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

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

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

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

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

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

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

102 To test whether the assembly of this complex in the myofilament fraction was BAG3-
103 dependent, we used mouse models of cardiomyocyte-specific heterozygous and homozygous
104 BAG3 deletion[24]. Western blot of myofilament-enriched LV tissue revealed myofilament levels
105 of HspB8, but not Hsp70, were significantly reduced in the partial absence of BAG3 (BAG3 +/-,
106 20% reduction of myofilament BAG3 vs. wild-type) and barely detectable in the BAG3 -/-
107 hearts (Fig. 3a). The same relationship was found in human DCM samples, where samples
108 with lowest myofilament BAG3 (≥20% decreased relative to non-failing) had significantly
109 reduced HspB8 levels but not Hsp70 levels (Fig. 3b-c). Notably, the E3 ubiquitin ligase for
110 Hsp70 clients, CHIP, was also significantly reduced with decreased BAG3 expression
111 (Extended Data Fig. 2). These data indicate that BAG3 is required for full assembly of the
112 CASA complex at the myofilament, either through directly mediating HspB8/CHIP localization
113 to the myofilament and/or serving to stabilize these proteins at the sarcomere and prevent their
114 degradation, which is supported by Fang et. al.[13].

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

125 Because myofilament ubiquitination increased in both human heart failure and our
126 mouse model of heart failure (Fig. 1), we next tested whether this was due to a failure of the
127 CASA complex to localize to the myofilament. However, Hsp70, HspB8, and CHIP all
128 significantly increased at the myofilament in the control-treated heart failure mice (Fig. 4a-b).
129 Both these results and immunofluorescence imaging of DCM patient cardiomyocytes
130 (Extended Data Fig. 4) indicated that the CASA complex targeting to the myofilament in
131 response to proteotoxic stress still occurs in heart failure. However, in the heart failure mice
132 that received rAAV9-BAG3, while BAG3 was significantly increased at the myofilament, Hsp70,
133 HspB8, and CHIP each decreased back to sham expression levels (Fig. 4a-b). Myofilament
134 levels of P62 also increased in the heart failure mice, indicating impaired autophagic flux[25], but
135 were reduced by BAG3 overexpression (Fig. 4b). These data suggest that in heart failure,
136 decreased CASA complex assembly at the sarcomere in end-stage heart failure (Figs. 1d and
137 3c) is preceded by impaired CASA clearance or turnover rate therein, thus explaining the
138 buildup of ubiquitinated myofilament proteins (Fig. 1k).

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

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

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

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

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

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

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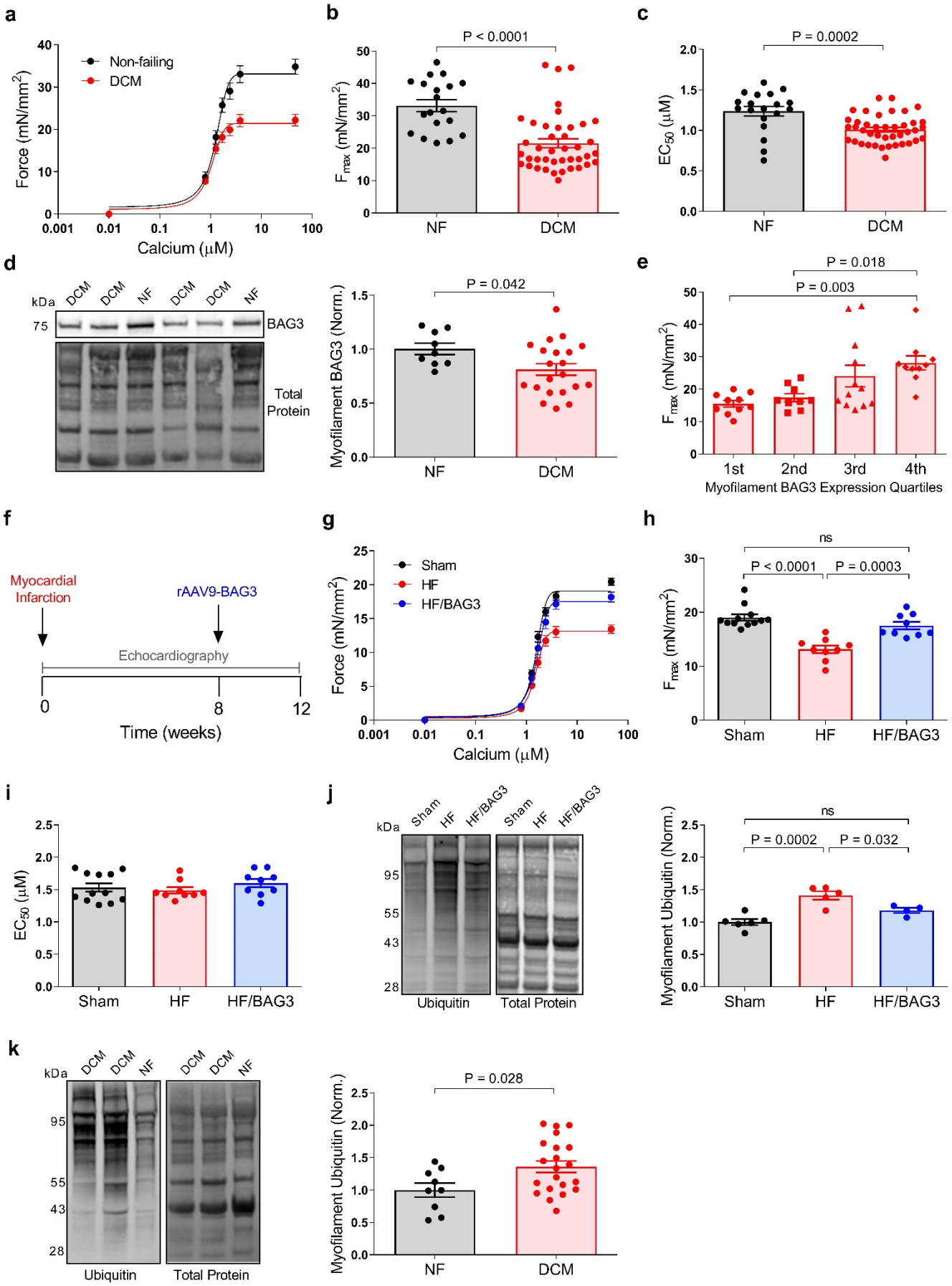

Fig. 1 | See next page for caption

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; 1st = lowest BAG3, 4th = 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.

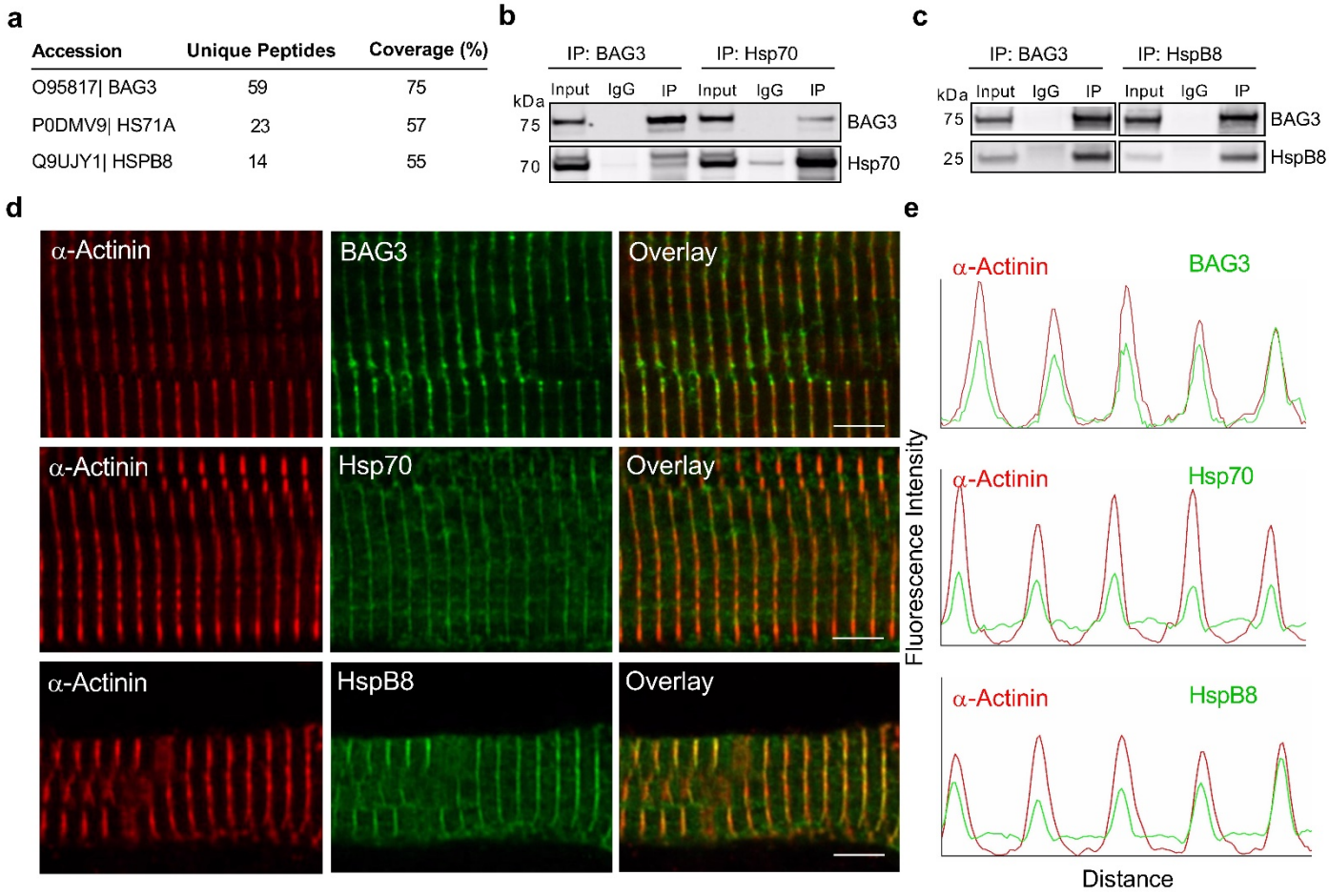

Fig. 2 | HspB8 and Hsp70 associate with BAG3 at the sarcomere Z-disc (**a**), mass spectrometry results for BAG3 immunoaffinity purification from myofilament-enriched human LV tissue. **b-c**, Western blot results of reciprocal co-immunoprecipitation experiments for BAG3/Hsp70 (**b**) and BAG3/HspB8 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**).

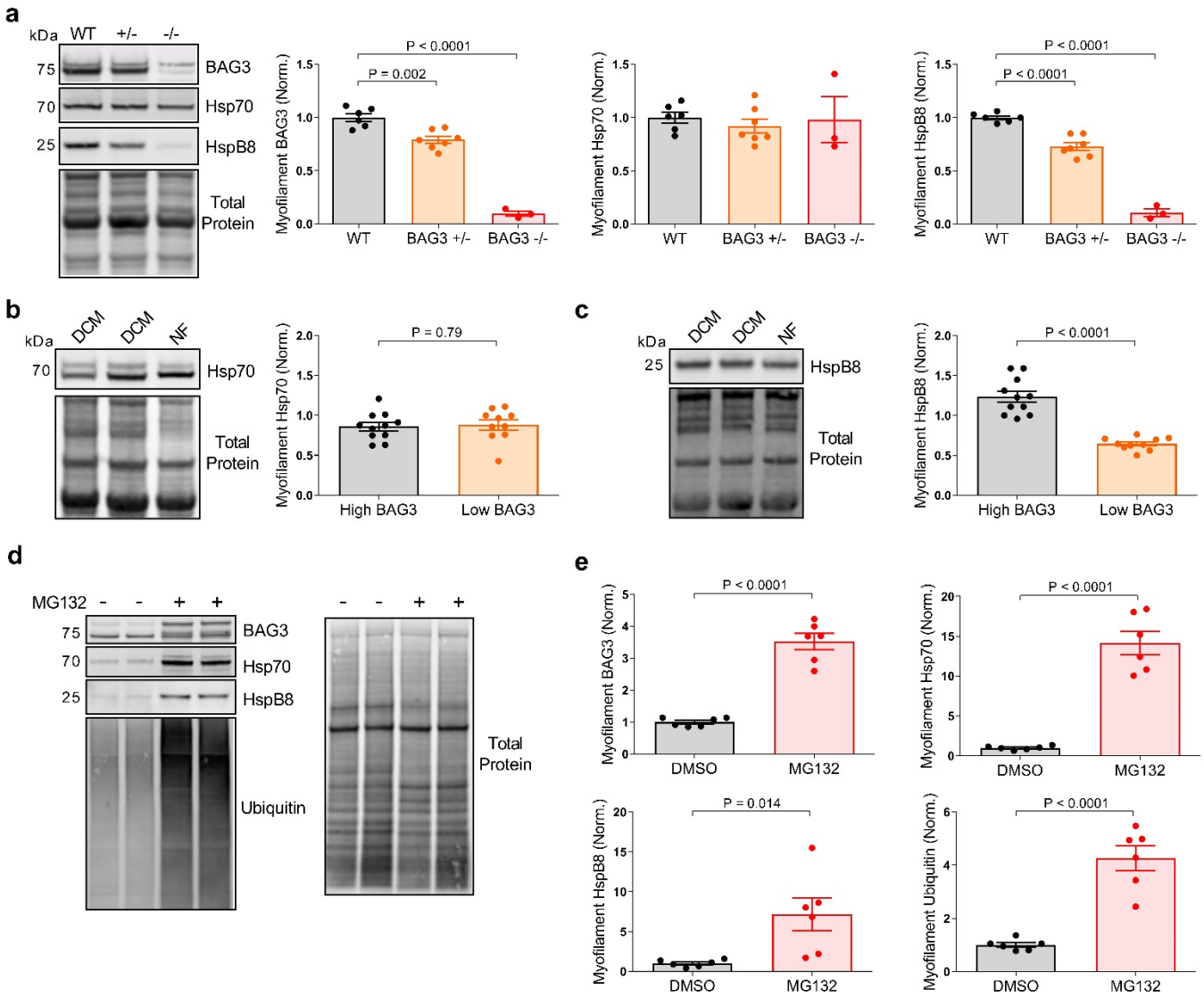

Fig. 3 | Assembly of the CASA complex at the myofilament is dependent on BAG3 and proteotoxic stress (**a**), Western blot in myofilament-enriched tissue for BAG3, Hsp70, and HspB8 in wild-type, BAG3 +/- and BAG3 -/- mouse LV normalized to total protein. Data are mean ± s.e.m.; n = 6 WT, 7 BAG3 +/-, 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.

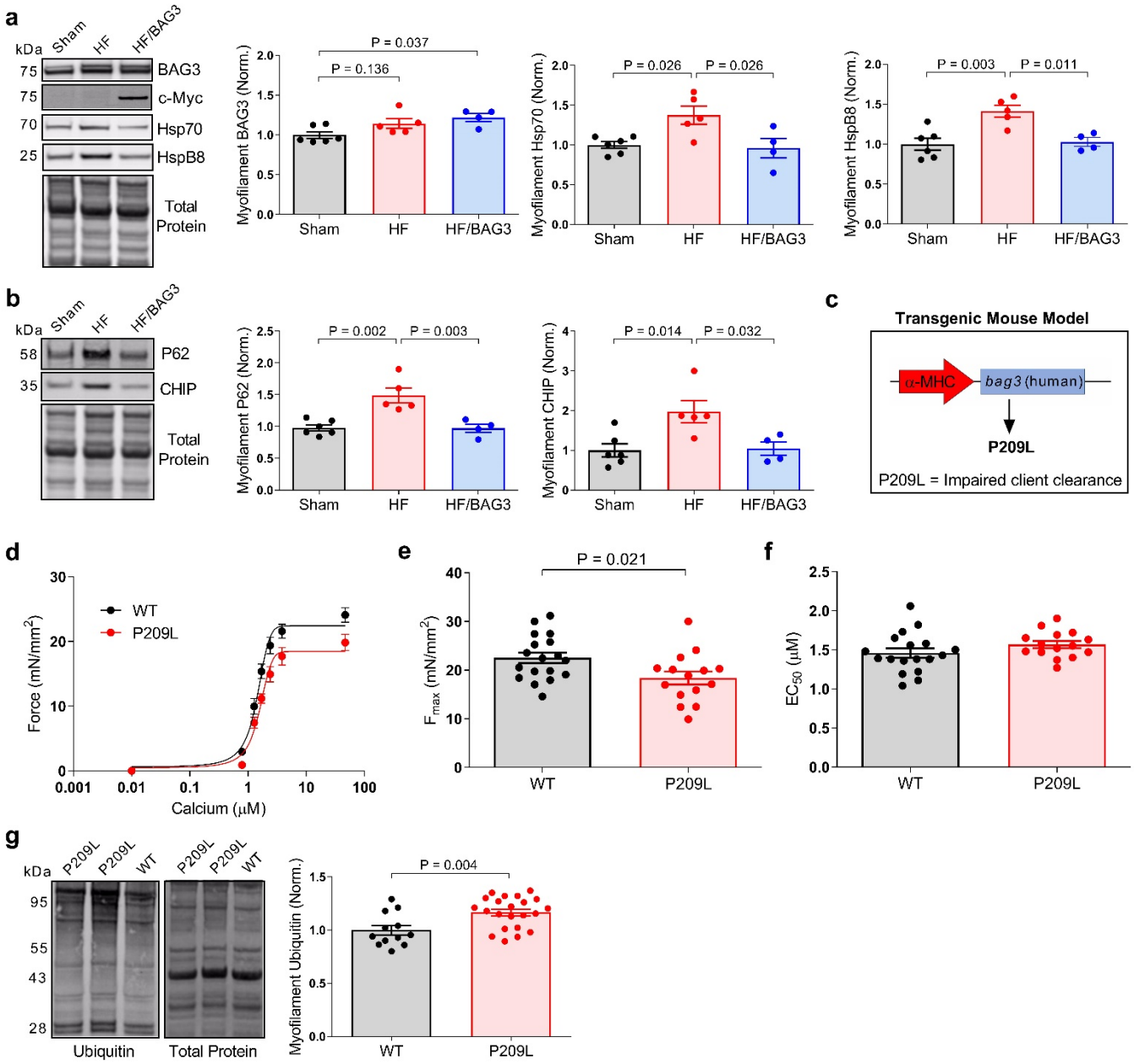

Fig. 4 | BAG3 overexpression in heart failure restores autophagy flux and the P209L BAG3 mutation impairs myofilament function. a-b, Western blot of total BAG3, myc-BAG3, Hsp70, and HspB8 (**a**) or P62 and CHIP (**b**) in myofilament-enriched LV tissue from sham, HF, and HF/BAG3 mice normalized to total 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 *α*MHC 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.