BAG3-dependent autophagy maintains sarcomere function in cardiomyocytes

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Compromised force-generating capacity is a hallmark of heart failure both at the organ[1] and single-cell level[2–6]. This is primarily due to changes at the sarcomere[7], the functional unit in cardiomyocytes responsible for contraction. However, the mechanisms of sarcomeric force depression in heart failure are incompletely understood. We show in human heart failure that myofilament BAG3 levels predict the severity of sarcomere dysfunction and in a mouse heart failure model, increasing BAG3 expression rescues contractile function. Further, myofilament ubiquitin increases in heart failure, indicating impaired protein turnover, but is reduced with increased BAG3 expression. Mass spectrometry revealed Hsp70, HspB8 associate with myofilament BAG3, forming a conserved selective autophagy complex, which localizes to the Z-disc. Assembly of this complex at the sarcomere is BAG3-dependent and triggered by proteotoxic stress, however, its clearance stalls in heart failure. Together, these findings identify BAG3-dependent autophagy as essential for functional maintenance of the cardiac sarcomere.

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Cardiomyocytes isolated from the left ventricle (LV) of dilated cardiomyopathy (DCM) heart failure patients (Extended Data Table 1) displayed a reduction in myofilament maximum calcium-activated force (F_{max}) and increased calcium sensitivity (decreased EC_{50} - calcium concentration required to elicit half maximal force) compared with non-failing donors (Fig. 1a-c). Changes in site-specific phosphorylation of myofilament proteins, most commonly troponin I, are widely attributed to this expected increase in calcium sensitivity found in heart failure[8]. The mechanisms underlying the decreased F_{max}, which describes the inherent force-generating capacity of a cardiomyocyte, are less well defined. One possible explanation for decreased F_{max} in heart failure is that there are fewer functional sarcomeres and thus, irrespective of calcium concentration, the ability to produce force is impaired[9]. However, the

factors that contribute to the decreased functionality of sarcomeres in heart failure remain to be fully elucidated.

Molecular chaperones are required to assemble and maintain highly ordered protein complexes like the sarcomere. These chaperones have roles in protein folding, stabilization, and degradation and are essential for sarcomere protein turnover[10]. The importance of maintaining sarcomere proteostasis in cardiomyocytes is accentuated by the fact that these cells are terminally differentiated and must survive for many years throughout which they experience constant mechanical stress. However, despite its evident importance, there is little mechanistic understanding of sarcomeric protein quality control. Bcl2-associated athanogene 3 (BAG3, Bis, CAIR-1), is a co-chaperone involved in protein quality control through promotion of ubiquitin-dependent macroautophagy[11,12]. Both decreased BAG3 expression[13,14] and BAG3 mutations[15] are associated with heart failure, most commonly DCM. In animal models, BAG3 deletion causes rapid progression into heart failure[13] and significant disruption of sarcomere structure[16,17], suggesting a role in maintaining sarcomere function. Intriguingly, one study with a large animal model of heart failure found increased myofilament BAG3 expression was associated with improved myofilament function[6] and BAG3 KO in iPSC-cardiomyocytes impaired contraction strength[18]. However, the functional role for BAG3 at the sarcomere remains to be fully elucidated.

BAG3 levels in the myofilament fraction were significantly depressed in DCM compared with non-failing donor controls (Fig. 1d), as has been described for whole LV BAG3 expression in heart failure[14]. Strikingly, the level of myofilament BAG3 was able to predict F_{max}, i.e., those patients with the lowest myofilament BAG3 had the most depressed force generating capacity (Fig. 1e). No such relationship existed between myofilament BAG3 and calcium sensitivity (Extended Data Fig. 1), or between whole LV BAG3 levels and F_{max} (Extended Data Fig. 1), suggesting the functional association is specific to the myofilament pool. We therefore

investigated whether increasing BAG3 levels in heart failure could restore myofilament function, using a mouse model of heart failure.

Eight-week-old mice received a myocardial infarction (MI) produced by permanent ligation of the left anterior descending coronary artery or sham surgery. Eight weeks post-surgery, the mice were randomly assigned to receive a recombinant adeno-associated virus serotype 9 (rAAV9) vector expressing the mouse *bag3* gene or GFP via retro-orbital injection. Four weeks after AAV injection, the mice were euthanized and myocardial was tissue collected (Fig. 1f). BAG3 overexpression in heart failure restored *in vivo* cardiac function, which was reported previously for this cohort by Knezevic et. al.[19].

Myofilament functional analyses were performed using skinned LV cardiomyocytes isolated from the infarct border zone. As in human heart failure, cardiomyocytes from the heart failure mice had a significant reduction in F_{max} compared to the sham animals (Fig. 1g-h). However, F_{max} was fully rescued by BAG3 overexpression, thus identifying for the first time a functional role for BAG3 at the myofilament. There were no detectable changes in myofilament calcium sensitivity between the three groups (Fig. 1i). To determine whether the functional effect was associated with restoration of sarcomere proteostasis, we used Western blot for ubiquitin in myofilament-enriched LV tissue. Unfortunately, more sophisticated approaches and current autophagy imaging techniques are not appropriate for assessing sarcomere-specific protein quality control, partly explaining our minimal understanding of the mechanisms involved. In both the mouse heart failure model (Fig.1j) and in human heart failure samples (Fig. 1k) we found an increase in myofilament protein ubiquitination, indicating impaired sarcomere protein quality control. However, the rAAV9-BAG3 treatment significantly decreased myofilament ubiquitin to levels comparable with the sham group. Together, these data highlight the functional significance of BAG3 at the myofilament and suggested recovery of sarcomere proteostasis as a potential mechanism for the restored contractile function.

To identify a mechanistic explanation for BAG3's functional effect and to determine its binding partners at the sarcomere, we immunoprecipitated BAG3 from myofilament-enriched human LV tissue and screened for BAG3-associated proteins by liquid chromatography tandem mass spectrometry. Among the top hits, mass spectrometry identified heat shock protein (Hsp) 70 (Hsp72, HspA1A) and HspB8 (Hsp22) (Fig. 2a). Reciprocal co-immunoprecipitation experiments confirmed the association of these Hsps with BAG3 in the myofilament fraction (Fig. 2b-c).

This is the first time the BAG3/Hsp70/HspB8 complex has been described in cardiomyocytes, although it has been observed in skeletal[20,21], and smooth muscle[22], where it is involved in a ubiquitin-dependent macroautophagy pathway termed chaperone-assisted selective autophagy (CASA). Importantly, the functional significance of the CASA complex has never been described in any muscle type. In CASA, BAG3 acts as a scaffold for Hsp70 and HspB8, which cooperate to clear misfolded proteins. To prevent aggregate formation, misfolded proteins are bound by HspB8 and then passed to Hsp70. CASA clients are subsequently ubiquitinated by the E3 ubiquitin ligase CHIP (carboxyl-terminus of Hsp70 interacting protein) and then removed through the actions of the autophagic ubiquitin receptor p62 (SQSTM1), which promotes the association of ubiquitinated proteins with the autophagosome membrane. Degradation of the complex and ubiquitinated cargo is achieved when the autophagosome fuses with the lysosome[23]. Using immunofluorescence microscopy on cardiomyocytes from the non-failing human LV we found that BAG3, Hsp70, and HspB8 each localized to the sarcomere Z-disc, as evident from their co-localization with the Z-disc protein α-actinin (Fig. 2d-e). Z-disc localization was also found for CHIP and P62 (Extended Data Fig. 2). The association of HspB8 and Hsp70 with BAG3 and their shared localization with CHIP and P62, together with the decreased myofilament ubiquitin upon BAG3

overexpression, suggests a role for BAG3 in mediating ubiquitin-dependent macroautophagy of sarcomere proteins through CASA.

To test whether the assembly of this complex in the myofilament fraction was BAG3-dependent, we used mouse models of cardiomyocyte-specific heterozygous and homozygous BAG3 deletion[24]. Western blot of myofilament-enriched LV tissue revealed myofilament levels of HspB8, but not Hsp70, were significantly reduced in the partial absence of BAG3 (BAG3 +/-, 20% reduction of myofilament BAG3 vs. wild-type) and barely detectable in the BAG3 -/- hearts (Fig. 3a). The same relationship was found in human DCM samples, where samples with lowest myofilament BAG3 (≥20% decreased relative to non-failing) had significantly reduced HspB8 levels but not Hsp70 levels (Fig. 3b-c). Notably, the E3 ubiquitin ligase for Hsp70 clients, CHIP, was also significantly reduced with decreased BAG3 expression (Extended Data Fig. 2). These data indicate that BAG3 is required for full assembly of the CASA complex at the myofilament, either through directly mediating HspB8/CHIP localization to the myofilament and/or serving to stabilize these proteins at the sarcomere and prevent their degradation, which is supported by Fang et. al.[13].

In addition to having BAG3-dependent assembly, the association of the CASA complex with the myofilament is enhanced in the context of proteotoxic stress. Neonatal rat ventricular myocytes (NRVMs) were isolated from zero to one-day-old rat pups and maintained in culture (Extended Data Fig. 3). To increase ubiquitinated protein load and thus induce proteotoxic stress, the proteasome inhibitor MG132 was added to the culture medium at 2 μM concentration for 24 hours. Proteasome inhibition resulted in a pronounced increase in myofilament protein ubiquitination (Fig. 3d-e). Similar results were obtained for BAG3, Hsp70, and HspB8 (Fig. 3d-e) which all increased significantly at the myofilament. Taken together with our previous data, these results implicate CASA as a stress-responsive protein quality control pathway for sarcomere proteins.

Because myofilament ubiquitination increased in both human heart failure and our mouse model of heart failure (Fig. 1), we next tested whether this was due to a failure of the CASA complex to localize to the myofilament. However, Hsp70, HspB8, and CHIP all significantly increased at the myofilament in the control-treated heart failure mice (Fig. 4a-b). Both these results and immunofluorescence imaging of DCM patient cardiomyocytes (Extended Data Fig. 4) indicated that the CASA complex targeting to the myofilament in response to proteotoxic stress still occurs in heart failure. However, in the heart failure mice that received rAAV9-BAG3, while BAG3 was significantly increased at the myofilament, Hsp70, HspB8, and CHIP each decreased back to sham expression levels (Fig. 4a-b). Myofilament levels of P62 also increased in the heart failure mice, indicating impaired autophagic flux[25], but were reduced by BAG3 overexpression (Fig. 4b). These data suggest that in heart failure, decreased CASA complex assembly at the sarcomere in end-stage heart failure (Figs. 1d and 3c) is preceded by impaired CASA clearance or turnover rate therein, thus explaining the buildup of ubiquitinated myofilament proteins (Fig. 1k).

Unlike in end-stage human heart failure where myofilament BAG3 levels decreased (Fig. 1d), BAG3 levels in the mouse heart failure model indicate no change or perhaps a mild increase compared with sham. The possible increase of myofilament BAG3 in response to the stress of heart failure is supported by earlier work in a large animal heart failure model[6]. While this appears to disagree with human heart failure in which we found decreased myofilament BAG3 (Fig. 1d), the apparent disparity may be due to the stage of disease, where these animal models represent an earlier, compensated heart failure and the human data are from the end-stage of the disease, in which proteotoxic stress response pathways are known to be impaired[26]. Nevertheless, our data support that increasing BAG3 levels at any stage of heart failure would prove beneficial both through elevation of myofilament BAG3 and restoration of CASA.

To directly assess the myofilament functional consequence of impaired CASA, we used a transgenic mouse model with cardiomyocyte-specific expression of human BAG3 with the missense proline to leucine mutation at amino acid 209 (P209L). Previous work with this model found that transgene-positive mice developed heart failure by 8 months of age and had elevated whole LV ubiquitin expression[27]. In humans, this mutation causes myofibrillar myopathy and restrictive cardiomyopathy that presents in early adolescence[28]. Recent work in *in vitro* systems found that the P209L mutation impaired client processing through CASA by impairing Hsp70 folding activity[29] and caused HspB8, P62, and Hsp70 to stall at the aggresome[30], thus preventing the clearance of the associated ubiquitinated clients. To determine the functional impact of the P209L mutation, we assessed myofilament function in cardiomyocytes from 8-month-old P209L mice and their wild-type counterparts. and found that cardiomyocytes from P209L mice had a significant reduction in F_{max} (Fig. 4c-d). Sarcomere proteostasis was also disrupted with the P209L mutation as evident from an increase in myofilament protein ubiquitination by 3 months of age (Fig. 4e). These data support that impaired sarcomeric CASA due to the P209L mutation has direct functional consequences for cardiomyocytes.

The mechanisms underlying compromised myofilament force-generating capacity in heart failure have long been unclear. To our knowledge, this is the first study to indicate impaired protein quality control at the sarcomere has a direct effect on myofilament function. Moreover, we show that BAG3 gene therapy fully rescues F_{max}, restores autophagy flux at the sarcomere, and decreases myofilament protein ubiquitination in mice with heart failure after myocardial infarction. We uncovered a BAG3-dependent selective autophagy complex at the sarcomere Z-disc and found that mobilization of this complex to the myofilament fraction is triggered by proteotoxic stress. Lastly, we show that the P209L BAG3 mutation which stalls client processing by CASA, has direct functional consequences for the myofilament. Our data

highlight for the first time the functional significance of protein quality control at the sarcomere and reveal the consequences of its disruption in heart failure. Altogether, these findings indicate impaired CASA is a primary mechanism for the depressed myofilament force-generating capacity in heart failure.

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Author contributions T.G.M and J.A.K designed the experiments. T.G.M., V.D.M., P.D., S.D., and E.P. performed the experiments. C.S.M provided human myocardial samples and revised the manuscript. M.S.W provided the $BAG3_{P209L}$ transgenic mouse strain and revised the manuscript. A.M.F provided myocardial tissue from the BAG3 overexpression mouse model and BAG3 +/- and BAG3 -/- mouse strains, provided scientific input, and revised the manuscript. J.A.K provided scientific input from the conception of the project idea through its completion, and substantially contributed to the data presentation, and analysis. T.G.M and J.A.K wrote the manuscript.

Competing interests A.M.F has equity in and is a director of Renovacor, Inc., a biotechnology company developing gene therapy for patients with BAG3 genetic variants. The other authors declare no competing interests.

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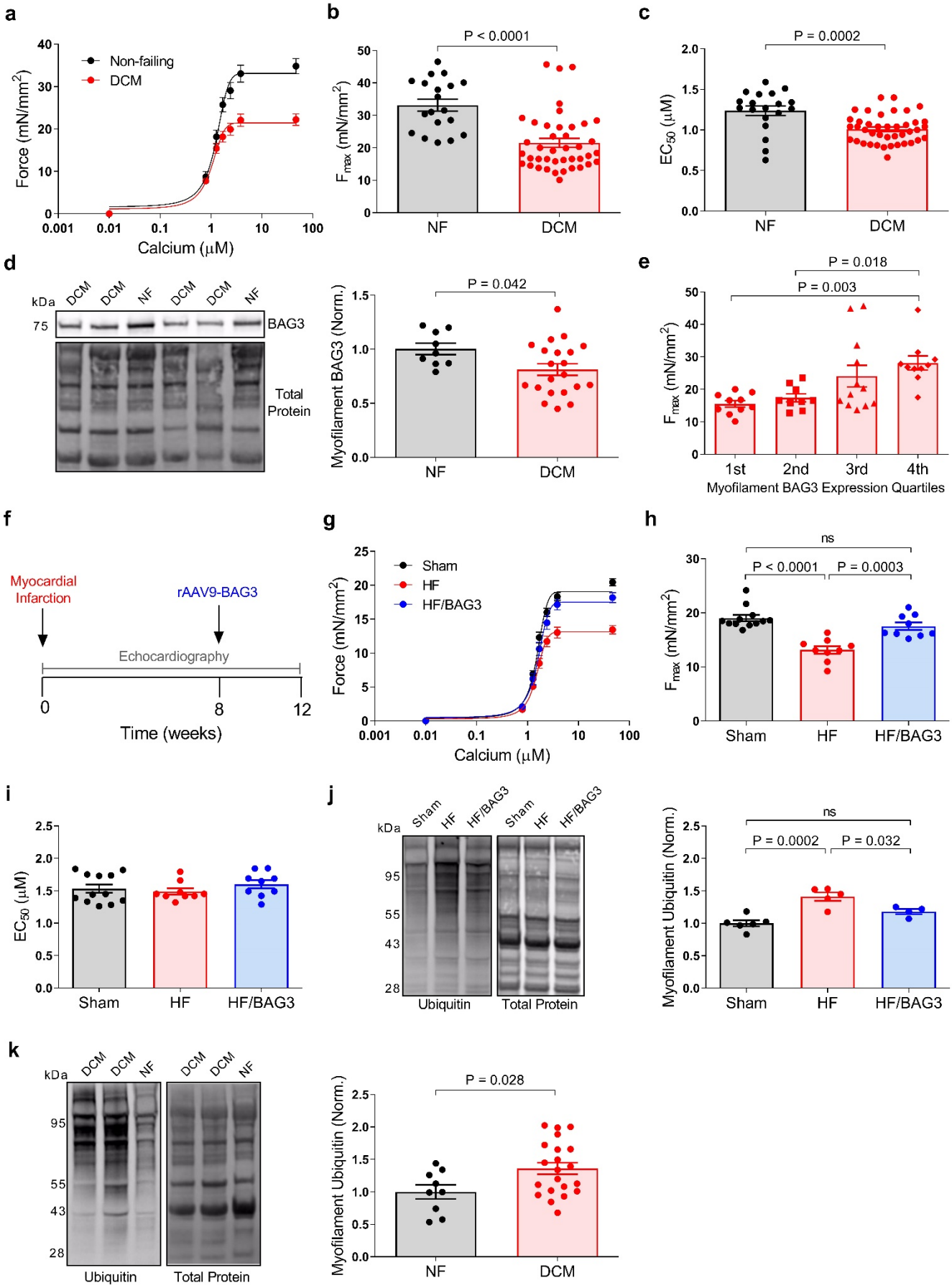

Fig. 1 | See next page for caption

Fig. 1 | Depressed myofilament force-generating capacity in heart failure is rescued by BAG3 overexpression. (**a**), myofilament force-calcium relationship for LV cardiomyocytes from non-failing (NF) and dilated cardiomyopathy (DCM) human samples (**b**), single cardiomyocyte maximal calcium-activated force (F_{max}) and (**c**), calcium sensitivity (EC_{50}). Data are mean ± s.e.m.; n = 19 NF from 6 patients, 41 DCM from 12 patients; unpaired t-test. **d-e,** Western blot of myofilament BAG3 normalized to total protein in NF and DCM samples. Data are mean ± s.e.m.; n = 9 NF, 21 DCM; unpaired t-test. (**d**) F_{max} values organized by quartile of BAG3 expression in DCM; 1^{st} = lowest BAG3, 4^{th} = highest BAG3; n = 12 DCM samples, 3-4 myocytes per sample for functional assessment; one-way ANOVA with Tukey post-hoc. (**f**), experimental paradigm: adult mice received myocardial infarction (MI) by LAD ligation to induce heart failure; 8 weeks after MI, rAAV9-BAG3 was injected and expressed for 4 weeks. (**g**), myofilament force-calcium relationship for LV cardiomyocytes from sham, HF, and HF/BAG3 mice. **h-i,** maximal calcium-activated force (F_{max}) (**h**) and calcium sensitivity (EC_{50}) (**i**) from the individual myocytes used for assessment. Data are mean ± s.e.m.; n = 12 sham from 4 mice, n = 9 HF and HF/BAG3, from 3 mice each; one-way ANOVA with Tukey post-hoc; ns = not significant. (**j**), Western blot for myofilament ubiquitin in sham, HF, and HF/BAG3 mice normalized to total protein. Data are mean ± s.e.m. n = 6 sham, 5 HF, and 4 HF/BAG3; one-way ANOVA with Tukey post-hoc. (**k**), Western blot for myofilament ubiquitin normalized to total protein in human NF and DCM samples. Data are mean ± s.e.m.; n = 9 NF, 21 DCM; unpaired t-test.

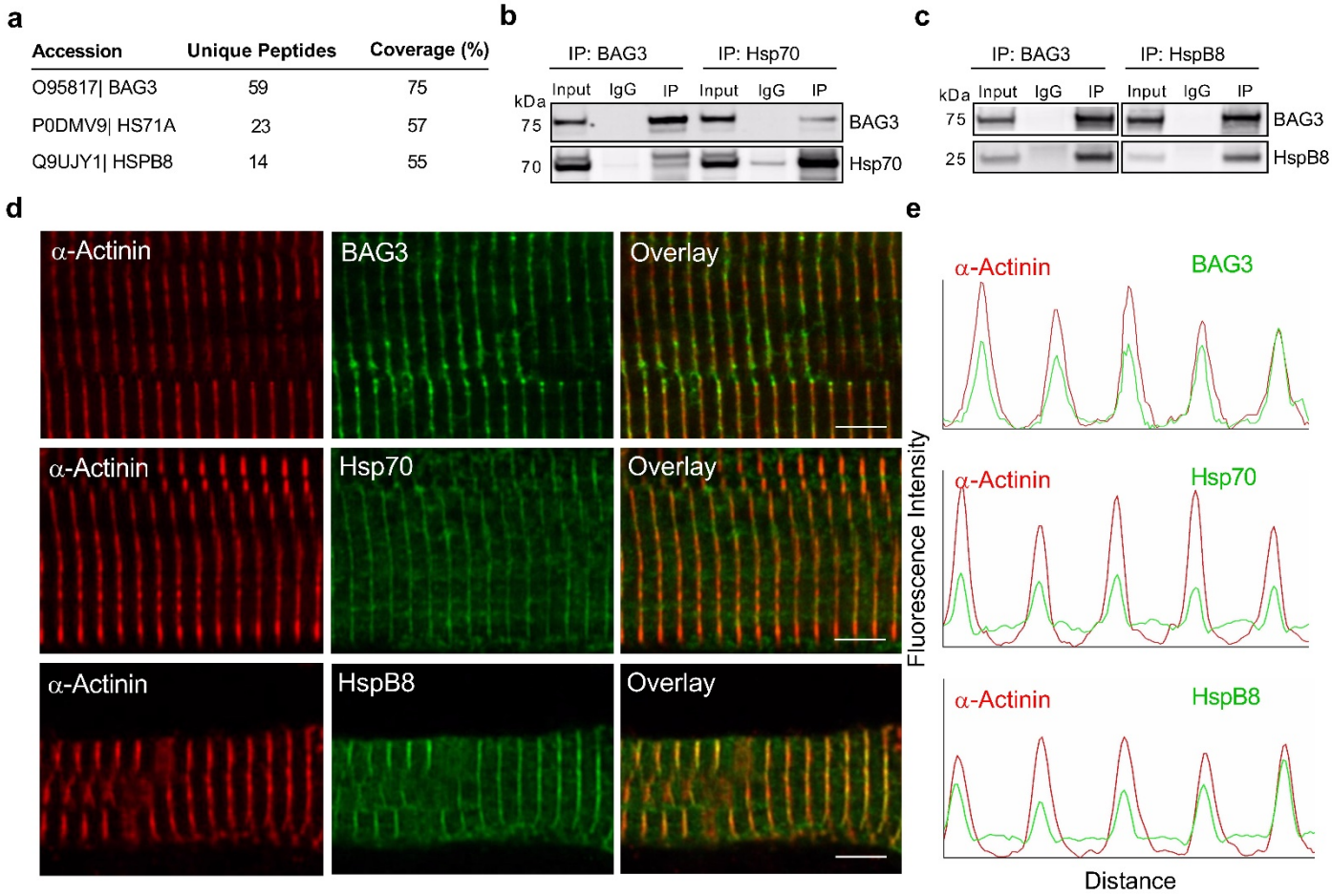

Fig. 2 | HspB8 and Hsp70 associate with BAG3 at the sarcomere Z-disc (**a**), mass spectrometry results for BAG3 immunoaffinity purification from myofilament-enriched human LV tissue. **b-c**, Western blot results of reciprocal co-immunoprecipitation experiments for BAG3/Hsp70 (**b**) and BAG3/HspB8 in myofilament-enriched human LV tissue (**c**). **d-e**, immunofluorescence images of human LV cardiomyocytes stained for BAG3, Hsp70, and HspB8 counterstained for the Z-disc protein α-actinin (63X magnification; scale bars = 5μm) (**d**) and quantitative line scan of fluorescence intensity signal overlap (ImageJ) (**e**).

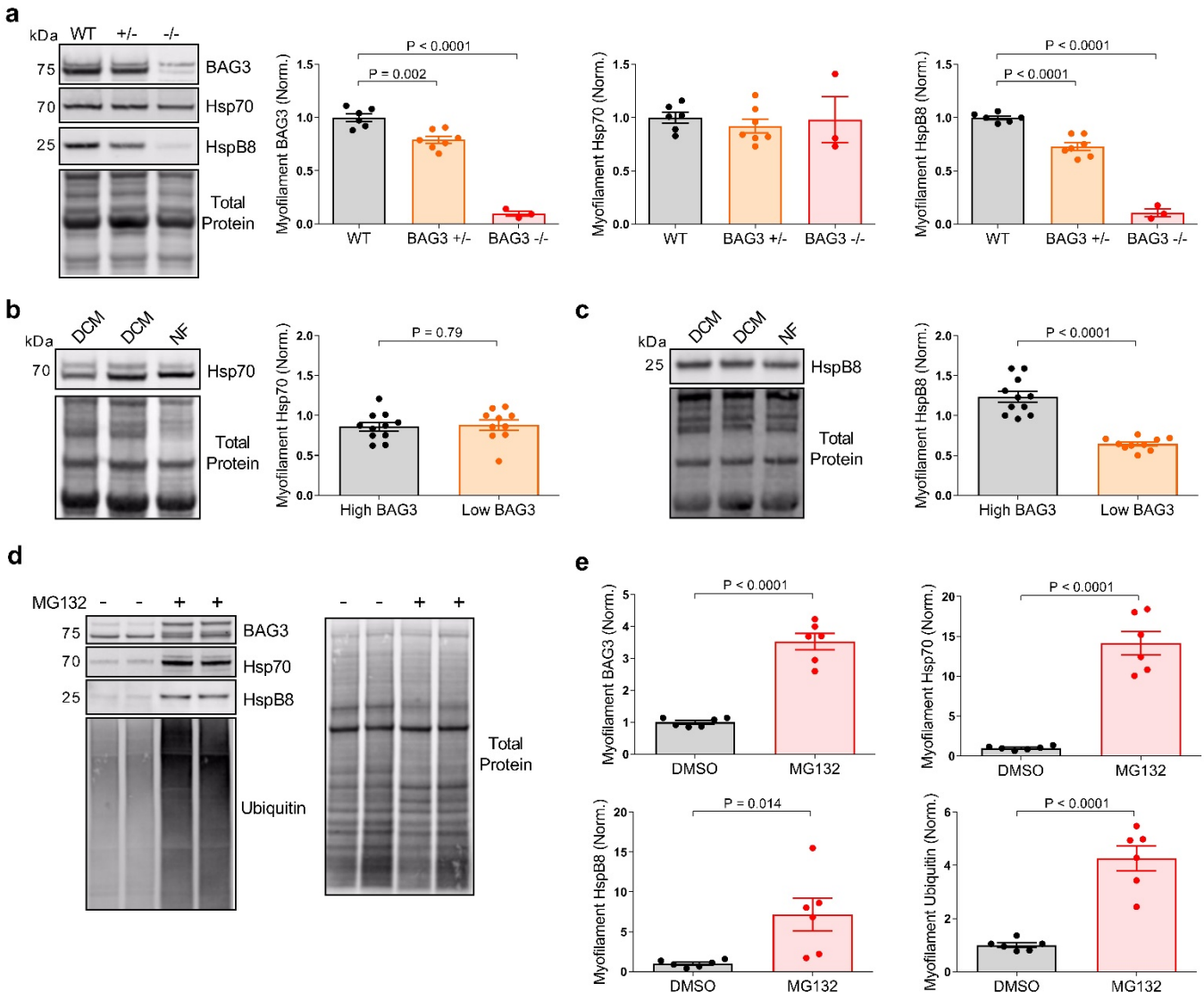

Fig. 3 | Assembly of the CASA complex at the myofilament is dependent on BAG3 and proteotoxic stress (**a**), Western blot in myofilament-enriched tissue for BAG3, Hsp70, and HspB8 in wild-type, BAG3 +/- and BAG3 -/- mouse LV normalized to total protein. Data are mean ± s.e.m.; n = 6 WT, 7 BAG3 +/-, 3 BAG3 -/-; one-way ANOVA with Tukey post-hoc. **b-c**, Western blot for Hsp70 (**b**) and HspB8 (**c**) in myofilament-enriched human LV with myofilament Hsp70/HspB8 expression in DCM samples grouped by high or low BAG3 expression; High ≥ 80% mean NF expression, Low < 80% mean NF expression; n = 11 High BAG3 and 10 Low BAG3; unpaired t-test. (**d**), Western blot for BAG3, Hsp70, HspB8, and ubiquitin in myofilament-enriched lysates from NRVMs treated with MG132 or DMSO control. Data are mean ± s.e.m.; n = 6 DMSO, 6 MG132 from 3 separate experiments; unpaired t-test.

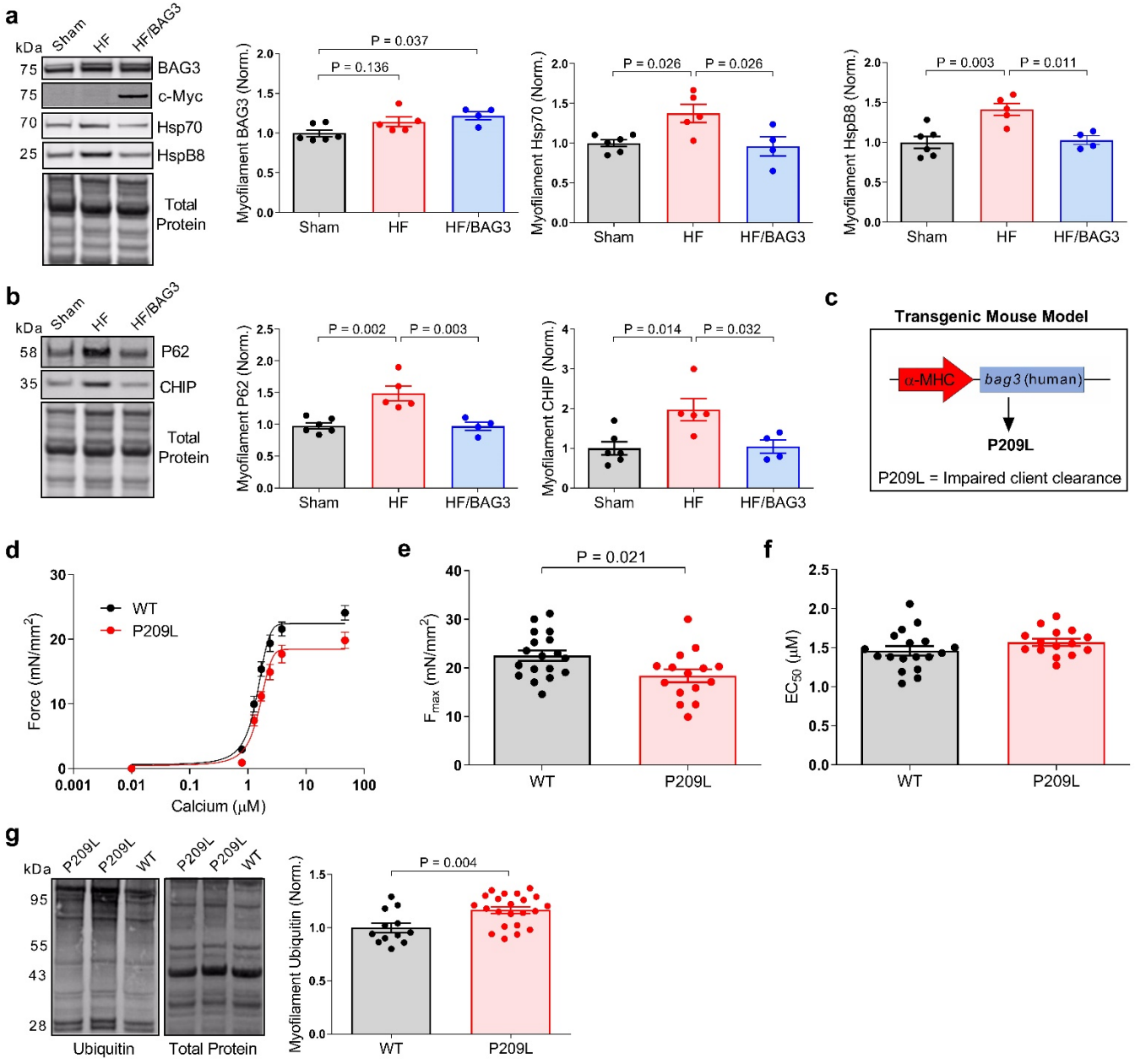

Fig. 4 | BAG3 overexpression in heart failure restores autophagy flux and the P209L BAG3 mutation impairs myofilament function. a-b, Western blot of total BAG3, myc-BAG3, Hsp70, and HspB8 (**a**) or P62 and CHIP (**b**) in myofilament-enriched LV tissue from sham, HF, and HF/BAG3 mice normalized to total myofilament protein. Data are mean ± s.e.m.; n = 6 sham, 5 HF, 4 HF/BAG3; one-way ANOVA, Tukey post-hoc. (**c**), transgenic mouse model with the CASA-disrupting $BAG3_{P209L}$ expression under the cardiomyocyte-specific αMHC promoter. (**d**), myofilament force-calcium relationship of LV cardiomyocytes from 8-month-old wild-type and $BAG3_{P209L}$ transgenic mice. (**e**), single cardiomyocyte maximal calcium-activated force (F_{max}) and (**f**) calcium sensitivity (EC_{50}) from the WT and $BAG3_{P209L}$ transgenic mice. Data are mean ± s.e.m.; n = 18 WT myocytes from 6 mice and 15 $BAG3_{P209L}$ myocytes from 5 mice; unpaired t-test. (**g**), Western blot for ubiquitin in myofilament-enriched LV tissue from 3-month-old WT and $BAG3_{P209L}$ transgenic mice normalized to total myofilament protein. Data are mean ± s.e.m.; n = 12 WT, 22 $BAG3_{P209L}$; unpaired t-test.