB8, HSPA8, and FLNC (Supplementary Fig. 8). The proteomics data underscored this, as we could find an enrichment of Bag3-interactors among the upregulated proteins in hearts from CAG-BAG3P209L mice. Thus, the mutant protein retains its ability to associate with partner proteins, including other proteostasis factors and chaperone clients. This is also supported by recent findings demonstrating that binding of hBag3P209L to Hsp70 and HspB8 is mostly unaffected and that both IPV domains need to be mutated to abolish HspB8 binding. This might also explain why Bag3P209L was able to rescue certain phenotypes associated with knockdown of Bag3 in zebrafish. However, when investigating the aggregation properties of hBag3P209L, we found them strongly increased in Bag3P209L CMs in vivo. This is most likely a key driver of the disease process and validates earlier in vitro findings and in vivo findings in zebrafish. It is further underscored by the FRAP experiments, which demonstrated reduced mobility of Bag3P209L, explaining the impairment of autophagic flow and hence its contribution to the accumulation of proteins. Thus, transgenic hBag3P209L-eGFP expression in our mice leads to co-aggregation and sequestration of endogenous Bag3 in CMs. This causes attenuation of its proteostasis function in muscle resulting in disintegration of myofibril architecture and a collapse of muscle cell protein homeostasis. The ensuing dysfunction of autophagy is corroborated by the increase and accumulation of p62 and LC3B. In addition, the formation of aggregates and lack of their clearing is a reliable sign of impaired protein quality control. Similar results were reported for a zebrafish model in which the bag3 gene was deleted and human Bag3P209L-eGFP was overexpressed. Also, small heatshock proteins, such as α-crystallin, HSPB6, and HSPB7 did accumulate, as has been described in a heart from a patient, and can therefore no longer fulfill their function. Taken together, our findings highlight the essential and multifaceted role of Bag3 in organizing the proteostasis machinery in striated muscle and to protect from mechanical stress. As a consequence, patients suffering from MFM6 should avoid physical exertion such as strenuous exercise. This could lead to progressive muscle damage, as has been observed in other MFM models.

To gain additional insight into pathophysiological processes and to explore its therapeutic potential, we have taken advantage of a gene therapy approach by using AAVs to achieve a specific knockdown of the hBag3P209L expression. These proof-of-principle experiments illustrated a strong rescue effect, as the phenotypic hallmarks of the disease, such as growth retardation and clinical signs of heart failure were strongly mitigated. At the cellular level, we found that in CMs only a few protein aggregates were detected and that their structural integrity was largely preserved, highlighting again the pathophysiological impact of protein aggregate formation for the disease process. Our AAV-based gene therapy approach demonstrated that it is possible to halt or even reverse the progression of this severe MFM by reducing expression levels of the pathological protein.

Application of this strategy to patients suffering from MFM6 would require the identification of an shRNA specific for the mRNA carrying the point mutation. This is critical, as loss of Bag3 leads to postnatal mortality, at least in mice, and has toxic effects in both zebrafish and isolated human CMs. The approach has proven challenging in the past and alternatively a micro-RNA based strategy could be used. A more sophisticated but also more complicated approach would involve the AAV-mediated delivery of CRISPR-based gene-editing tools, such as prime-editing tools for correction of the point mutation. Such a strategy should be developed and tested in CMs derived from patient-specific iPS-cells before application in the clinic.

**Methods**

**Generation of αMHC-BAG3WT-eGFP and αMHC-BAG3P209L-eGFP constructs.**

Human Bag3WT and Bag3P209L cDNAs were cloned in fusion with the eGFP cDNA under the control of the CAG promoter. By restriction of CAG-hBag3WT-eGFP with EcoRI, blunting, and subsequent digestion with NotI a 2468 bp fragment containing the hBag3WT-eGFP cDNA was isolated and inserted into the AgeI (blunted) and NotI sites of an αMHC-plasmid. The exchange of a fragment in the hBag3WT-eGFP DNA with the equivalent region of the hBag3P209L cDNA containing the mutation resulted in αMHC-hBag3P209L-eGFP. All vectors were verified by sequencing.
**Generation of Tg(CAG-flox-hBAG3<sup>P209L</sup>-eGFP) and Tg(CAG-flox-hBAG3<sup>WT</sup>-eGFP) constructs.** Human BAG3<sup>WT</sup>-eGFP and BAG3<sup>P209L</sup>-eGFP driven by the ubiquitous CAG promoter were cloned into the pDonor Rosa26 vector from Addgene (Plasmid #37200). Initially, a hygromycin-stop-cassette flanked by loxP-sequences was inserted downstream of the CAG promoter in the CAG-hBAG3WT-eGFP and CAG-hBAG3P209L-eGFP plasmid, respectively, by using the Quick&Easy Conditional Knockout Kit-loxP from Gene Bridges. The resulting plasmids were digested with SnaBI and MunI and 5763 bp fragments containing the 3′ part of the CAG promoter, the loxP flanked stop cassette and the BAGWT-eGFP or BAGP209L expression cassettes were exchanged against the 2233 bp SnaBI-MunI fragment of a CAG-eGFP-Neo pDonor Rosa26 vector.

All vectors were verified by sequencing.

**Generation and cultivation of transgenic mESC clones.** The cultivation of G4 hybrid ESCs<sup>27</sup> was performed in Knockout-Dulbecco’s modified Eagle’s medium (DMEM high glucose, supplemented with 15% v/v fetal calf serum (FCS), 0.1 mM...
Fig. 7 AAV-based gene therapy approach with shRNA against hBAG3fl-209L rescues key features of the disease in vivo. a Body weight was found to be increased in CAG-BAG3fl-209L mice 3 weeks after a single intraocular injection of AAV/h10 hBAG3 shRNA at P15, when compared to CAG-BAG3fl-209L mice treated with AAV/h10 scr. shRNA. Mean ± SEM, n = 6 CTRL and 8 CAG-BAG3fl-209L mice. Two-sided Student’s t-test, scr. shRNA = scrambled shRNA. b Immunoblot analysis and quantification of protein expression revealed a decrease in the amount of hBAG3 (BAG3 upper band) in CAG-BAG3fl-209L mice treated with AAV/h10 hBAG3 shRNA in comparison to mice treated with AAV/h10 scr. shRNA. This correlated inversely with the amount of mCherry expression; for normalization of expression levels β-actin was used. Mean ± SEM, n = 6 hearts per group. Two-sided Student’s t-test, scr. shRNA = scrambled RNA. c–e Sections of hearts from 5-week-old CAG-BAG3fl-209L mice which were treated with either AAV/h10 BAG3 shRNA or AAV/h10 scr. shRNA were stained for hBAG3 shRNA. Mean ± SEM, n = 12 CTRL and 14 CAG-BAG3fl-209L mice. Two-sided Student’s t-test, scr. shRNA = scrambled shRNA.

Generation of transgenic mice. Animal experiments were conducted according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. For the generation of transgenic mouse lines, transgenic mESCs with the respective integrated targeting construct and a karyotype of 40 chromosomes were aggregated with diploid morula stage CD-1 embryos28. For genotyping of the transgene mESCs started 2 days after electroperoration by adding 165 µg/ml G418 to the medium. Restless colonies were isolated and cultivated onto neomycin-resistant mouse embryonic fibroblasts before they were propagated and analyzed for BAG3WT/BAG3fl-209L-eGFP expression.

Protein expression and purification. BAG3WT and BAG3fl-209L cDNAs were cloned in pET-M11 (Novagen) and human HSPA8 and a human FLNC cDNA fragment corresponding to its lg2-like domains 19–21 into pET-28a (Novagen). His-tagged proteins were expressed and purified using Ni-NTA-agarose (Qagen) as described by the manufacturer. An additional washing step with MgATP (2 mM ATP, 1 mM MgCl2, 20 mM MOPS pH 7.2, 100 mM KCl) was added before elution. Rat HSPA8 was expressed in Sf9 insect cells following infection with a recombinant baculovirus carrying the corresponding coding region and subsequently purified on an ATP-sequphosphate (Sigma-Aldrich). IPV2wt and IPV2P209L encoding cDNA fragments (corresponding to aa 128–285 of human HAG3) and PPPY encoding cDNA fragments (corresponding to aa 337–670 of human TSC1) were cloned into the BD FACS™ Sorter 1.0.1.654.4 software. Gating for eGFP was set using cDNAs from wild-type mice (Supplementary Figure 12).

ECochardiography. Cardiac morphology and function were determined by echocardiography from parasternal and apical views during isoflurane anesthesia (1.5 vol% in oxygen) using a VisualSonics Vevo2100 ultrasound system and Vevo LAB version 3.3.3 software for analysis. Stroke volume was calculated from 2D images (stroke volume = LVIDd × ejection fraction)12. Pulmonary artery pressure was derived from non-invasive measurement of pulmonary artery flow as described before41. The investigator was blinded to genotype.

Echocardiography. Cardiac morphology and function were determined by echocardio-
Heat denaturation. To examine the thermal stability of BAG3 WT and BAG3 P209L, 30 µl of purified protein (2 mg/ml) was incubated at 0, 60, or 70 °C for 15 min and denatured protein sedimented at 100,000 x g for 30 min at 4 °C. Proteins in the supernatant and insoluble proteins were resuspended in an equivalent volume of SDS loading buffer. Supernatant and pellet fractions were analyzed by SDS-PAGE, and stained gels were photographed using a Bio-Rad Chemidoc imaging system. Uncropped blots are presented in the Source data.

Histology, immunofluorescence staining, and microscopy. 10 µm thick cryosections were cut on a cryotome CM 3050S (Leica). Sirius red and Fast Green staining was performed using standard histology protocols. For staining of fixed cells and heart sections the following antibodies were used (for 2 h at RT in 0.2% Triton X in PBS, 5% donkey serum or overnight at 4 °C in 0.2% Triton X in PBS, 5% donkey serum): α-actin (1:200, Sigma-Aldrich), BAG3 (1:200, Proteintech, recognizing both human and mouse BAG3), desmin (1:100, DAKO), FLNC (1:1000, Biogene), SYNO2 (1:200, Invitrogen), titin (1:50, T122140), cCAP5 (1:50, Cell Signaling Technology), CD45 (1:200, Merck Millipore), CD68 (1:100, ebioscience), vimentin (1:400, Merck Millipore), HSPB8 (1:100, Abcam). After extensive washing, all sections were postfixed with 4% PFA, 5% donkey serum or overnight at 4 °C in 0.2% Triton X in PBS, 5% donkey serum. Cells and heart sections the following antibodies were used (for 2 h at RT in 0.2% Triton X in PBS, 5% donkey serum): α-actin (1:200, Sigma-Aldrich), BAG3 (1:200, Proteintech, recognizing both human and mouse BAG3), desmin (1:100, DAKO), FLNC (1:1000, Biogene), SYNO2 (1:200, Invitrogen), titin (1:50, T122140), cCAP5 (1:50, Cell Signaling Technology), CD45 (1:200, Merck Millipore), CD68 (1:100, ebioscience), vimentin (1:400, Merck Millipore), HSPB8 (1:100, Abcam). After washing secondary antibodies conjugated to Cy2, Alexa Fluor 488, Cy3 or Alexa Fluor 647, sections were incubated with Alexa Fluor 488, 594 or 647-conjugated secondary antibodies (Invitrogen) at 37 °C for 1 h. Postfixed cardiac sections were mounted in water; B, 0.1% formic acid in acetonitrile) and introduced into a high-resolution Q-TOF mass spectrometer (LEO 4800 QTOF, Bruker Daltonics, Bremen, Germany) for non-targeted proteomics analysis. For target-specific antibody validation, peptide sequences from mouse endogenous BAG3 or eGFP mouse BAG3 transgenic protein were identified and used to design antibodies for Western blotting (Abcam). For quantification of protein expression levels, Western blots were scanned using the Molecular Imaging Research software. ImageJ package Fiji was used to determine relative optical densities of bands using ImageJ software.

Tissue RNA-Seq analysis. For RNA-Seq experiments, DNA-free total RNA was isolated from total hearts from 6 CAG-BAG3 P209L and 6 control mice using the RNeasy Kit (Qiagen) including on-column DNAse digestion. RNA quality was analyzed by an Agilent Bioanalyzer (Agilent). For library preparation the Trio RNA-Seq Library Preparation kit for mouse (TECAN) was used, starting with 50 ng of total RNA. Thirteen PCR cycles were used for library amplification and libraries with an average fragment size of 380 bp were sequenced on a NextSeq 500 in paired-end mode (75 bp, Illumina). For bioinformatics analysis, we used the Galaxy platform (Freiburg Galaxy Project34). RNA sequencing reads were mapped using RNA STAR35 followed by counting reads per gene by using featureCounts36. Differentially expressed genes were identified by DESeq237. For data visualization, normalized gene and cluster analysis heatmap and Volcano plot (Freiburg Galaxy Project34) was used. Gene ontology analysis was performed by ClueGO (two-sided hypergeometric test) by using the Kegg pathway and GO-term databases with a significance interval for pathways of p < 0.05. p-values were corrected for multiple-testing by the Bonferroni step down method.

Single-cell RNA-Seq analysis. Single CMs from Langendorff dissociated mouse hearts (n = 2 control hearts and n = 2 CAG-BAG3 P209L) were manually picked into each well of 96-well plates which were filled with 9.5 µl of lysis buffer (0.25 µl RNase Inhibitor, 0.05 µl 10% Triton, 2.5 µl 10 mM DNTP, 6.7 µl H2O). Single-cell mRNA sequencing procedure was carried out at the Stanford Functional Genomics Facility by following the SMART-seq2 protocol38. Briefly, the single cells were lysed, and their mRNA was reverse transcribed into cDNA and pre-amplified. After a cleanup procedure, the cDNA was quantified and used to make single-cell libraries following the manufacturer protocol in Illumina Nextera DNA library kit. The single-cell libraries were further sequenced on an Illumina HiSeq 4000 platform using 2 × 75 bp mode. The sequencing reads for each cell were mapped to a reference database with the mouse genome and human BAG3 and eGFP transcript sequences using STAR2.7.0f with the default parameters. After a cleanup procedure, the cDNA was quantified and used to make single-cell libraries following the manufacturer protocol in Illumina Nextera DNA library kit. The single-cell libraries were further sequenced on an Illumina HiSeq 4000 platform using 2 × 75 bp mode.

Proteome sample preparation and data acquisition. Heart samples from the mouse were harvested, weighed, and frozen in liquid N2 until use. For proteome analysis about 100 mg of heart sample was homogenized using a TissueLyser LT (Qiagen) in 1 ml of 50 mM Tris buffer (pH 6.8) containing 8 M Urea, 2% SDS, 1.5% Triton-X100, 1 mM DTT, 1 mM PMSF and 1:100 (v/v) protease inhibitor cocktail. The protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Scientific) and 100 µg proteome was reduced using 10 mM DTT at 37 °C for 30 min followed by carboxymethylmethylation using 50 mM chloroacetamide at room temperature (RT) in dark for another 30 min. The reaction was quenched with 50 mM DTT at RT for 20 min. Proteins were purified using paramagnetic SP3 beads and the digested protein was fractioned using paramagnetic SP3 beads. Desalted peptides were desalted by reverse-phase solid phase extraction using self-packed C18 STAGE tips, before loading to the nano-LC system (Dionex NCS-3500 RS) operated in two column-parallel setup (PharmaFluidics µPAC C18 trap column and a 50 cm PharmaFluidics µPAC C18 analytical column). 500 ng peptide were separated at a flow rate of 600 nl/min with a binary gradient from 0 to 32% eluent B (A, 0.1% formic acid in HPLC-grade water; B, 0.1% formic acid in acetonitrile) and introduced into a high-resolution Q-TOF mass spectrometer (Impact-II, Bruker) using a CaptiveSpray ion source. The HESI II (v3.2, Bruker Daltonics) was used for data acquisition in positive ion mode in a mass range from 200 to 1750 m/z with an acquisition rate of 4 Hz for MS1 spectra and the top 17 most intense ions were selected for fragmentation. A dynamic exclusion window of precursors selected within 30 s was applied unless the signal to noise ratio improved more than 3-fold. Fragmentation spectra were acquired between 5 Hz for low-intensity precursor ions (~500 cts) and 20 Hz for high intensity (>5 kcts) ions, each with stepped parameters, each with 50% of the acquisition time dedicated for each precursor: 100 µs transition time, 7 eV collision energy and a collision RF of 1300 Vpp followed by 100 µs transition time, 9 eV collision energy and a collision RF of 1700 Vpp.
contaminants listed in MaxQuant, the human BAG3 P209L–GFP sequence, and reverse-decode sequences were used for target-decode database searches with an FDR of 0.01 as the protein and peptide level. Label-free quantification (LFQ) and the “match between runs” features were enabled. Trypsin was set as digestion enzyme, oxidation (M), and acetylation (protein N-term) were set as variable modifications while carbamidomethylation of cysteine was set as fixed modification. The LFQ data were analyzed using Perseus\(^\text{v}\) version 1.6.10.0, filtering for proteins quantified in at least both biological replicates/animals per genotype, followed by imputation of missing values using Perseus standard settings and determination of significant changes with a two-sample t-test with Benjamini–Hochberg FDR < 0.05 to correct for multiple hypothesis testing.

Immunoblot analysis of mouse hearts. For protein isolation, hearts were mechanically homogenized in urea buffer (8 M urea, 2% SDS, 1.5% Igepal, 0.05 M Tris-HCl pH 8.6) and protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fischer Scientific). SDS-PAGE was performed in a 12.5% polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane using the semi-dry blotting method. The following primary antibodies were used: Anti-alpha B-crystallin (1:5000, Enzo Life Science), anti-HPSPB (1:1000, Abcam), anti-HPSP8 (1:1000, Abcam) anti-CL3B (1:1000, Thermo Fischer Scientific), anti-p62 (1:2000, Invitrogen) 

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Study approval. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were following the ethical standards of the institution at which the studies were conducted and were approved by the responsible government animal care and use office, the Landesamt für Natur, Umwelt und Verbraucherschutz, LANUV (84-02.04.2012.A146 and 81-02.04.2019.A062).

Housing and husbandry of mice. All mice are housed under Specific-Pathogen-free (SPF) conditions in individually ventilated cages in the animal facility of the University Clinic Bonn. Our surveillance program follows the Federation of European Laboratory Animal Science Associations (FELASA) guidelines. A maximum of 5 mice are housed in Type 2 L cages on a 12/12 h light/dark cycle and mice have ad libitum access to food and water. The enclosures for laboratory rodents have a standardized air temperature of 22 °C, 50–70% humidity and up to 15-fold air exchange. Bedding material is added in all mating cages and small houses and toys are routinely added to enrich the environment of the mice. Access to the facilities is restricted to qualified personnel that received appropriate training in animal handling and experimentation. The facility has appointed a certified veterinarian, who regularly monitors the sanitary status of the facilities and the health status of all the animals kept.

Reporting summary. Further information on research design is available in the Nature Data Reporting Summary linked to this article.

Data availability

MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository\(^\text{v}\) with the project accession number PXD002116. Single cell RNA-Seq data have been deposited to the GEO database with the GEO accession number GSE166862. Tissue RNA-Seq data have been deposited to the SRA database with the BioProject accession number PRINA700583. Source data are provided with this paper. All other source data are provided upon request.

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### References

1. Selcen, D. et al. Mutation in BAG3 causes severe dominant childhood muscular dystrophy. *Ann. Neurol.* 65, 83–89 (2009).
2. Arndt, V. et al. Chaperone-assisted selective autophagy is essential for muscle maintenance. *Carr. Biol.* 20, 143–148 (2010).
3. Ulbricht, A., Arndt, V. & Höhfeld, J. Chaperone-assisted proteinostasis is essential for mechanotransduction in mammalian cells. *Commun. Integr. Biol.* 6, e24925 (2013).
4. Homma, S. et al. BAG3 deficiency results in fulminant myopathy and early lethality. *Am. J. Pathol.* 169, 761–773 (2006).
5. Meister-Brockema, M. et al. Myopathy associated BAG3 mutations lead to proteome aggregation by stalling Hsp70 networks. *Nat. Commun.* 9, 5342 (2018).
6. Adriaenssens, E. et al. BAG3 Pro209 mutants associated with myopathy and neuropathy relocate chaperones of the CASA-complex to aggresomes. *Sci. Rep.* 10, 8755 (2020).
7. Fang, X. et al. P209L mutation in Bag3 does not cause cardiomyopathy in mice. *Am. J. Physiol. Heart Circ. Physiol.* 316, H392–H399 (2019).
8. Quintana, M. T. et al. Cardiomyocyte-specific human Bcl2-associated anthanogene 3 haploinsufficiency, and activates p38 signaling. *Am. J. Pathol.* 186, 1989–2007 (2016).
9. Konersman, C. G. et al. BAG3 myopathy results from insufficient chaperones and activates p38 signaling. *Am. J. Pathol.* 186, 1989–2007 (2016).
10. Biankin, S. et al. A histological survey of green fluorescent protein expression in ‘green’ mice: implications for stem cell research. *Pathology* 39, 247–251 (2007).
11. Naguve, S. F. et al. Recommendations for the evaluation of left ventricular diastolic function by echocardiography: an update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *Eur. Heart J. Cardiovasc. Imaging* 17, 1321–1360 (2016).

12. Tournoux, F. et al. Validation of noninvasive measurements of cardiac output in mice using echocardiography. *J. Am. Soc. Echocardiogr.* 24, 465–470 (2011).

13. Stark, C. et al. BioGRID: a general repository for interaction datasets. *Nucleic Acids Res.* 34, D535–D539 (2006).

14. Schänzer, A. et al. Dysregulated autophagy in restrictive cardiomyopathy due to Pro209Leu mutation in BAG3. *Mol. Genet. Metab.* 123, 388–399 (2018).

15. Fuchs, M. et al. Identification of the key structural motifs involved in HspB8/HspB6-Bag3 interaction. *Biochem. J.* 425, 245–255 (2010).

16. Hu, C., Basutti, R. W. & Lipszutz, G. S. RH10 provides superior transgene expression in mice when compared with natural AAV serotypes for neonatal gene therapy. *J. Gene Med.* 12, 766–778 (2010).

17. Odgerel, Z. et al. Inheritance patterns and phenotypic features of myofibrillar myopathy associated with a BAG3 mutation. *Neuromuscul. Disord.* 20, 438–442 (2010).

18. Kushnaha, S. S., Fallon, J. T. & Fuster, V. Restrictive cardiomyopathy. *N. Engl. J. Med.* 336, 267–276 (1997).

19. Ruparelia, A. A., Oorschot, V., Vaz, R., Ramm, G. & Bryson-Richardson, R. J. Zebrasilf model of BAG3 myofibrillar myopathy suggest a toxic gain of function leading to BAG3 insufficiency. *Acta Neuropathol.* 128, 821–833 (2014).

20. Ruparelia A. A. et al. Metformin rescues muscle function in BAG3 myofibrillar myopathy mouse model. *Autophagy* https://doi.org/10.1080/15548627.2020.1833500 (2020).

21. Chevesi, F. et al. Myofibrillar instability exacerbated by acute exercise in filaminopathy. *Hum. Mol. Genet.* 24, 7207–7220 (2015).

22. Ruparelia, A. A., Oorschot, V., Ramm, G. & Bryson-Richardson, R. J. FLNC myofibrillar myopathy results from impaired autophagy and protein instability. *Hum. Mol. Genet.* 25, 2131–2142 (2016).

23. Feldman, A. M. et al. BAG3 regulates contractility and Ca2+ homeostasis in adult mouse ventricular myocytes. *J. Mol. Cell Cardiol.* 92, 10–20 (2016).

24. Schwarz, D. S. et al. Designing siRNA that distinguish between genes that differ by a single nucleotide. *PloS Genet.* 2, e140 (2006).

25. Acunzo, M. et al. Selective targeting of point-mutated KRAS through artificial microRNAs. *Proc. Natl Acad. Sci. USA* 114, E4203–E4212 (2017).

26. Anzalone, A. V. et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576, 149–157 (2019).

27. George, S. H. et al. Developmental and adult phenotyping directly from mutant embryonic stem cells. *Proc. Natl Acad. Sci. USA* 104, 4455–4460 (2007).

28. Nagy, A., Rossant, J., Nagy, R., Bramow-Newbery, W. & Roder, J. C. Derivation of completely cell culture-derived mice from early–passage embryonic stem cells. *Proc. Natl Acad. Sci. USA* 90, 8442–8448 (1993).

29. Louch, W. E., Sheehan, K. A. & Wol ska, B. M. Methods in cardiology autophagy isolation, culture, and gene transfer. *Hum. Gene Ther.* 12, 388–398 (2001).

30. Wol ska, B. M. & So lano, E. J. Method for isolation of adult mouse cardiac myocytes for studies of contraction and microfluorimetry. *Am. J. Physiol. 271*, H1250–H1255 (1996).

31. Thi bault, H. B. et al. Noninvasive assessment of murine pulmonary arterial pressure: validation and application to models of pulmonary hypertension. *Circ. Cardiovasc. Imaging* 3, 157–163 (2010).

32. Fürst, D. O., Osborn, M., Nave, R. & Weber, K. The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z line extends close to the M line. *J. Cell Biol.* 106, 1563–1572 (1988).

33. Leber, Y. et al. Filamin C is a highly dynamic protein associated with fast repair of myofibrillar microdamage. *Hum. Mol. Genet.* 25, 2776–2788 (2016).

34. Afgan, E. et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res.* 46, W537–W544 (2018).

35. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21 (2013).

36. Li, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923–930 (2014).

37. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550 (2014).

38. Picelli, S. et al. Full-length RNA-seq from single cells using Smart-seq2. *Nat. Protoc.* 9, 171–181 (2014).

39. Butler, A., Hoffman, P., Smibert, P., Papanicolaou, A. U. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* 36, 411–420 (2018).

40. Tyanova, S. et al. The Perseus computational platform for comprehensive analysis of (pro)teomics data. *Nat. methods* 13, 731–740 (2016).

41. Perez-Riverol, Y. et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* 47, D442–D450 (2019).

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**Author contributions**

K.K., A.O., and K.G.R. performed molecular biology, cell culture, imaging, and immunohistochemistry experiments and data analysis. W.A.L. and A.U. designed, performed, and analyzed (immuno)electron microscopy experiments. C.G. was involved in molecular biology experiments and data analysis. D.O.F., J.S., and P.F.M.d.v.d.V. performed FRAP, heat denaturation experiments, and immunoblotting. W.R. and W.B. were involved in small animal surgery and treadmill exercise experiments. M.K. and P.F.H. performed proteomics analysis. J.D. and J.H. designed and performed immunoprecipitation, in vitro binding studies, and partial trypsin digestion experiments. A.L. and L.H. performed echocardiography analysis. M.H. and S.T. were involved in the generation of transgenic mice and M.H. performed RNA-Seq analysis. S.M.W. and G.L. performed single-cell RNA-Seq experiments and analysis. M.H. and B.K.F. designed the study and wrote the manuscript (together with D.O.F.).

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