Supplemental material

Full Materials and methods

Mice
bag3-deficient mice (*bag3*-/-) were described previously[1]. All animal experiments were performed according to NIH guidelines, and Public Health Service Policy on Humane Care and Use of laboratory Animals and the USDA Animal Welfare Act.

Antibodies
A polyclonal antibody that recognizes BAG3 was described previously[1]. Mouse monoclonal antibodies for FLAG and α-actinin (sarcomeric) were purchased from SIGMA (SIGMA, St Louis, MO). Antibodies for CapZα (BD Transduction Laboratories, San Jose, CA) and actin (Neomarker, Fremont, CA) were purchased from the indicated manufacturer. All other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Antisera against recombinant CapZβ1, β2 and pan β were kindly provided by Dr. John A. Cooper (Washington University). The antiserum was applied to a GST column equilibrated with TBS (25 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl), followed by a CapZ GST column, and the antibody bound to the recombinant protein was eluted in glycine elution buffer (200 mmol/L glycine-HCl, pH 2.0, 150 mmol/L NaCl). The eluted antibody was immediately neutralized with 1/10 volume of 2 mol/L Tris-HCl (pH 8.0), and dialyzed in PBS overnight.

Histology and Immunofluorescence
Dissected hearts were immersed in Tyrode Solution (100 mmol/L NaCl, 30 mmol/L KCl, 1 mmol/L $MgCl_2$, 4 mmol/L $NaHCO_3$ 10 mmol/L HEPES, 5.5 mmol/L glucose, pH 7.4) for 5 minutes and fixed with 4% paraformaldehyde overnight. Frozen sections (10 mm) of heart tissue from *bag3*$^{-/-}$ or *bag3*$^{+/+}$ mice were stained with hematoxylin and eosin (H&E) (Richard Allen Scientific). The sections were fixed with ice-cold methanol for 10 minutes. For staining of cultured cells, cells were washed with PBS, and fixed with 4% paraformaldehyde in PBS for 5 minutes. After three 5-min washes with PBS, the fixed samples were incubated in 0.2% Triton X-100 in PBS for 3 minutes and blocked with 2% BSA in PBS for 1 hour. Various primary antibodies used for staining were incubated with the samples for 1 hour at room temperature. After washing with PBS, the samples were incubated with Alexa Fluor conjugated antibodies (Alexa Fluor 594 goat anti-mouse IgG, Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR)). Actin filaments were stained using rhodamine-phalloidin (165nmol/L; Molecular Probes). After washing with PBS, samples were mounted using Vectashield with Dapi (4', 6-diamidino-2-phenylindole: Vector Laboratories, Burlingame, CA).

Immunoblotting
Extracted proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biosciences), and probed with the indicated antibodies. Signals were visualized using ECL Plus reagents (Amersham Pharmacia Biosciences). Protein concentrations were measured using the BCL protein assay reagent (PIERCE, Rockford, IL).

Plasmid Construction
cDNAs for full-length human BAG3, Hsc70, mouse capping protein α1 (accession number, U10406), capping protein β1 (accession number, BC085506) and capping protein β2 (accession number, NM_009798) were amplified by reverse transcriptase-PCR and subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) with Flag- or Myc- epitope tags at the N-terminus. For expression of GST fusion proteins in *E. coli* strain BL21, these cDNAs were also subcloned into pGEX-6P-1 (Amersham Biosciences, Uppsala, Sweden). Gene silencing using short hairpin RNA (shRNA) and adenoviral vector were described

previously[1]. Adenoviruses were produced using Adeno-X Expression System Kit (BD Biosciences Clontech). Adenoviral vector encoding β-galactosidase was used as a control. For the FRET assay, CapZβ and Hsc70 were subcloned into pcDNA3-CFP and pcDNA3-YFP, respectively (Addegene, Cambridge, MA). For the expression of BAG3 in Sf9 insect cells, BAG3 cDNA was also subcloned into pFastBacHT B (Invitrogen, Carlsbad, CA).

Cell culture

Cardiomyocytes were isolated from neonatal Sprague-Dawley rats (Harlan, Indianapolis, IN) using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corporation, Lakewood, NJ). Isolated cardiomyocytes were cultured on fibronectin-coated plates (0.5μg/ml fibronectin, SIGMA). Cardiomyocytes and HEK293 cells were cultured at 37 ºC in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS supplemented with penicillin and streptomycin. Mirus was used for transfection (Mirus, Madison, WI). Sf9 cells were cultured at 28 ºC in Sf-900 II SFM (Invitrogen) containing 5% FBS, penicillin and streptomycin.

Proteins

Hsp70 proteins purified from bovine brain were purchased from SIGMA (H9776). GST-CapZα and GST-CapZβ were expressed in *E. coli* strain BL21, and lysed in lysozyme lysis buffer (50 mmol/L Tris, pH 8.0, 2 mmol/L EDTA, 100 mmol/L NaCl, 1% Triton X-100, 200 mmol/L NaSCN, 1mg/ml lysozyme) supplemented with a mixture of protease inhibitors (CompleteTM, Roche Diagnostics). After brief sonication, the cell lysate was centrifuged at 18,000 x g for 15min at 4 ºC, and the supernatant was mixed with GSH-sepharose 4B (Amersham Pharmacia Biosciences) for 2 hrs. The beads were then washed four times with lysis buffer. The expression and purification were confirmed by immunoblot assay. BAG3 protein was expressed as a His-tagged protein in Sf9 cells using BAC-TO-BAC Baculovirus Expression Systems (Invitrogen, Carlsbad, CA). His-tagged BAG3 was purified with Ni-NTA His-Bind Superflow (Novagen, WI).

In vivo binding

For *in vivo* association, HEK293 cells were transiently transfected with various plasmids. 48 hours after transfection, cells were lysed in immunoprecipitation buffer (20 mmol/L Tris, pH7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L NaF, 2 mmol/L Na_3VO_4, 2 mmol/L PMSF, and 1% TritonX-100) supplemented with a mixture of protease inhibitors (CompleteTM, Roche Diagnostics). Pre-cleared lysates were subjected to immunoprecipitation with anti-Flag M2 agarose affinity gel (SIGMA). Precipitated proteins were eluted from the beads by boiling in SDS sample buffer, and separated by SDS-PAGE. Immunoblot assays were performed using the indicated antibodies.

In vitro binding

To assay binding *in vitro*, a GST pull-down was performed. GST and GST fusion CapZα and CapZβ were expressed in BL21 cells, induced by 1 mmol/L isopropyl-1-thio-β-galactopyranoside, and purified using glutathione-Sepharose beads (Amersham Pharmacia Bioscience, Uppsala, Sweden). Purified Hsp70 proteins were incubated with GST, GST-CapZα and CapZβ with or without purified BAG3 in immunoprecipitation buffer followed by precipitation with glutathione-Sepharose beads (Amersham Pharmacia Bioscience). Bound proteins were eluted from beads by boiling in SDS sample buffer, separated by SDS-PAGE, and visualized by immunoblotting using anti-Hsp70 antibody.

2D electrophoresis

Two dimensional isoelectric focusing was used to identify and separate CapZ isoforms (CapZβ1, β2 and α) and Hsc70 that co-migrate in a regular two-dimensional gel[2]. Immunoprecipitated sample or cell lysate was applied to an isoelectric focusing polyacrylamide tube gel (1.5mm inner diameter) with 5% pH ampholytes 3.0/10(Pharmalyte,Pharmacia LKB Biotechnology Inc.), and run overnight. Then, an appropriate portion of gel was grafted horizontally onto the top of a SDS-polyacrylamide slab gel (12.5%) and the second dimension was run. Electrophoretic transfer was carried out from polyacrylamide gels to PVDF membranes, followed by immunoblotting as described above.

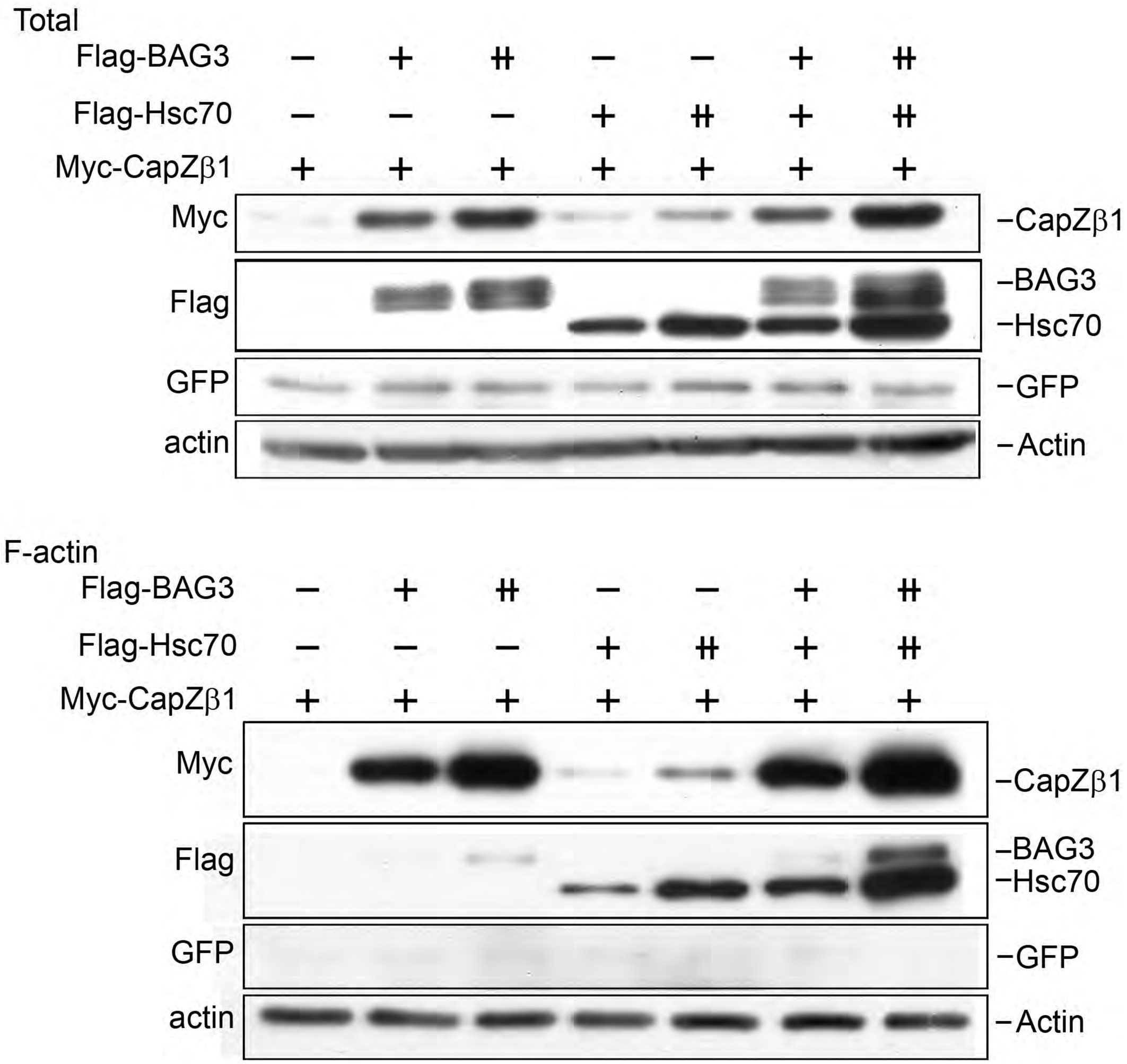
Fractionation of cytoskeletal components
For sedimentation of cytoskeletal components, cells were homogenized in F-actin stabilization buffer (50 mmol/L PIPES, pH 6.9, 50 mmol/L NaCl, 5 mmol/L $MgCl_2$, 5 mmol/L EGTA, 1 mmol/L ATP, 5% glycerol, 0.1% Nonidet P-40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% β-mercaptoethanol, 2 mmol/L Na_3VO_4, 10 mmol/L NaF, 2 mmol/L PMSF, and 1:50 protease inhibitor cocktail). After centrifugation at 100,000 x g for 60 minutes at 37ºC, the supernatant was transferred to a new tube to represent the "G-actin fraction". The pellet fraction was resuspended in solubilization buffer (10 mmol/L Tris, pH8.0, 150 mmol/L NaCl, 2% SDS, 10 mmol/L NaF, 2 mmol/L PMSF, and 1:50 protease inhibitor cocktail). The resuspended samples were sonicated, and boiled to complete solubilization and represented the "F-actin fraction". The samples were subjected to immunoblot assay to detect CapZ proteins.

Measurement of myofibril lengths
Myofibril length was measured using Image J. Cardiomyocytes were fixed, and F-actin and the Z-disc were stained with Rhodamine-Phalloidin and anti-actinin antibody, respectively. Myofibril length was specified by the staining pattern of Rhodamine-phalloidin and sarcomeric structure. The longest 10 sets of myofibrils in more than 10 cardiomyocytes from each group were measured, and normalized.

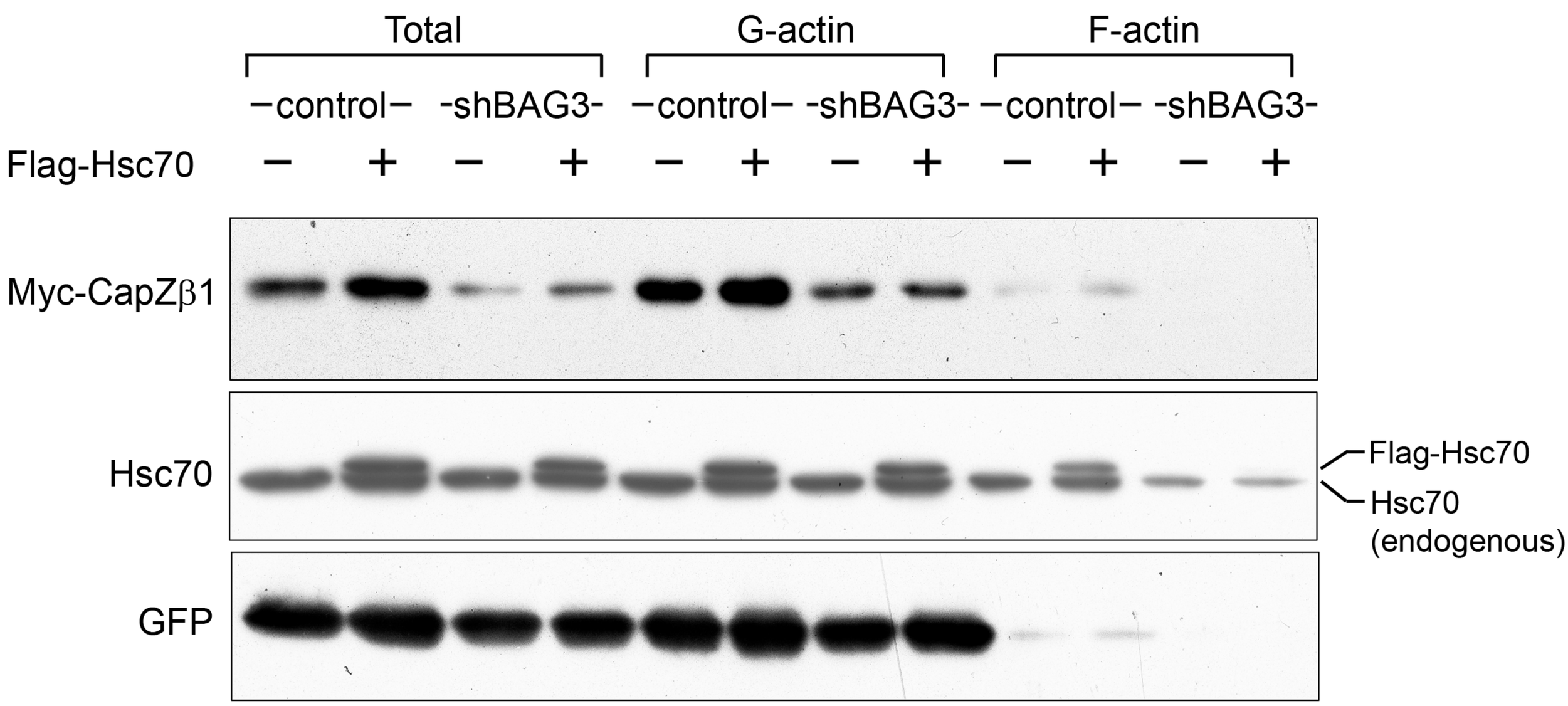
Statistics
Data are expressed as mean +/- S.E. A paired Student's t-test was used to analyze differences between two groups, and p vales of <0.05 or <0.01 were considered significant. "n.s" stands for not significant. Image J is a public domain, Java-based image processing program developed at the National Institutes of Health (NIH).

References:

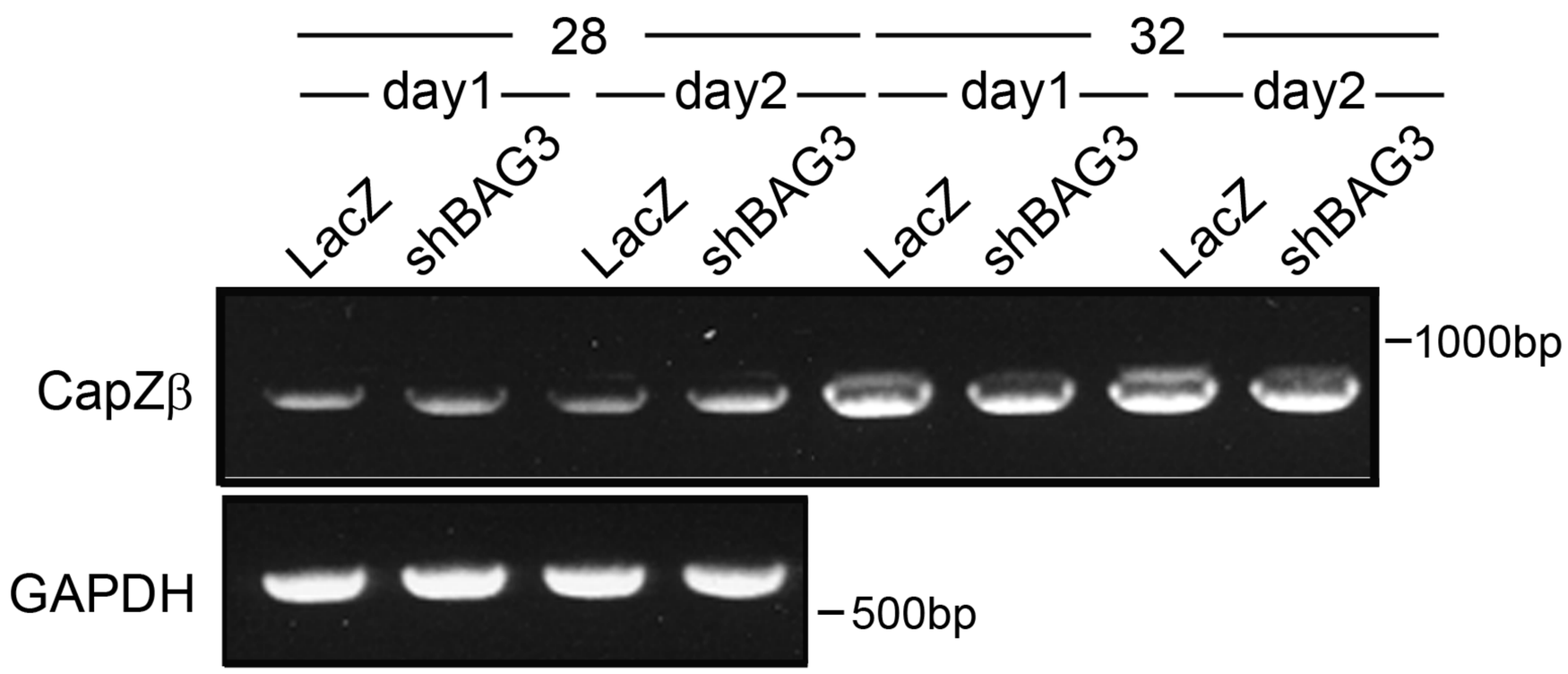
1. Homma S, Iwasaki M, Shelton GD, Engvall E, Reed JC, Takayama S. BAG3 deficiency results in fulminant myopathy and early lethality. *Am J Pathol.* 2006;169:761-773.
2. Schafer DA, Korshunova YO, Schroer TA, Cooper JA. Differential localization and sequence analysis of capping protein beta-subunit isoforms of vertebrates. *J Cell Biol.*1994;127:453-465.



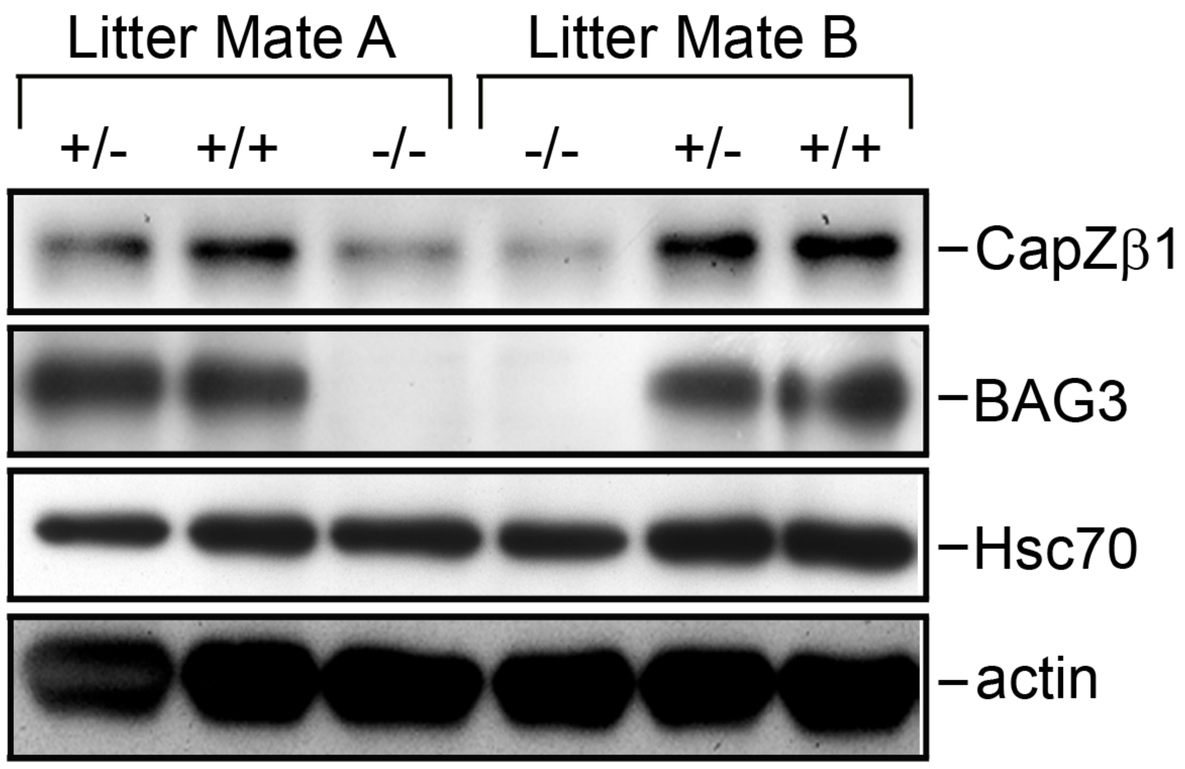
Online Figure I. BAG3 has a synergistic effect with Hsc70 on CapZβ stability and localization. The plasmids for 1 Myc-tagged CapZβ1(0.2 μg) were transfected to HEK293 cells ($4x10^5$) with plasmids for BAG3, Hsc70 or both at the indicated amount (+: 0.05 μg, ++: 0.15 μg). Two days after transfection, cells were harvested and applied to the fractionation assay. Localization of CapZβ1 to F-actin-rich fraction was maximal when cells expressed both BAG3 and Hsc70. Although BAG3 and Hsc70 did not affect each other's expression, BAG3 increased Hsc70 localization to the F-actin-rich fraction in addition to CapZβ1.

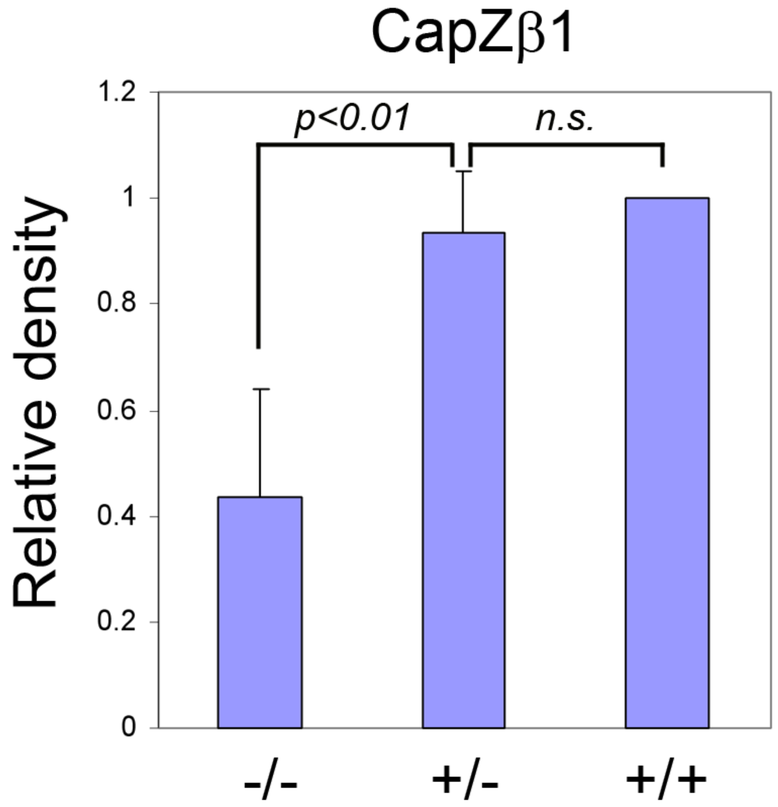


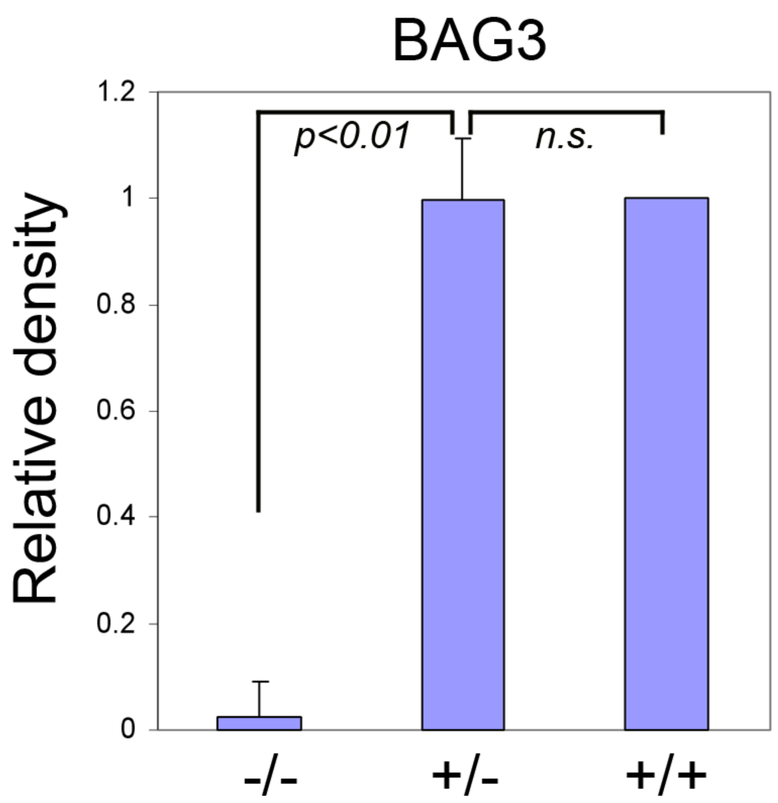
Online Figure II. BAG3 is indispensable for the function of Hsc70 on CapZβ1. Myc-tagged CapZβ1 was expressed with or without Flag-tagged Hsc70 in *bag3* knockdown (shBAG3) or control (control) HEK293 cells. Two days later, a fractionation assay was performed on the cell lysates to investigate the stabilization (Total) and translocalization (G-actin and F-actin) of CapZβ1. The plasmid for GFP protein was also transfected together as a transfection control. Hsc70 expression was constant in the total lysate and G-actin fractions of either the control or *bag3* knockdown cells. In contrast, very low amounts of Hsc70 or CapZβ1 were present in the F-actin fraction of *bag3* knockdown cells as compared to control cells.



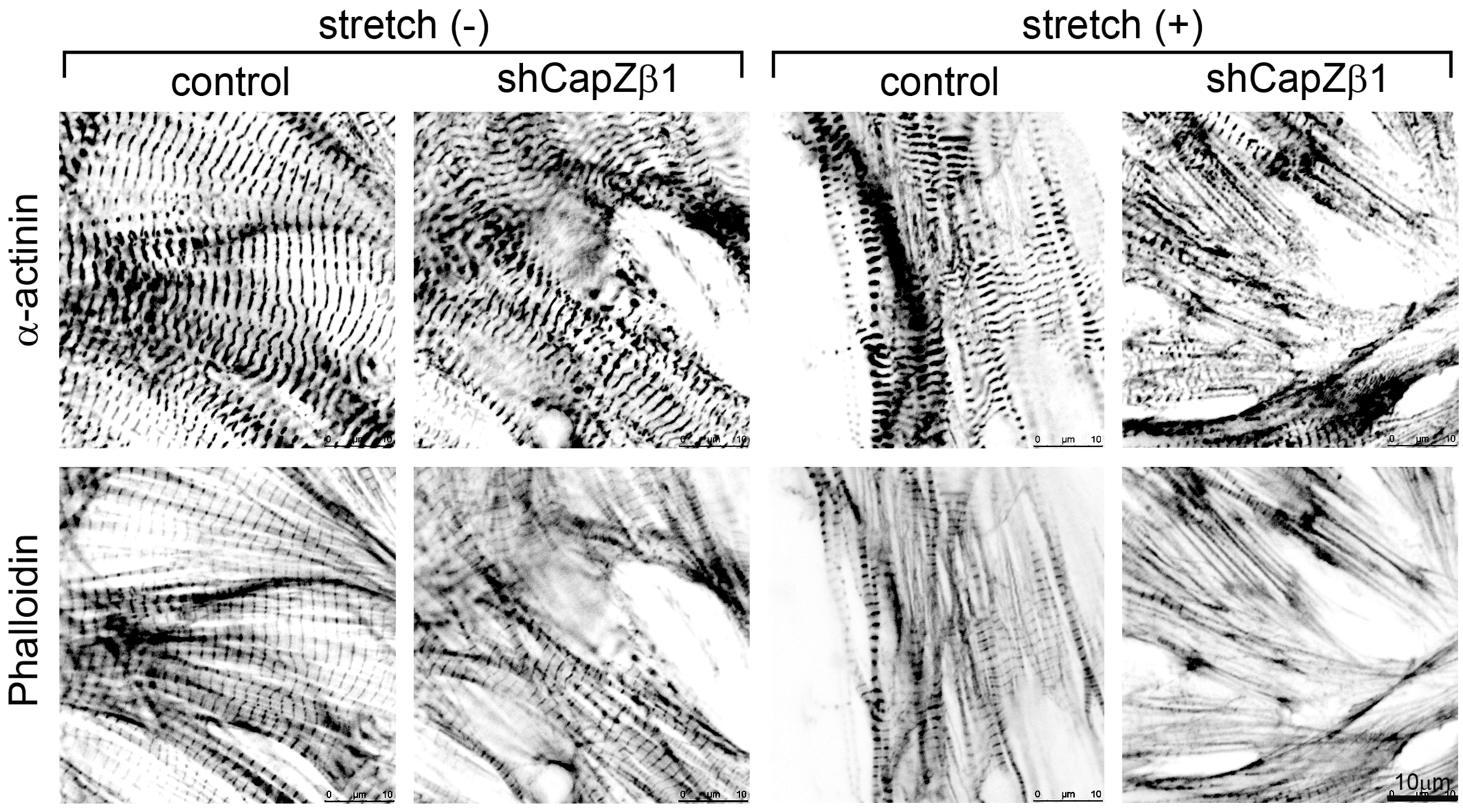
Online Figure III. Transcriptional expression of CapZβ1 is similar for *bag3* knockdown and control cardiomyocytes. Cardiomyocytes were infected with LacZ or shBAG3 knockdown adenovirus, and cells were harvested on the first or second day post-infection. Total RNA was extracted from cardiomyocytes with LacZ or shBAG3 adenovirus, and RT-PCR was performed to monitor CapZβ1 expression at the RNA level. PCR samples were collected after 28 cycles (28) or 32 cycles (32), and loaded onto an agarose gel. Primer set for GAPDH was used for a loading control (GAPDH).



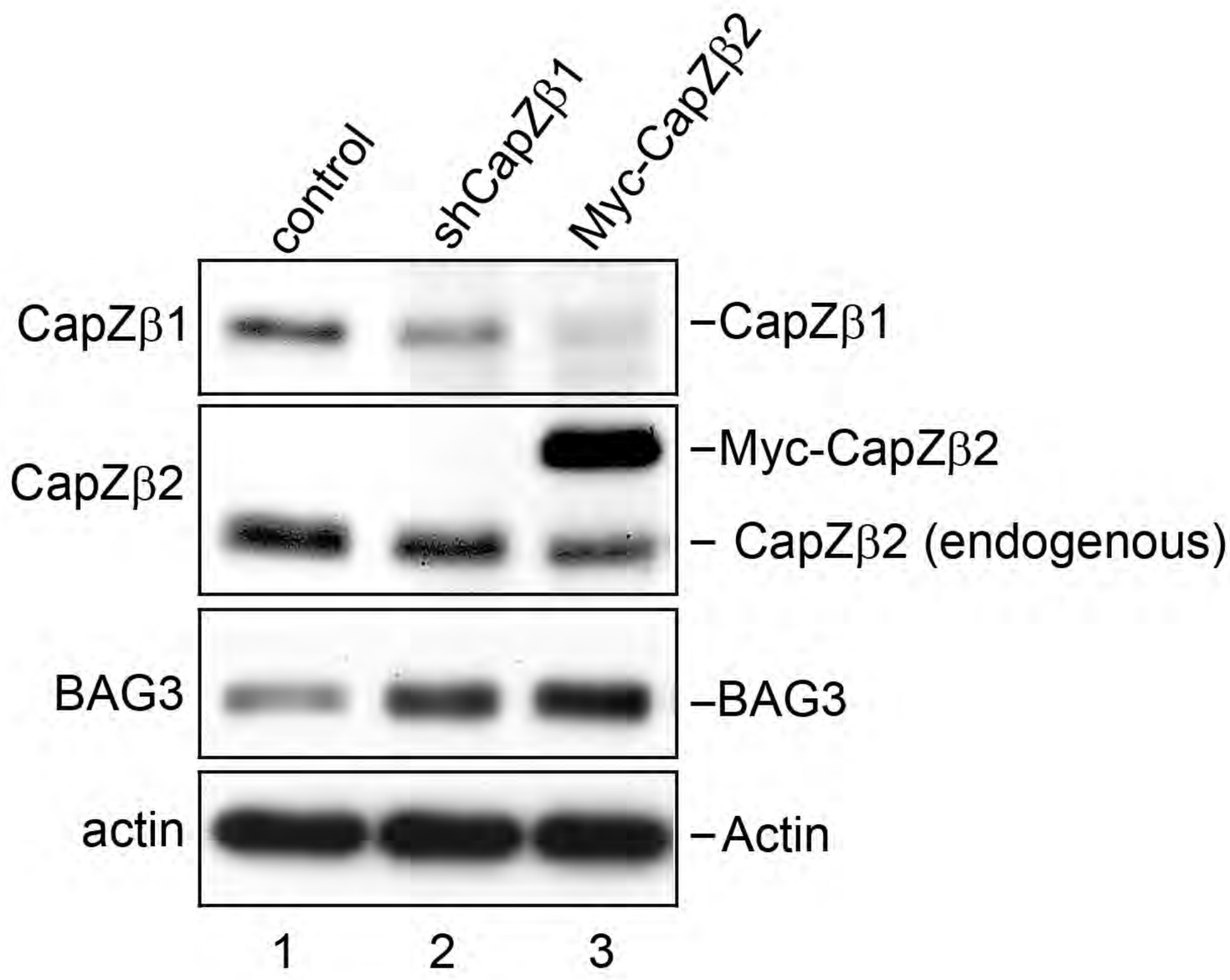


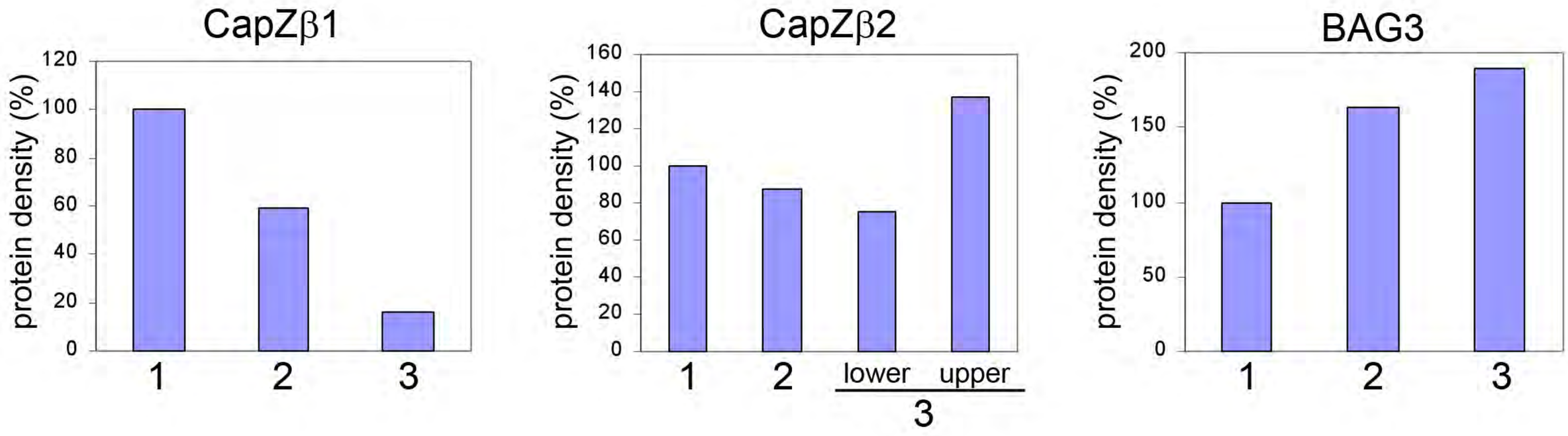


Online Figure IV. Destabilization of CapZβ1 in *bag3*-/- mice. Tissue extracts were prepared from hearts of 14 day old wild type (+/+), heterozygous (+/-) or knockout (-/-) mice from two different litters (A and B) and used for immunoblot assays. CapZβ1, BAG3, Hsc70, and actin were detected using the indicated antibody. Signal intensity was determined by densitometry analysis using ImageJ and normalized with respect to actin. The results for CapZβ1 (upper panel) and BAG3 (lower panel) represent the average of signals from seven independent immunoblots.

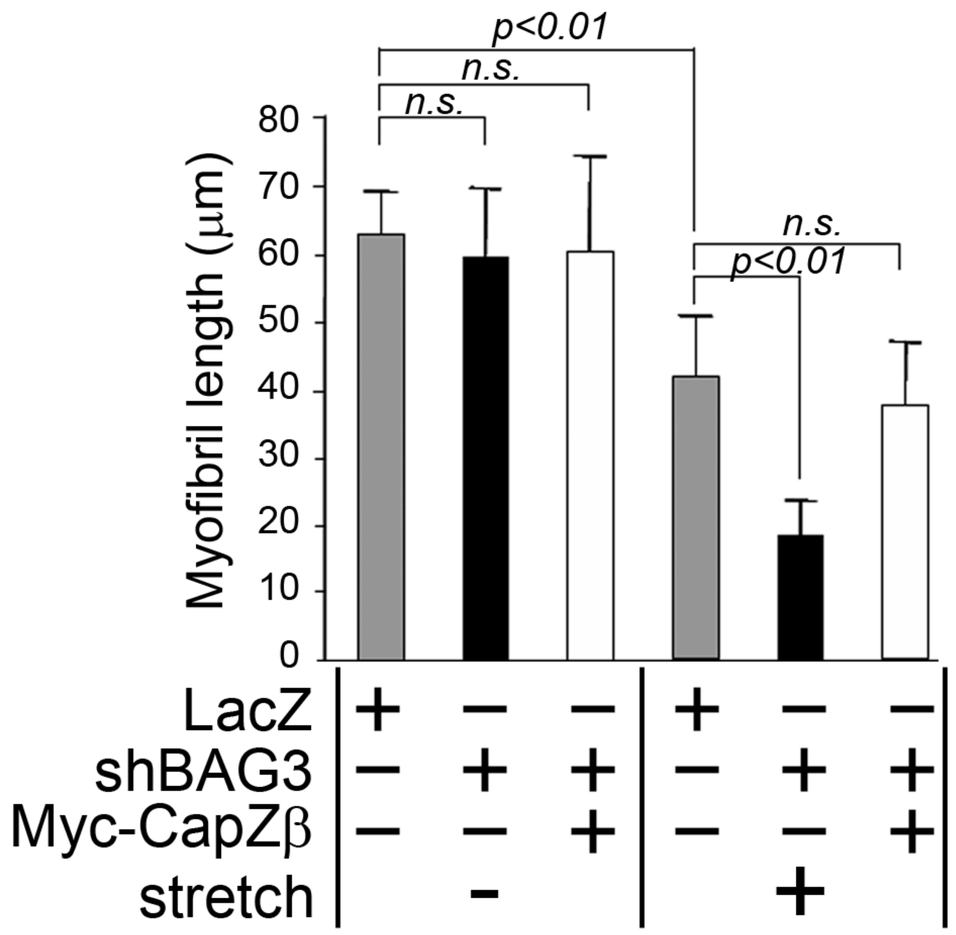


Online Figure V. Knockdown of CapZβ1 causes myofibrillar breakdown in cardiomyocytes. Adenovirus for LacZ (control) or CapZβ1 knockdown (shCapZβ1) were infected to cardiomyocytes, and mechanical stretch was applied for 2 hours. Z-disc and F-actin structures were stained using anti-α-actinin antibody (α-actinin: upper panels) and rhodamine-phalloidin (Phalloidin: lower panels). Moderate cytoskeletal disruption was observed in about 20% of shCapZβ1 cardiomyocytes, with similar cytological changes as shown in this figure.





Online Figure VI. Expression of CapZβ1 and CapZβ2 proteins in CapZβ1 knockdown or CapZβ2 overexpressing cardiomyocytes. Cardiomyocytes were infected with adenovirus carrying LacZ (control), shCapZβ1 or Myc-tagged CapZβ2 (Myc-CapZβ2). Two days later, cells were lysed, and an immunoblot assay performed with anti-CapZβ1 antibody. The same membrane was blotted with anti-CapZβ2, BAG3, and actin antibodies. The intensity of each band was calculated with ImageJ software and shown as bar graph.



Online Figure VII. Myofibrillar fragmentation is inhibited by overexpression of CapZβ1 in *bag3* knockdown cardiomyocytes. After mechanical stress, cardiomyocytes were fixed and stained with Phalloidin-Rhodamine to measure myofibril length (Figure 4E). The length of myofibril in cardiomyocytes was calculated using NIH image J and statistically analyzed.

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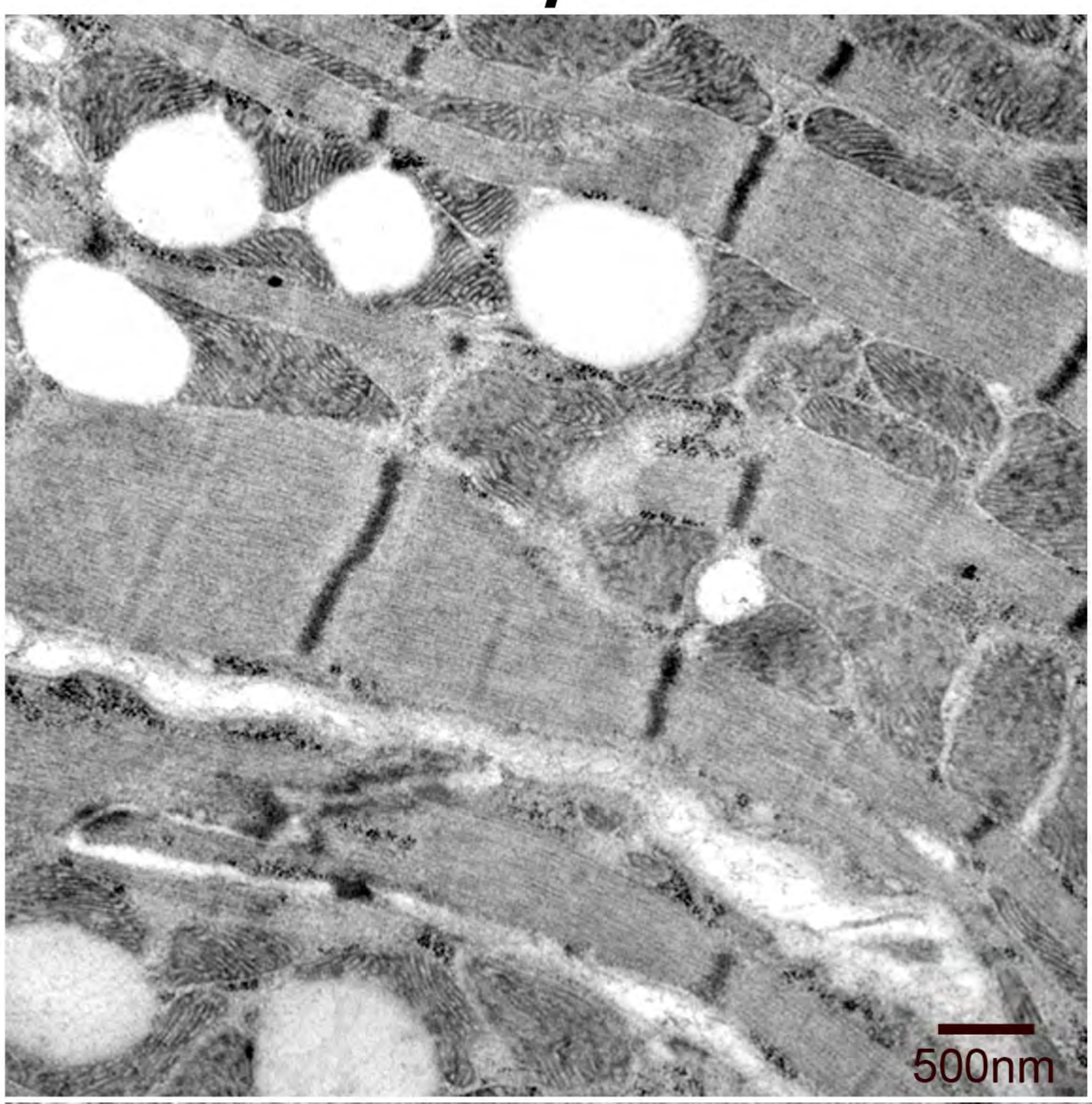
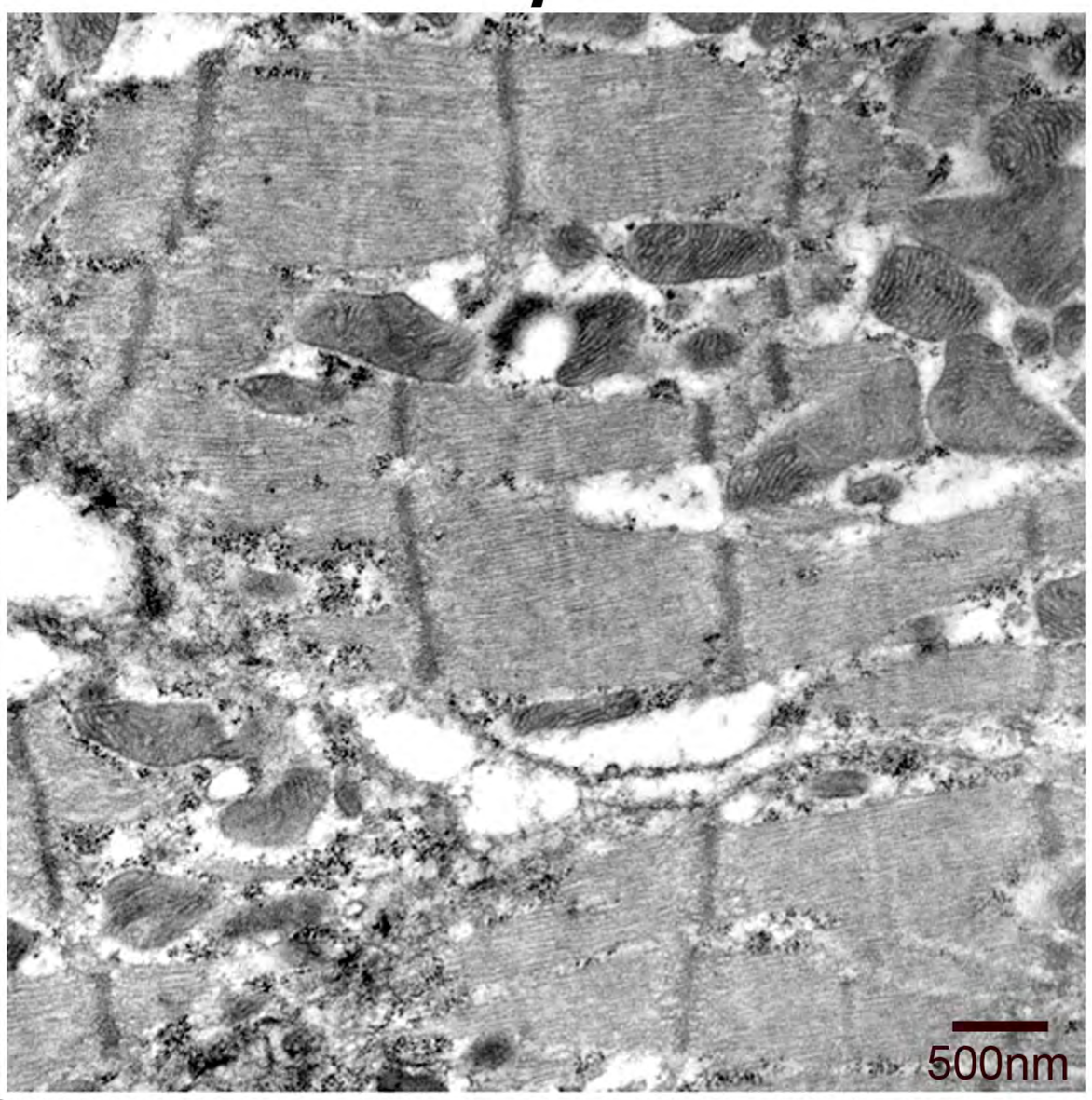
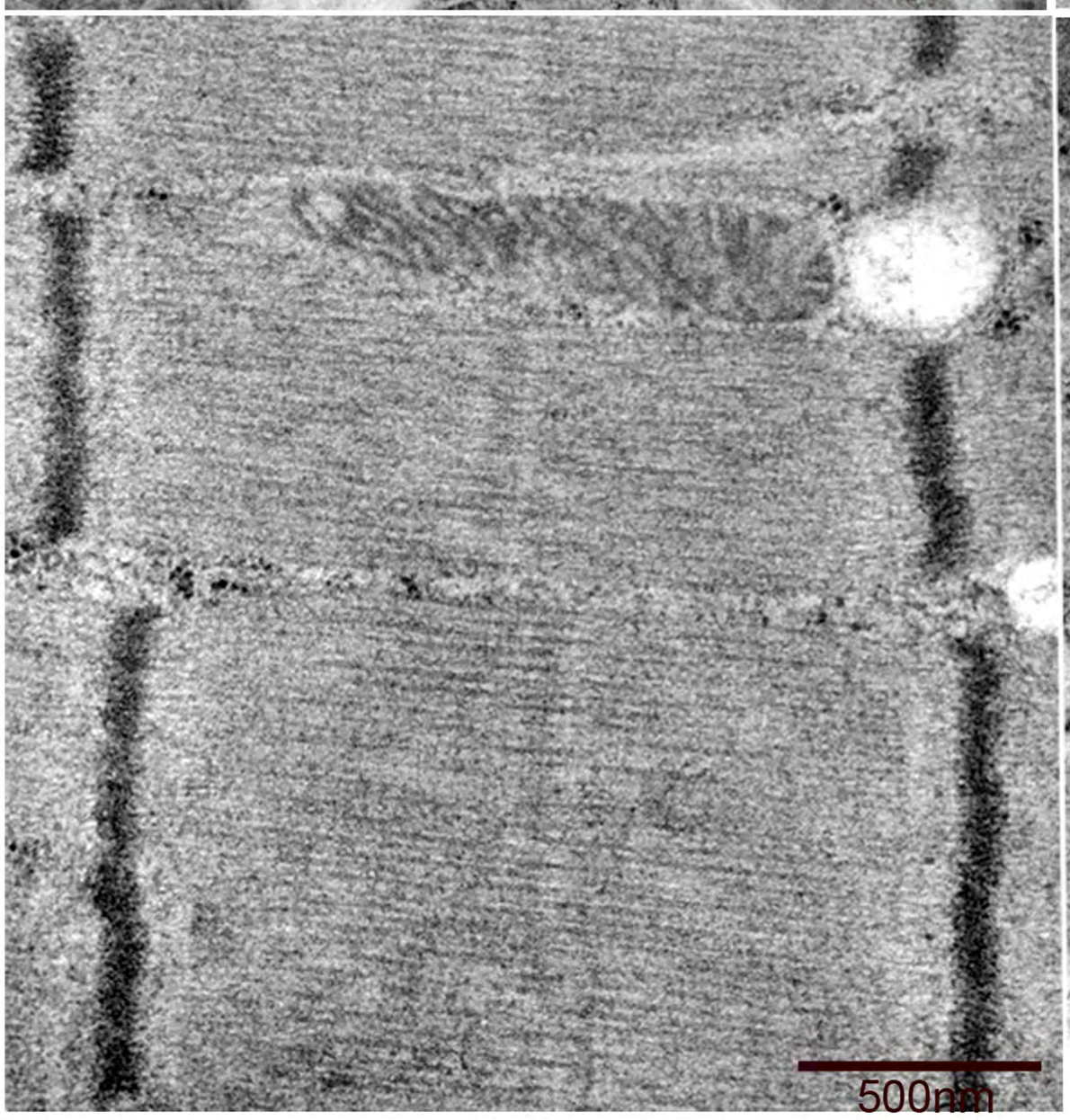
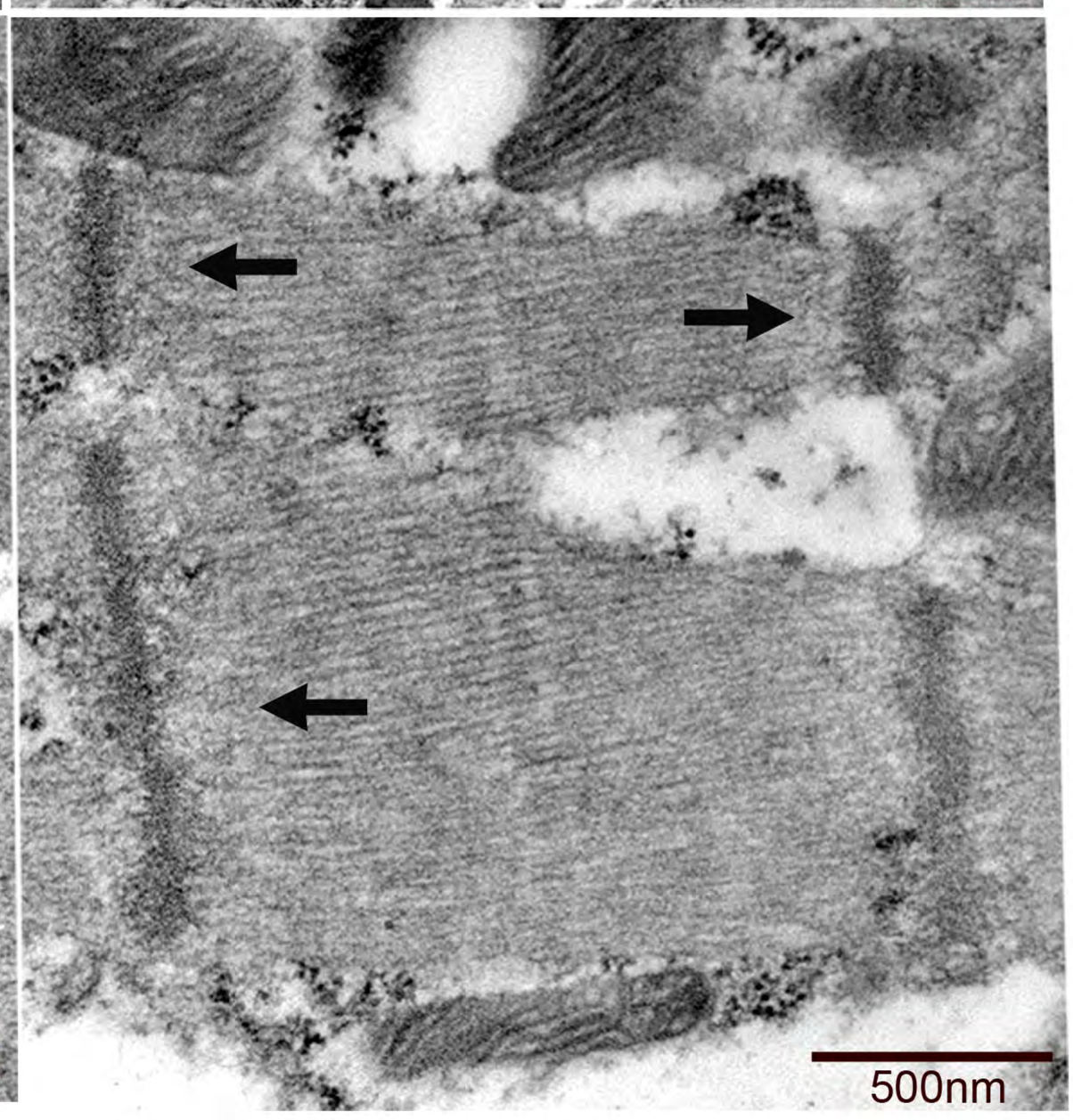
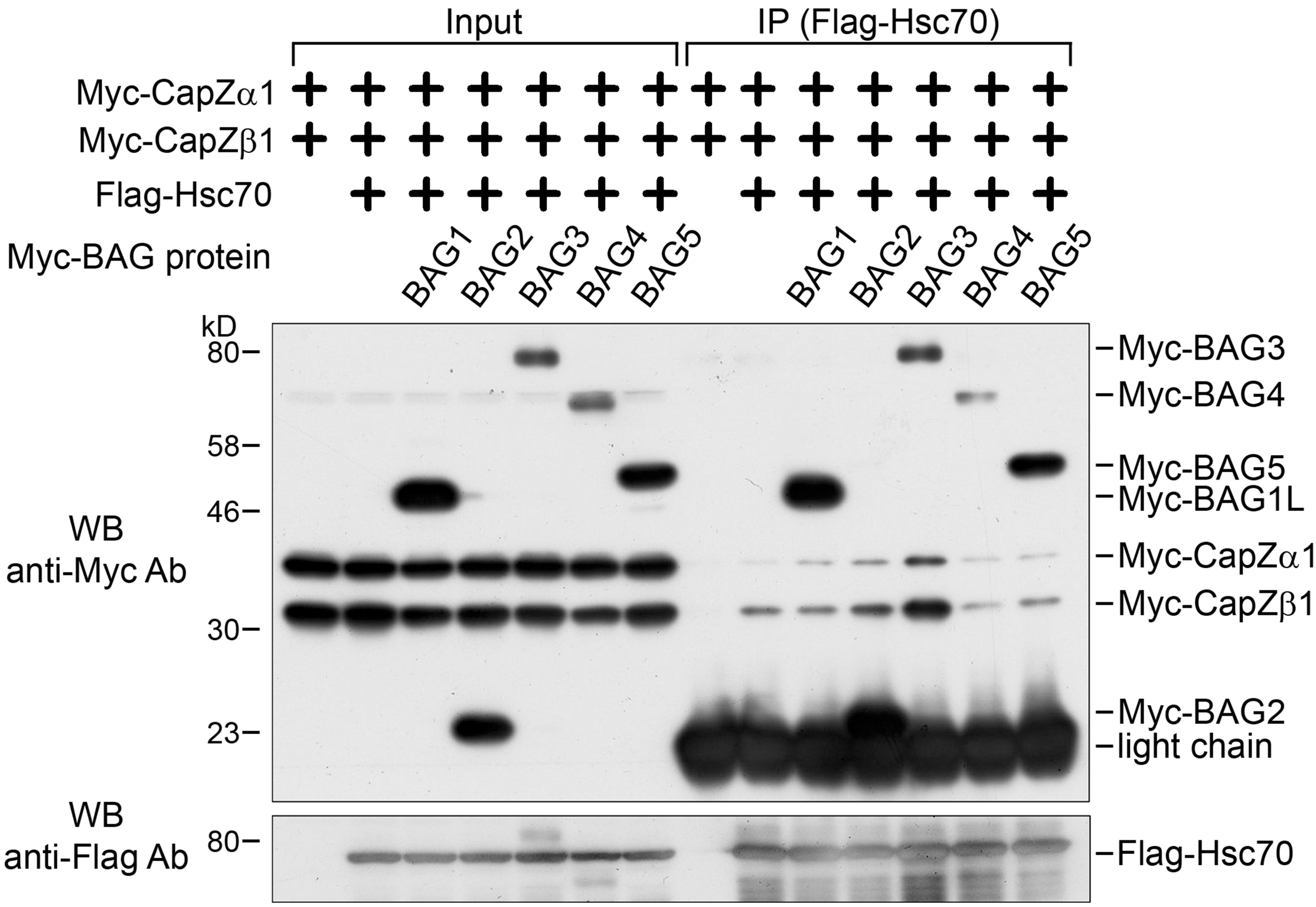




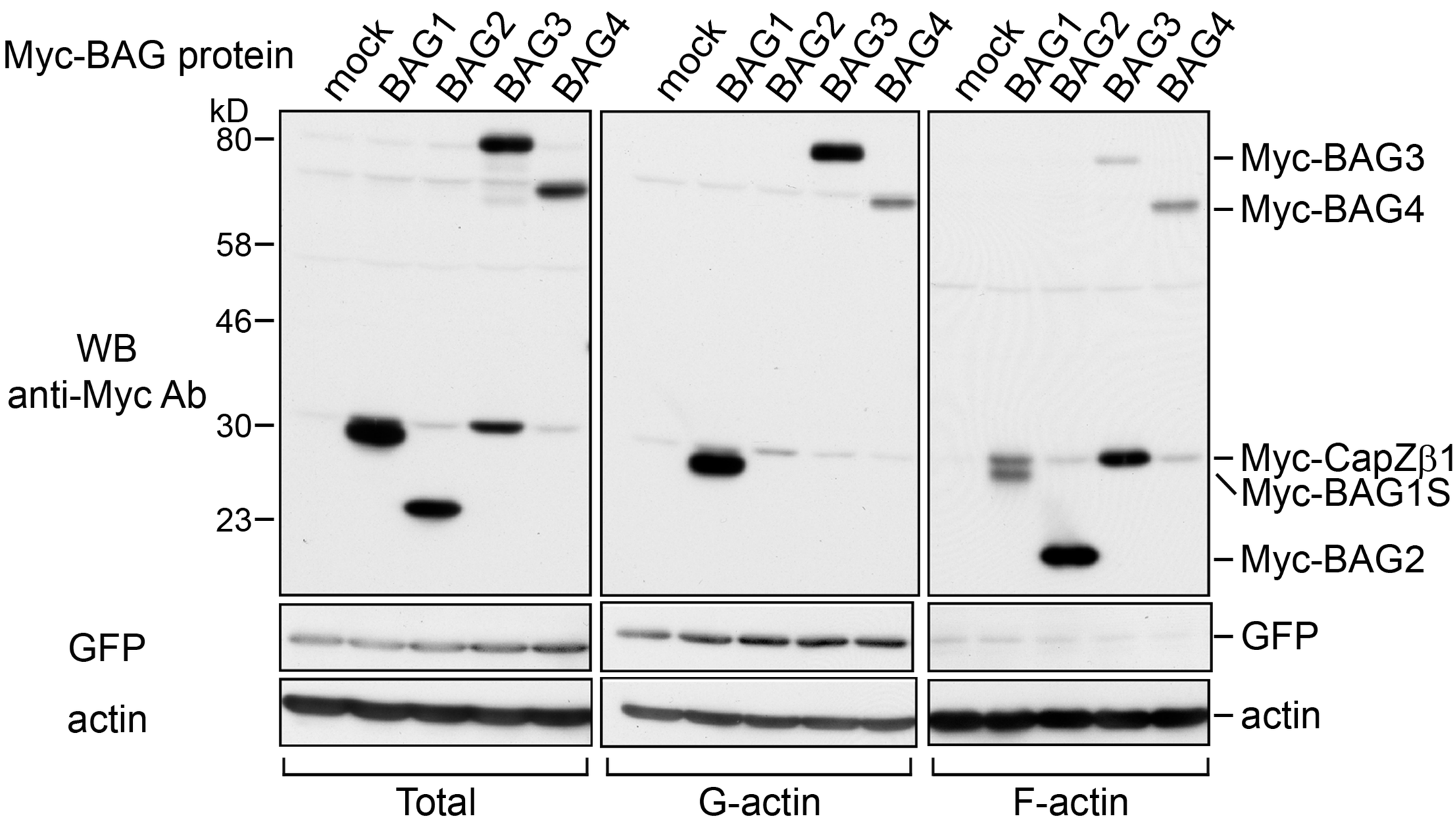




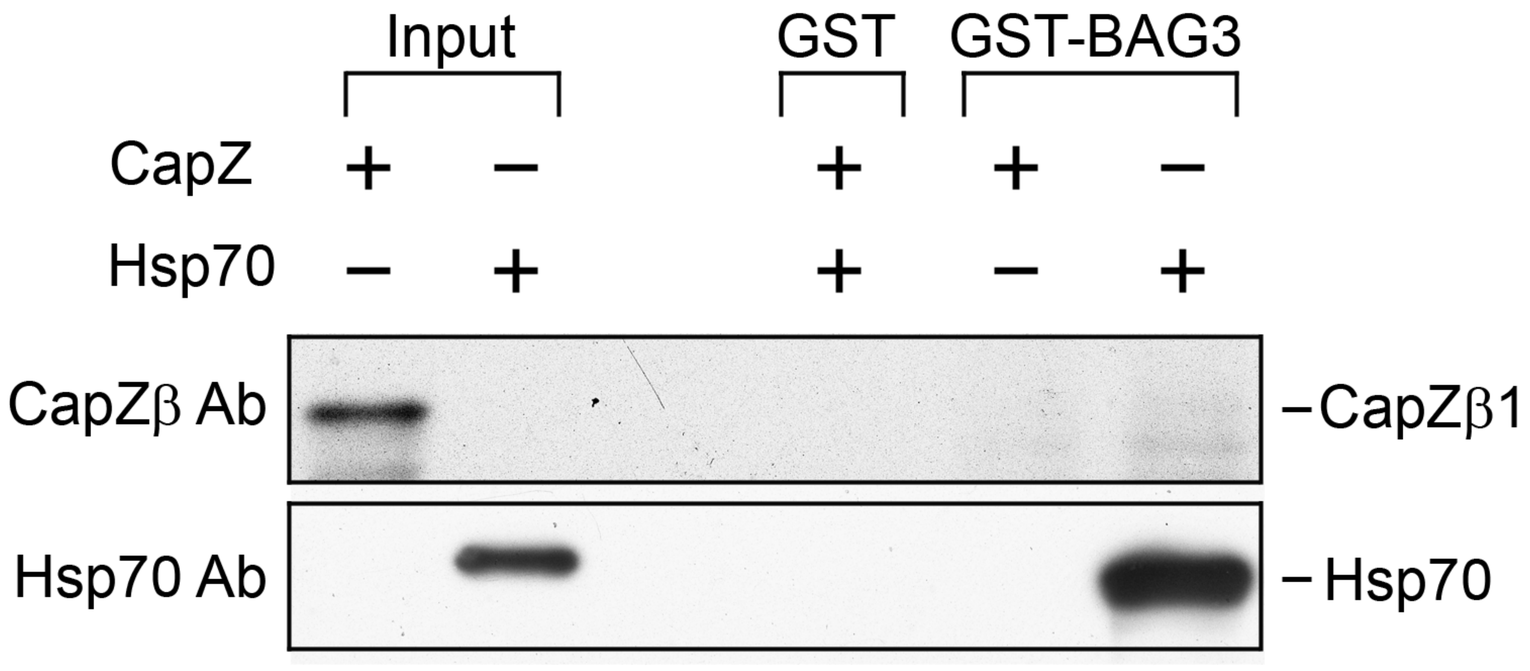
Online Figure VIII. Ultrastructure of sarcomere from papillary muscles of *bag3*+/- and -/- mice. Electron microscopic examination was performed using papillary muscles from 12 day old mice (*bag3*-/- n=2, *bag3*+/- n=1).



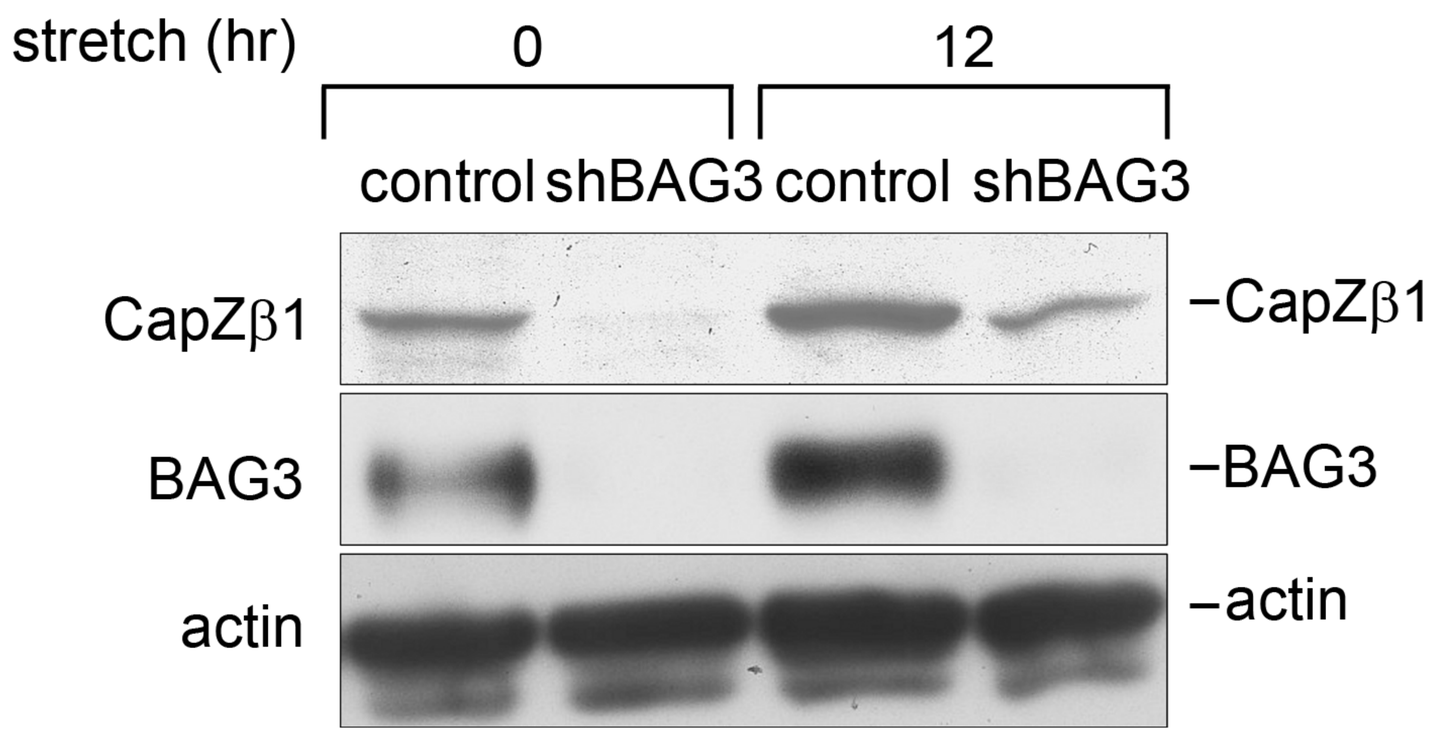
Online Figure IX. BAG3 specifically enhances the interaction of Hsc70 and CapZ proteins. Myc-tagged CapZα1 and CapZβ1 were expressed in HEK293 cells with or without Flag-tagged Hsc70 and the Myc-tagged BAG member (BAG1-5). Two days after transfection, cells were harvested and an immunoprecipitation assay was performed with anti-Flag antibody. Immune complexes were separated by SDS-PAGE, and an immunoblot assay was performed with anti-Myc antibody to detect Myc-tagged CapZα1 and CapZβ1 or Myc-tagged BAG proteins. Co-purified Flag-tagged Hsc70 was confirmed with anti-Flag antibody (Hsc70). Only BAG3 increases interaction between Hsc70 and CapZ proteins.



Online Figure X. BAG3 specifically promotes CapZβ1 stability and translocation to F-actin fraction. Myc-tagged CapZβ1 was expressed with BAG proteins (BAG1, 2, 3, 4 and 5) in HEK293 cells. Two days after transfection, cells were lysed in F-actin stabilization buffer, and the cell lysate was fractionated into G-actin and F-actin fractions by ultracentrifugation. Fractionated samples were loaded onto SDS-PAGE, and an immunoblot assay was performed with anti-Myc antibody to detect Myc-tagged CapZβ1 (CapZβ1) and Myc-tagged BAG protein (BAG1-4). To monitor the transfection efficiency, a plasmid for GFP expression was also co-transfected, and detected with anti-GFP antibody (GFP). Actin was used for a loading control (actin).



Online Figure XI. BAG3 does not bind to CapZβ1 directly. GST or GST-BAG3 was mixed with purified recombinant CapZα and CapZβ or Hsp70, and a pulldown assay was performed. The protein complexes were separated by SDS-PAGE, and western blotting was performed with anti-CapZβ antibody (CapZβ) or Hsp70 antibody (Hsp70).



Online Figure XII. Upregulation of CapZβ1 expression in response to mechanical stress. Cardiomyocytes were cultured on an elastic membrane, and adenovirus for lacZ (control) or *bag3* knockdown (shBAG3) was infected to cardiomyocytes. 24 hours after infection, culture medium was changed to serum-free medium, and the cells were incubated for additional 24 hours. A 20% static mechanical stress was applied to cells for 12 hours, and the cells were harvested. An immunoblot assay was performed with anti-CapZβ1 antibody or anti-BAG3 antibody to monitor the expression of endogenous CapZβ1 or BAG3, respectively. Anti-actin antibody was used for a loading control (actin). After applying mechanical stress, upregulation of CapZβ1 expression was observed in control adenovirus infected cardiomyocytes, but not in the *bag3* knockdown cardiomyocytes.