Supplementary Online Content

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This supplementary material has been provided by the authors to give readers additional information about their work.

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eMethods.

Sequencing

Genomic DNA from each patient was PCR amplified using primers designed using NCBI's primer Blast algorithms (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and were optimized for amplification and sequencing performance. PCR products are analyzed by agarose gel electrophoresis and purified using Exonuclease III and Shrimp Alkaline Phosphatase (Promega, Madison, WI) prior to Sanger sequencing. Products were sequenced with the PCR primers using fluorescent dideoxy terminator method of cycle sequencing (ABI Prism BigDye Terminator v3.1 Cycle Sequencing kits using 1/16 of the "standard" protocol). Reaction clean up was carried out using Applied Biosystems XTerminator purification system, following Applied Biosystem's protocols. Sequencing reactions were run on a 3730xl DNA Analyzer (Applied Biosystems Division, Foster City, CA) following Applied Biosystems' protocols. Sequence data was analyzed using Sequencher Software, version 5.4.6 (Gene Codes, Ann Arbor, MI). SNPs were identified using the software's "Call Secondary Peaks" function. All data was manually inspected.

BAG Exon 2 and BAG Exon 3
Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Amplification was carried out in 10µl reaction containing 40ng DNA, 0.15µM each primer, 0.15mM each dNTPs (Life Technologies, Rockville, MD), 1X Buffer II (10 mM Tris-HCl pH 8.3, 50 mM KCl), 1.5mM MgCl2, and 0.5U AmpliTaq DNA Polymerase (Perkin Elmer, Foster City, CA). Thermocycling was carried out in a Veriti thermocycler (Applied Biosystems). Samples were denatured for 2 minutes at 96ºC followed by 40 cycles of 94ºC for 20 seconds, 61ºC for 20 seconds, and 72ºC for 20 seconds, then a final extension at 72ºC for 10 minutes.
BAG Exon 1 and BAG Exon 4
Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Amplification was carried out in 10µl reaction containing 40ng DNA, 0.15µM each primer, 0.15mM each dNTPs (Life Technologies, Rockville, MD), HotStar1X PCR buffer (Tris-HCl pH 8.7, KCl, 1.5 mM MgCl2, $(NH_4)_2SO_4$), and 0.5U HotStarTaq DNA Polymerase (Qiagen, Valencia, CA) Thermocycling was carried out in a Veriti (Applied Biosystems). Samples were denatured for 15 minutes at 95ºC followed by 45 cycles of 94ºC for 20 seconds, 63ºC (BAG Exon 1) or 61ºC (BAG Exon 4) for 30 seconds, and 72ºC for 30 seconds, then a final extension at 72ºC for 10 minutes.
GRCF Sanger Sequencing protocol:
The GRCF Sanger sequencing master mix formula is the following: 110 ul BigDye v3.1; 500 ul 5x Sequencing buffer; 100 ul of distilled water. Reactions are set up with 8 ul of mastermix, 2 ul of template/primer combination and cycled following Applied Biosystems cycling recommendations, but for 35 cycles instead of the recommended 25 cycles, in a Life Technologies Veriti 96W Thermal Cycler 0.2 Ml version.

Genotyping of Patient DNA

Targeted single nucleotide variant (SNV) genotyping was performed to confirm the results of the GRAFH cohort and for assessment of each of the identified SNPs in cohorts A-E using an Applied Biosystems QuantStudio 7 real-time PCR System (Applied Biosystems, Foster City, CA) and SNP-specific reagents obtained from Applied Biosystems (rs144041999, Assay ID: C_160577079_10; rs144692954, Assay ID: C_160606790_10; rs34656239, Assay ID: C__25628833_10). All reactions were carried out according to the manufacturer's protocol and the output was analyzed using Applied Biosystems QuantStudio Real-Time PCR Software. PCR products were analyzed by agarose gel electrophoresis and purified using Exonuclease III and Shrimp Alkaline Phosphatase (Promega, Madison, WI) prior to Sanger sequencing. Products were sequenced using the fluorescent dideoxy terminator method of cycle sequencing (ABI Prism BigDye Terminator v3.1 Cycle Sequencing kits using 1/16 of the "standard" protocol). Reaction clean-up was carried out using Applied Biosystems XTerminator purification system, following Applied Biosystem's protocols. Sequencing reactions were run on a 3730xl DNA Analyzer (Applied Biosystems Division, Foster City, CA) following Applied Biosystems' protocols. Sequence data was analyzed using Sequencher Software, version 5.4.6 (Gene Codes, Ann Arbor, MI). SNVs were identified using the software's "Call Secondary Peaks" function. All data was manually inspected

Determination of Linked Variants

To determine whether two BAG3 SNVs (P63A - rs144041999 and P380S - rs144692954) were arranged in cis on the same chromosome or in trans, the BAG3 locus from exons 2-4 was amplified, cloned and sequenced using methods as shown in figure 8. In brief, primers were designed to PCR out from patient DNA the portion of BAG3 containing both the P63A and P380S SNP (Figure e1A and e1B). The 7345 nucleotide PCR product was then inserted into a plasmids for sequencing (Figure e1C). Sequencing primers were then used on each amplified PCR product specific to where the P63A and the P380S mutations occurred (Figure e1D).

Protein Extraction and Immunoblotting from Tissue

Cardiac tissue was placed in RIPA buffer and homogenized with 0.5mm diameter zirconium oxide beads (Thomas Scientific) in a Bullet Blender BBX24 according to manufacturer's instructions. After tissue homogenization the suspension was centrifuged at 13,000g for 5 min at 4°C and supernatant containing protein was collected into a new pre-chilled tube. Protein concentration was determined by colorimetric Bradford assay from Bio-Rad according to manufacturer's protocol and samples with equal amount of protein were used for analysis by Western Blot.

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Western Immunoblots: were performed as described previously (1). In brief, snap frozen left ventricles were lysed in buffer and homogenized in a Bullet Blender. 25µg of reduced protein lysates were run on SDS-PAGE gels and transferred to Odyssey nitrocellulose membranes for blotting. Blots were then incubated with a primary antibody and secondary antibodies prior to scanning with an Odyssey scanner and quantification using Image Studio software. Primary antibodies used were: BAG3 (Cat. # 10599-1-AP, Protein Tech, Rosemont, IL) Secondary antibodies were: goat anti-mouse IRDye 800 (Cat. #926-32210; LiCor Biosciences, Lincoln, NE) and IRDye 680 goat anti-rabbit (Cat. #926-68071; Licor Biosciences, Lincoln, NE).
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RNA Extraction and qRT-PCR Analysis

Total RNA was extracted using the ThermoFisher Scientific *mir*Vana RNA isolation kit according to manufacturer's instructions. Complementary DNA was generated using a reverse transcription protocol. Generation of cDNA product was verified using primers for the housekeeping gene GAPDH. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of target genes was normalized to GAPDH. All primer sequences used are listed in Table 1. LightCycler 480 SYBR Green I Master Mix with specific primers and cDNA was used for qRT-PCR 20ul total reaction mixture. The qRT-PCR reaction was performed as previously described (5) in a 96-well plate on an Applied Biosystems StepOne Real Time PCR System. All samples were in triplicates, and amplification curves and Cp values were obtained for analysis. Results were expressed as mean ± SEM.

BAG3 Variant Plasmid Construction

Plasmid pTU31, generated by Joseph Rabinowitz at Temple University, was used as the backbone for cloning of the BAG3 variants. Wild type BAG3 was inserted under a CMV promoter and each of the variants, p.Pro63Ala, p.His83Gln, p. Pro380Ser, p.Pro63Ala + p.Pro380Ser, and p. Ala479Val, were made using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara CA) according to manufacturer instructions with the primers listed in etable 2. p.Pro63Ala + p.Pro380Ser was made by sequential mutations of each site. p.160Aladup was synthesized by GenScript (Piscataway Township, NJ) in pcDNA3.1 plasmid.

Cell Culture, Infection or Transfection, and Hypoxia/Re-oxygenation

AC-16 cells are human ventricular cells that have been fused with SV40 transformed fibroblasts.(4) Autophagy flux was measured in the AC16 cells(1) using an autophagy reporter construct described in detail previously.(2,3) Wild type BAG3 was inserted into a plasmid under a CMV promoter and each of the SNVs of interest were then generated. The p.160Aladup variant was synthesized by GenScript (Piscataway Township, NJ). Transformed human ventricular cells (AC16)(4) were cultured at 37°C with 5% CO_2 in Dulbecco's modified eagle medium with 5% FBS until 60-70% confluent, The cells were then transfected with plasmids containing c.null, c.BAG3, c.187C>G+c.1138C>T (p.P63Ala+P380S), c.249C>A,(p.H83Q) c.474_476dupGGC,(p.A160dup) or c.1436C>T (p.A479V) and co-transfected with the autophagy reporter construct adenovirus-red fluorescent protein-green fluorescent protein-LC3 (Adv-RFP-GFP-LC3). Each transfection was carried out for 36 hours after which cells were subjected to 4 hours of hypoxia (5% CO_2/1% N_2O_2) followed by 2 hours of re-oxygenation or normoxia (5% CO_2:21% 0_2). The cells were imaged with confocal microscopy and the number of LC3 puncta were counted as we previously described.(10) Expression levels of each plasmid were assessed by western blot and were equivalent.

Cell Harvest and Protein Extraction

In a separate group of experiments protein was collected from the AC16 cells for determination of expression levels. Two days post-infection or transfection cells were washed in 1X PBS and lysed in lysis buffer supplemented with mammalian protease inhibitor cocktail. Suspension was collected in Eppendorf tubes and in order to reach complete lysis, cells were vortexed every 2 minutes for 10 minutes, remaining on ice in between. After vortexing, samples were centrifuged at 13,000g for 5 min. The supernatant was collected and used for protein analysis. Protein concentration was determined by colorimetric Bradford assay from Bio-Rad according to manufacturer's protocol and samples with equal amount of protein were used for analysis by Western Blot.

Measurement of Autophagic Flux

Autophagy flux was measured in AC16 cells, primary cells from human ventricular tissue that were fused with transformed human fibroblasts,(1) using the autophagy reporter construct adenovirus-red fluorescent protein-green fluorescent protein-microtubule-associated protein 1A/1B light chain 3 (Adv-RFP-GFP-LC3) method as described in detail previously.(2,3) Transformed human ventricular cells (AC16)(4) were cultured at 37°C with 5% CO_2 in Dulbecco's modified eagle medium with 5% FBS until 60-70% confluent, The cells were then transfected with the autophagy reporter construct adenovirus-red fluorescent protein-green fluorescent protein-LC3 (Adv-RFP-GFP-LC3). (LC3-microtubule-associated protein 1A/1B-light chain 3). The next day, cells were plated on Thermo Scientific™ Nunc™ Lab-Tek™ Chambered Coverglass and transfected with indicated BAG3 expressing plasmid along with the vector control using Lipofectamine 3000 reagent. Post 36 hrs of transfection, cells were placed in hypoxia chamber for 4 hrs and then 2hrs reoxygenation. The confocal images were obtained at 488 and 561 nm excitation using a 100×oil objective (510 Meta; Carl Zeiss, Inc.). Images were analyzed and quantified using ZEN 2010 software. Autophagic flux was determined by evaluating the punctuated pattern of LC3 as described earlier (2, 3).

Measurement of Cell death using Annexin V/PI staining

In a second group of experiments apoptosis was assessed using Annexin V/Propidium Iodide staining as we have described previously.(5,6) AC-16 cells were plated on Thermo Scientific™ Nunc™ Lab-Tek™ Chambered Coverglass and transfected with

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indicated BAG3 expressing plasmid along with the vector control using Lipofectamine 3000 reagent. Post 36 hrs of transfection, cells were placed in hypoxia chamber for 12 hrs and then 2hrs reoxygenation. Cells were stained with Alexa Fluor 488 Annexin V/Propidium iodide solution for 30 min. Cells were washed with DPBS, and confocal images were obtained at 488 and 561 nm excitation using a 40×oil objective (510 Meta; Carl Zeiss, Inc.). Images were analyzed and quantified using ZEN 2010 software (4). At least 20 cells per field and 10 fields per condition were counted. The investigator was blinded to the experimental group.

Assessment of LV Function by Echocardiography

To confirm the pathogenicity of the p.P63A+P380S variant in vivo, we generated an adeno-associated virus serotype nine-BAG3$^{p.P63A+o,P380S}$ (AAV9-BAG3$^{p.P63A+p.P380S}$) that was driven by the CMV promoter. In an initial experiment, we injected $1x10^{12}$ particles of the AAV9-BAG3 (wild-type) into the retro-orbital plexus of 9-10 week-old cBAG3$^{+/-}$ mice.(7) After documenting our ability to restore normal of near-normal LV function in the mice, we then injected AAV9-BAG3$^{P63A+P380S}$ into the extra-orbital plexus as described previously.(8) Mice were followed echocardiographically as described previously.(3,9)

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Primer Name	Primer Sequence 5'-3'	Primer Name	Primer Sequence 5'-3'	Base Pairs
BAG3 Exon 1-F	CCGGGAACACTCACTCGG	BAG3 Exon 1-R	CACTCCCCGCCGCCT	831
BAG3 Exon 2-F	CAATGCCAAGCGCCACAG	BAG3 Exon 2-R	TGCTGCAGGCTAGACCCA	550
BAG3 Exon 3-F	GAGGAGGTGCACAGCAGAA	BAG3 Exon 3-R	CTCCTGCACCCCTGGAGA	551
BAG3 Exon 4-F	GGGGTGATCAATGGAAGCCT	BAG3 Exon 4-R	AGTGTTTTGCCTCCACCCA	1179

eTable 1. Primers used for BAG3 exon sequencing

Variant	Forward Primer 5'-3'	Reverse Primer 5'-3'
Pro63Ala	ATTGGCAGAGGATGCAGTCTCCTTGGTTA	GCCCCAAGGAGACTGCATCCTCTGCCAAT
His83Gln	GGGGTACACAGGTTGGCCTTCCCTAGC	GCTAGGGAAGGCCAACCTGTGTACCCC
Pro380Ser	GGACAGCAGAAGAGCCAGGGCTGGG	CCCAGCCCTGGCTCTTCTGCTGTCC
Ala479Val	CACCGTCTCTCCTGACCTGACGCACATCG	CGATGTGCGTCAGGTCAGGAGAGACGGTG

eTable 2. Primers used for generation of BAG3 variant plasmids

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eTable 3. Sequencing results

Exon 1:

Nucleotide	Amino Acid	rs Number	Type	Allele Frequency (AA)	Allele Frequency (EA)
c.25A>G	p.Met9Val	rs137965903	NS	0.005463	0.000

Exon 2:

Nucleotide	Amino Acid	rs Number	Type	Allele Frequency (AA)	Allele Frequency (EA)
c.187C>G	p.Pro63Ala	rs644041999	NS	0.01904	0.00007117
c.212G>A	p.Arg71Gln	rs35434411	NS	0.004	0.031
c.231G>A	p.Pro77Pro	rs143752613	S	0.0152	0.000031
c.249C>A	p.His83Gln	rs151331972	NS	0.0104	0.000039
c.386G>A	p.Arg123Gln	rs199991063	NS	0.000	0.00006268
c.451T>C	p.Cys151Arg	rs2234962	NS	0.03632	0.2163
c.474_476dup GGC	p.Ala160dup	rs139438727	II	0.02684	0.000
c.498C>T	p.His166His	rs111682654	S	0.0009147	0.0000147

Exon 3:

Nucleotide	Amino Acid	rs Number	Type	Allele Frequency (AA)	Allele Frequency (EA)
c.605C>T	p.Pro202Leu	rs556000090	NS	0.0001979	0.000
c.606G>T	p.Pro202Pro	rs74157690	S	0.01419	0.00003251
c.870C>G	p.Pro290Pro	rs74157690	S	0.002040	0.00001582

Exon 4:

Nucleotide	Amino Acid	rs Number	Type	Allele Frequency (AA)	Allele Frequency (EA)
c.1002T>G	p.Pro334Pro	rs3858339	S	0.1666	0.08729
c.1138C>T	p.Pro380Ser	rs144692954	NS	0.01091	0.00006314
c.1220C>T	p.Pro407Leu	rs3858340	NS	0.1655	0.08714
c.1296A>G	p.Val432Val	rs196295	S	0.7531	0.7836
c.1436C>T	p.Ala479Val	rs34656239	NS	0.003286	0.000
c.1503C>A	p.Val501Val	rs147277075	S	0.003121	0.00001578
c.1587C>T	p.Ala529Ala	rs149358702	S	0.005414	0.00001579

eTable 3: Variants found within the coding regions of the BAG3 gene. NS – Non-synonymous; S – Synonymous; II – In frame insertion, AA – African ancestry, EA – European ancestry

eTable 4. Patient Demographics

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Parameter	BAG3 SNV (N=51)	No BAG3 SNV (N=458)	p-Value
Age	55.6 ± 13.4	54.5 ± 13.6	0.578
Sex (% Male)	58.8	61.1	0.748
Heart Rate	72.4 ± 12.9	75.5 ± 13.3	0.119
Systolic BP	126.6 ± 15.3	124.1 ± 18.5	0.348
Diastolic BP	76.8 ± 10.9	76.7 ± 11.2	0.930
EF at Baseline	30.6 ± 9.5	30.4 ± 10.2	0.918
Change in EF at 6 Months	4.6 ± 9.8	4.3 ± 10.2	0.851
LVEDD at Baseline	6.3 ± 1.3	6.4 ± 1.2	0.594
Change in LVEDD at 6 Months	-0.2 ± 1.1	-0.2 ± 1.0	0.897
NYHA III or IV (%)	80.4	80.3	0.983
ICD (%)	35.7	34.0	0.896
Diabetes (%)	24.4	37.5	0.083
Medications:			
Beta Blockers (%)	88.2	82.3	0.287
ACE Inhibitors or ARBs (%)	98.0	92.8	0.234
Aldosterone Antagonists (%)	33.3	35.4	0.773
Hydralazine and Isosorbide Dinitrate (%)	54.1	44.9	0.292

eTable 4: There were no significant demographic differences between patients carrying a BAG3 variant and those not carrying a BAG3 variant.

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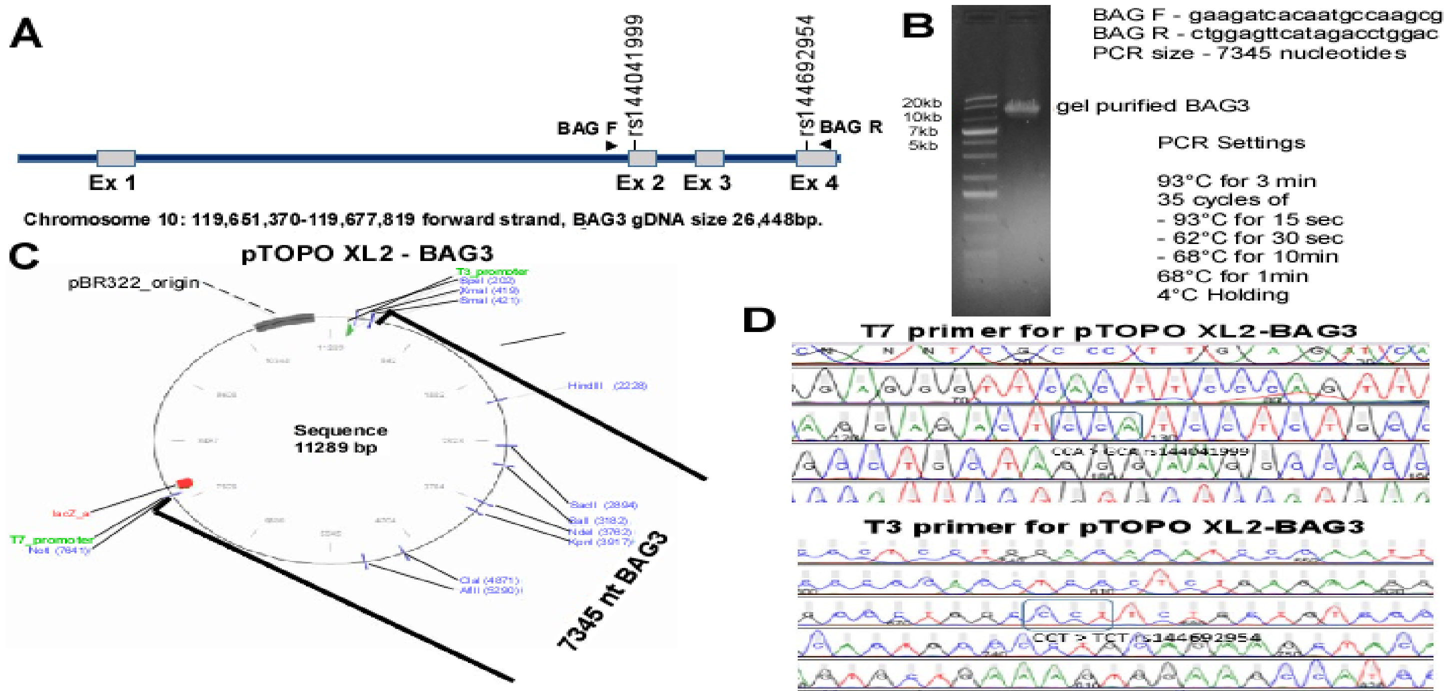

eFigure 1: Methods used to determine configuration of P63A and P380S SNPs. **A**) Representation of primer recognition sites for PCR of BAG3 **B**) Image of gel purified BAG3 and PCR parameters to get 7345 nucleotide product between primers shown in previous panel **C**) Construction of plasmids containing BAG3 PCR product **D**) Primers used to sequence plasmids to determine cis conformation of the two simultaneously occurring SNPs.

eFigure 1: Determination of linked variants

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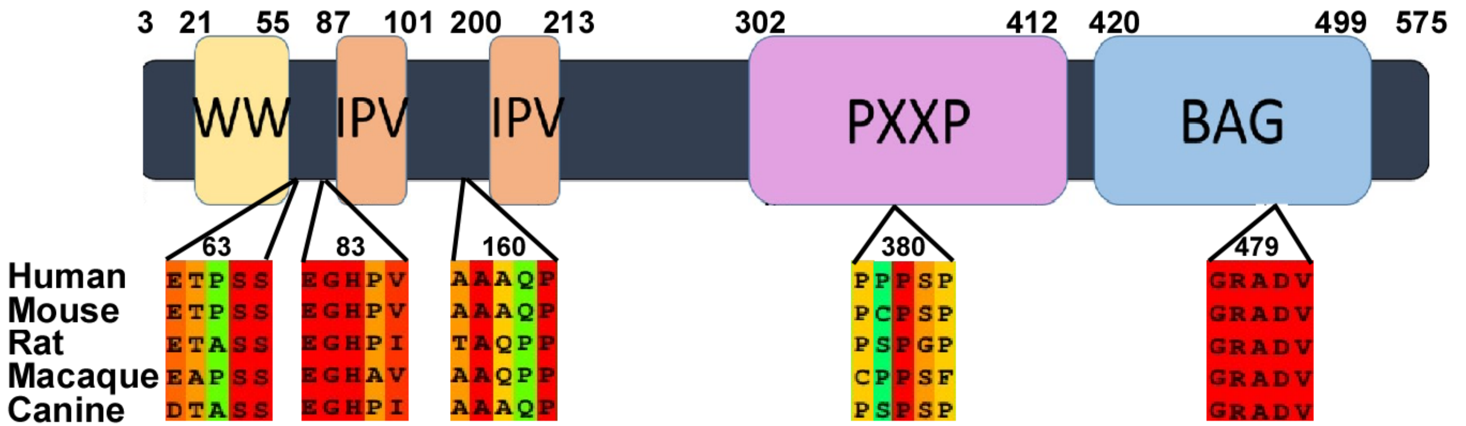
eFigure 2:Conservation of BAG3 variant sites



eFigure 2: Diagram of BAG3 showing protein binding domains and sites of single non-synonymous variants, the single 3 nucleotide insertion and the double heterozygote non-synonymous variants. Blocks indicate conservation of amino acids across multiple mammalian species. The WW domain interacts with the PXXP region to modify the 3-dimensional structure of BAG3 and is also involved in chaperone-assisted selective autophagy (CASA); IPV motifs interact with small heat shock proteins HspB8 and HspB6 to support macroautophagy; the PXXP region facilitates binding to Src homology 3 (SH3) domain to promote migration and metastasis of cancer cells and also binds dynein complex to facilitate intracellular protein transport to perinuclear aggresomes; and the BAG domain interacts with Bcl-2 resulting in inhibition of apoptosis.

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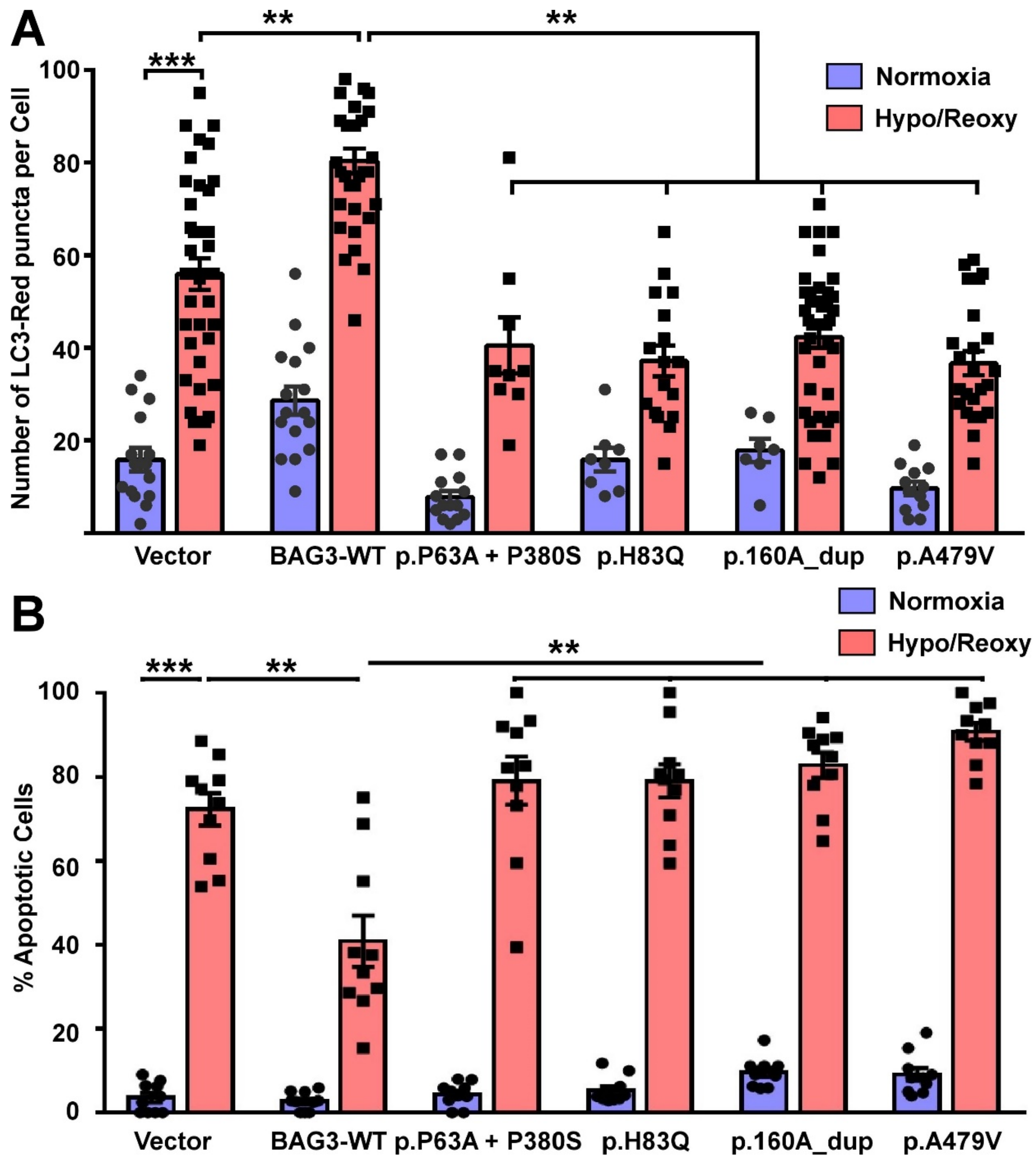
eFigure 3: Quantification of autophagy and apoptotic cell death in AC16 cells with BAG3 variant expression



eFigure 3: Quantification of LC3 puncta per cell or percent apoptotic cells per field. Representative images shown in figure 3. eFigure 3A shows quantification of images from between 8 and 42 cells per condition (each point represents one cell). eFigure 3B is the quantification of images presented as the percentaged of green and/or red cells per field.

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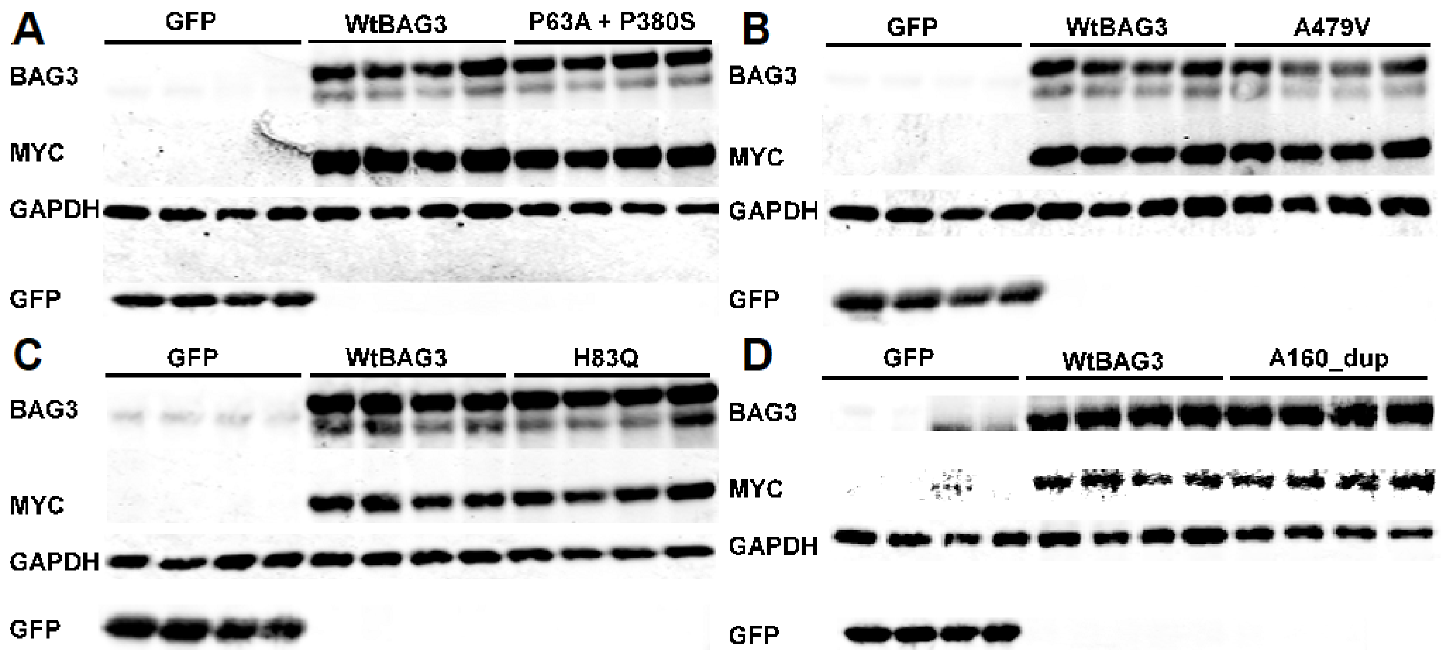
eFigure 4: Plasmid expression in AC16 cells



eFigure 4: Expression of variant plasmid and wild-type BAG3 plasmid in AC16 cells was equivalent as shown by Myc expression levels, assessed using western blotting. AC16 cells were transfected using Lipofectamine 3000 for 48 hours with GFP plasmid, wild-type BAG3 plasmid or A) P63A + P380S plasmid, B) A479V plasmid, C) H83Q plasmid or D) A160_dup plasmid.

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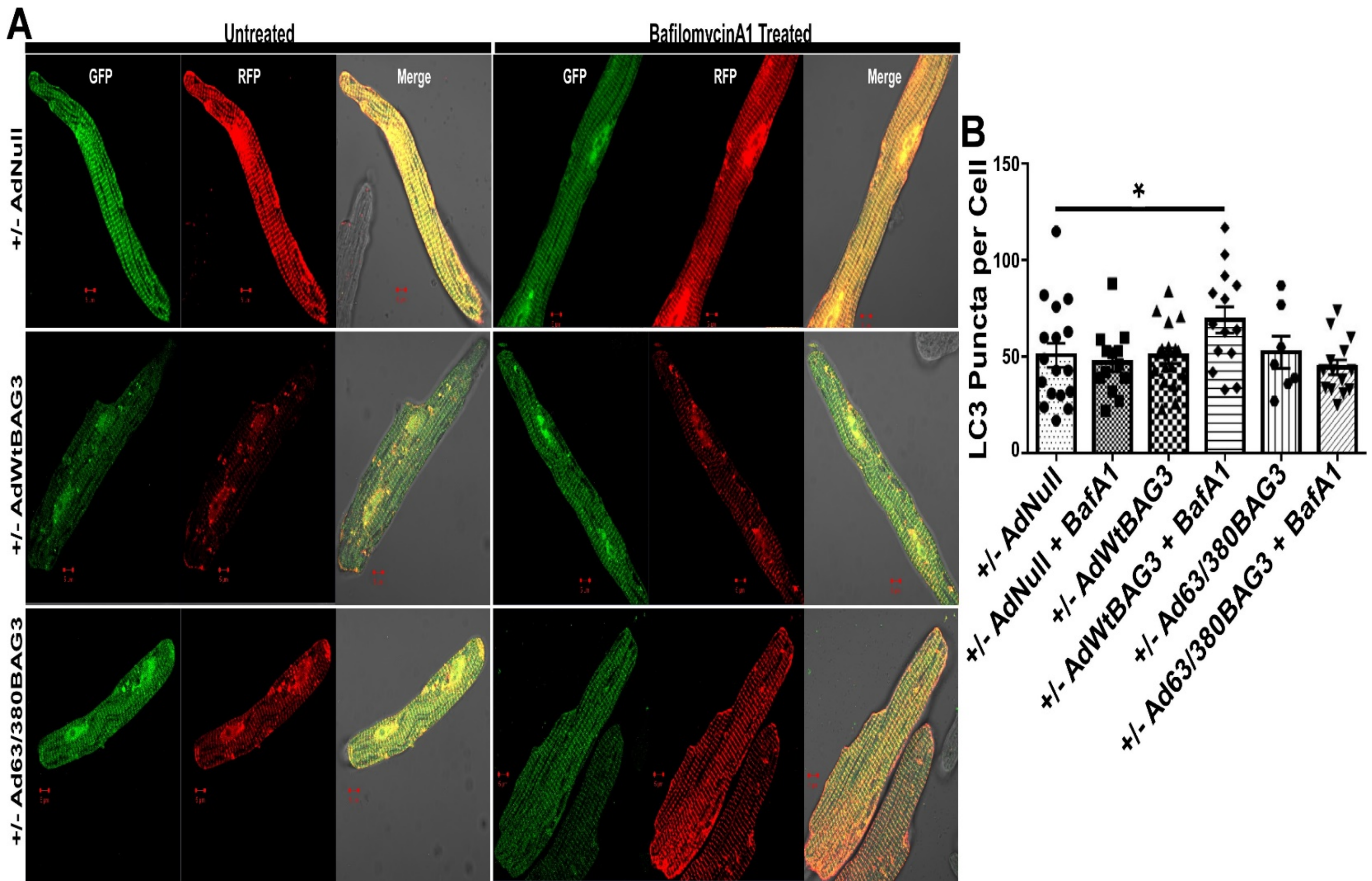
eFigure 5: Autophagy in adult ventricular cardiomyocytes expressing P63A + P380S BAG3 expression



eFigure 5: Adult cardiomyocytes isolated from BAG3 haplo-insufficient mice (+/-) infected with the LC3 reporter construct and either AdNull, AdWtBAG3 or AdP63A + P380SBAG3 for 48 hours prior to treatment with bafilomycinA1. eFigure 5A shows representative confocal live cell images. eFigure 5B shows quantification of LC3 puncta (total) present with and without BafilomycinA1 treatment with expression of either AdNull, AdWtBAG3 or AdP63ABAG3. *P<0.0001

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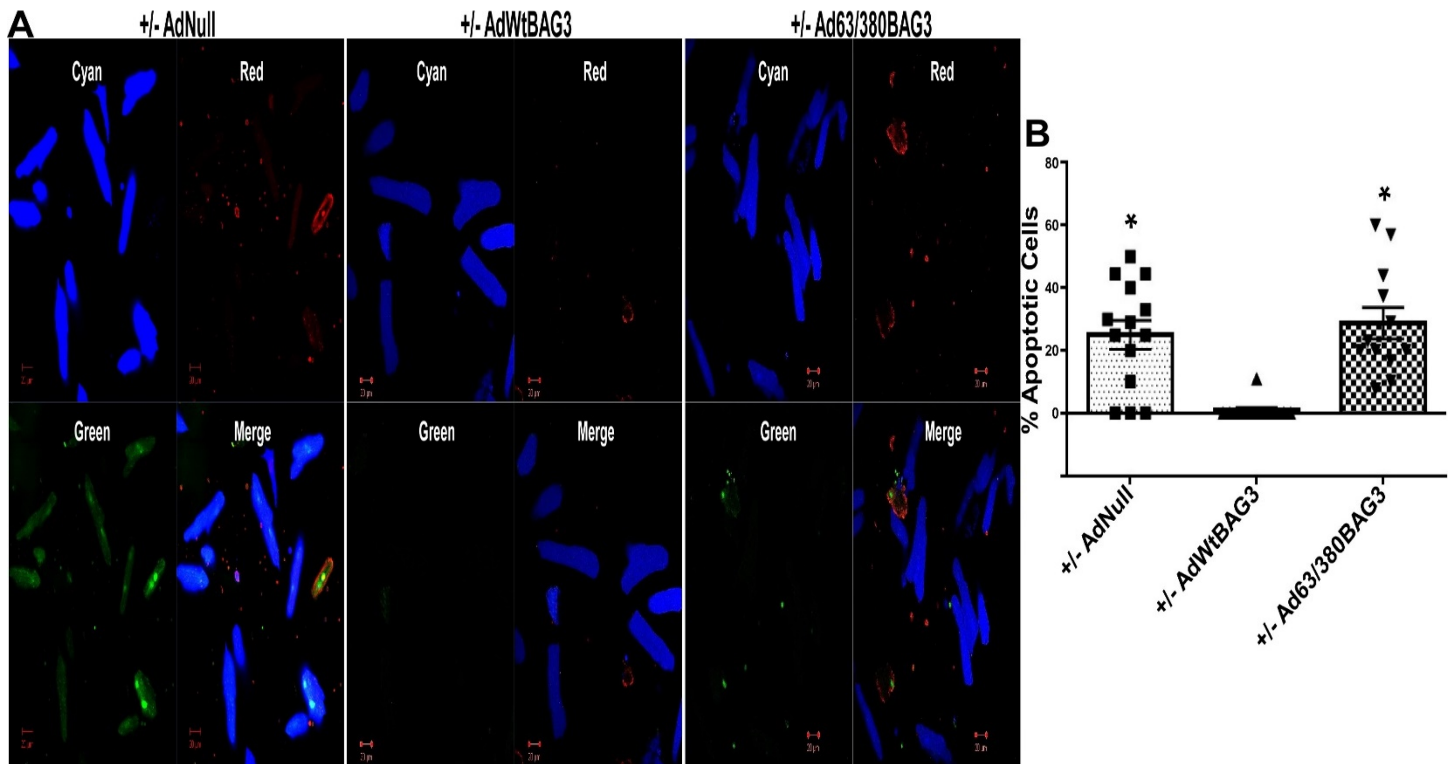
eFigure 6: Apoptotic cell death in adult ventricular cardiomyocytes expressing P63A + P380S BAG3 expression



eFigure 6: Adult cardiomyocytes isolated from BAG3 haplo-insufficient mice (+/-) infected with either AdNull, AdWtBAG3 or AdP63A + P380SBAG3 for 48 hours prior to staining. eFigure 6A shows representative confocal images of adult cardiomyocytes stained for AnnexinV (Red), nuclear permeability (Green) or DNA (cyan) after infection with either AdNull, AdWtBAG3 or AdP63ABAG3. eFigure 6B shows quantification of percent apoptotic cells per field. *P<0.0001 By contrast with AC16 cells, we did not expose the adult myocytes to hypoxia/re-oxygenation because the extent of apoptosis was such that there were too few lives cells to count.

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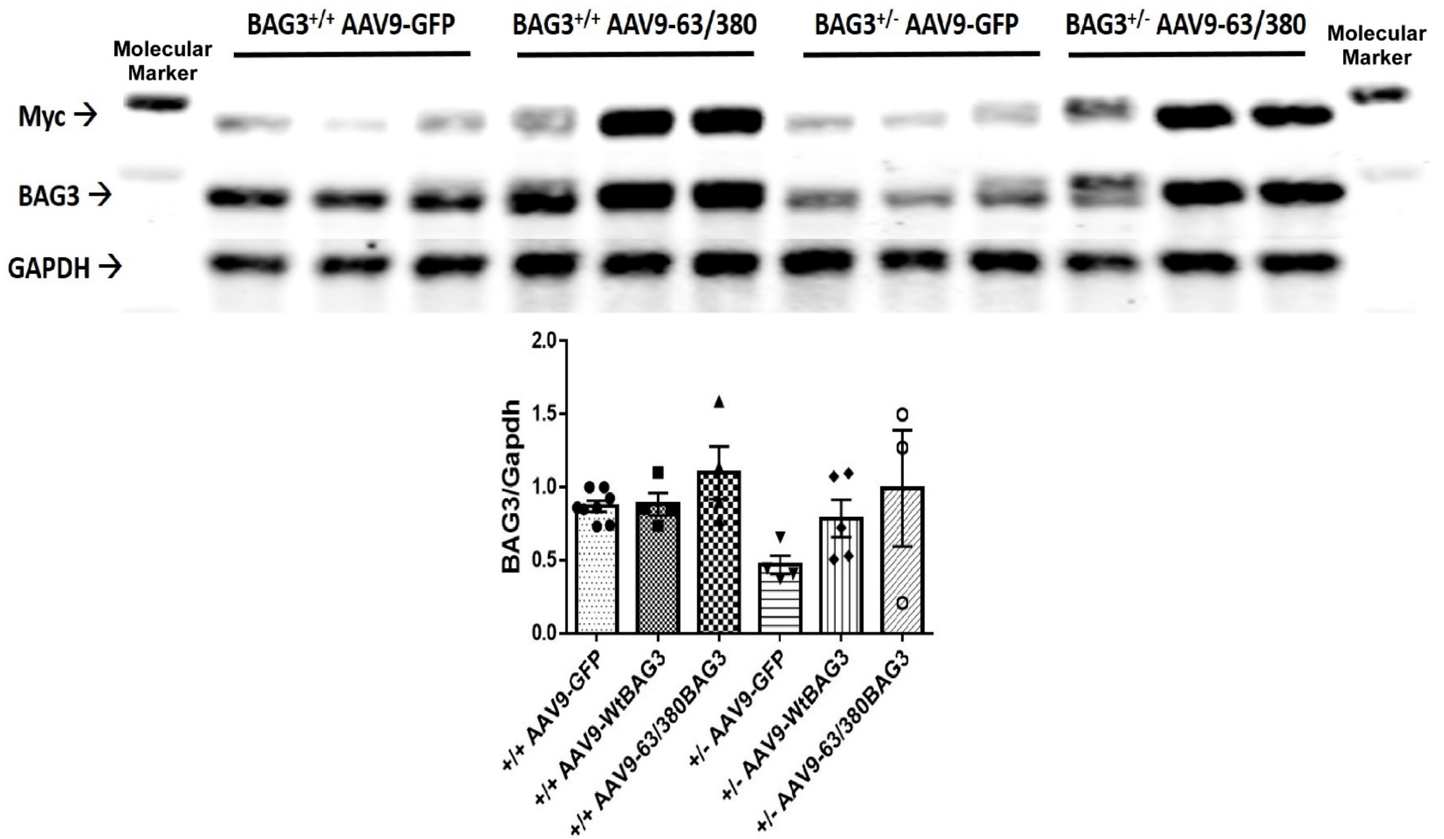
eFigure 7: BAG3 protein levels in BAG3$^{+/+}$ and cBAG3$^{+/-}$ mice transduced with AAV9-GFP or AAV9-BAG3$^{p.P63A+P380S}$



eFigure 7: BAG3 protein levels in mice transduced with rAAV9-GFP, rAAV9-BAG3$^{Wild\ Type}$ or rAAV9-BAG3$^{p.P63A+P380S}$. eFigure 7A is a representative blot of myc-BAG3 and GAPDH in left ventricular myocardium from mice presented in figure 4. eFigure 7B shows quantification of BAG3 levels in the cohort of mice whose functional data is shown in figure 4. cBAG3$^{+/+}$: rAAV9-GFP, n=8; rAAV9-BAG3$^{Wild\ Type}$, n=4; rAAV9-BAG3$^{p.P63A+P380S}$, n=4. cBAG3$^{+/-}$: rAAV9-GFP, n=4; rAAV9-BAG3$^{Wild\ Type}$, n=5; rAAV9-BAG3$^{p.P63A+P380S}$, n=3. The levels of BAG3 in the cBAG3$^{+/-}$ mice are approximately half of the level seen in cBAG3$^{+/+}$ mice, consistent with haplo-insufficiency. Furthermore, the levels of BAG3 in the cBAG3$^{+/+}$ and cBAG3$^{+/-}$ mice injected with rAAV9-BAG3$^{Wild\ Type}$ appeared to be comparable; however, the small size of the cohort precluded statistical comparisons.

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