ANIMAL MODELS

BAG3^{P215L/KO} Mice as a Model of BAG3^{P209L} Myofibrillar Myopathy

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BCL-2-associated athanogene 3 (BAG3) is a co-chaperone to heat shock proteins important in degrading misfolded proteins through chaperone-assisted selective autophagy. The recurrent dominant BAG3-P209L mutation results in a severe childhood-onset myofibrillar myopathy (MFM) associated with progressive muscle weakness, cardiomyopathy, and respiratory failure. Because a homozygous knock-in (KI) strain for the mP215L mutation homologous to the human P209L mutation did not have a gross phenotype, compound heterozygote knockout (KO) and KI mP215L mice were generated to establish whether further reduction in BAG3 expression would lead to a phenotype. The KI/KO mice have a significant decrease in voluntary movement compared with wild-type and KI/KI mice in the open field starting at 7 months. The KI/KI and KI/KO mice both have significantly smaller muscle fiber cross-sectional area. However, only the KI/KO mice have clear skeletal muscle histologic changes in MFM. As in patient muscle, there are increased levels of BAG3-interacting proteins, such as p62, heat shock protein B8, and α B-crystallin. The KI/KO mP215L strain is the first murine model of BAG3 myopathy that resembles the human skeletal muscle pathologic features. The results support the hypothesis that the pathologic development of MFM requires a significant decrease in BAG3 protein level and not only a gain of function caused by the dominant missense mutation. (*Am J Pathol* 2020, 190: 554–562; <https://doi.org/10.1016/j.ajpath.2019.11.005>)

Muscular dystrophy is a heterogeneous group of diseases characterized by progressive muscle weakness. Myofibrillar myopathies (MFMs) are a subset of muscular dystrophy that share pathologic features that may also present with cardiac muscle involvement and neuropathies. Symptoms and age at onset vary among the different MFMs, although most forms affect adults.¹ Histologically and structurally, they all share similar features: Z-disk streaming and disruption, aggregation of Z-disk-associated proteins, abnormal fiber size,¹ and mitochondrial abnormalities.^{2,3} In MFM, the broken-down filaments of the myofibers aggregate with membranous organelles and ectopic proteins.^{1,4} Mutations in the genes *DES*, *MYOT*, *FLNC*, *LDB3*, *FHL1*, *CRYAB*, and *BAG3* cause MFMs and their proteins to localize to the Z-disk.⁵

BCL-2-associated athanogene 3 (BAG3) is one of six BAG proteins in humans. The BAG proteins share the BAG domain that binds to the ATPase domain of heat shock protein (HSP) 70 and as such are considered to be co-chaperones to HSPs and pro-survival in nature.⁶ BAG3 in particular is important to muscle and its maintenance because of its role in chaperone-assisted selective autophagy (CASA), an ubiquitin-dependent system operating at the Z-disk that leads to the autophagic degradation of damaged cytoskeletal proteins.⁷ It fulfills this role through the aforementioned association with HSP70, as well as HSPB8 and HSPB6 at BAG3's

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Ile-Pro-Val motifs. BAG3 also plays a role in the regulation of the stability of actin in conjunction with heat shock cognate protein 70 through interaction with the actin capping protein CapZ (a barbed-end actin capping *protein* from the Z line of skeletal muscle).⁸ Furthermore, it also plays an antiapoptotic role because of its association with B-cell lymphoma 2, an antiapoptotic protein localized to the mitochondria.⁹

The recurrent proline to leucine mutation in one of the Ile-Pro-Val motifs, P209L, results in a severe, childhood, autosomal-dominant MF. Morphologically similar to other MFs, these motifs display Z-disk streaming, aggregates of Z-disk proteins, abnormal fiber size, ragged red fibers (RRFs), abnormal mitochondrial morphologic features, and regenerating and splitting fibers.¹⁰ Clinically, affected patients begin experiencing symptoms in childhood, such as toe walking, that progress into more generalized muscle weakness, with spinal rigidity and a peripheral neuropathy in many patients. Respiratory insufficiency and cardiomyopathy usually develop by the second decade of life, often necessitating heart transplant and eventually leading to death.^{10,11} Because of the severity of the disease, the P209L mutation is usually *de novo*, with only one published case of it being inherited from a mosaic father.¹¹ This finding makes this disease extremely rare, with only 19 published cases.^{10–18}

One of the initial hypotheses concerning the mechanism of disease is that mutated BAG3 protein impairs autophagy,¹⁹ resulting in aggregates of misfolded proteins. This theory, however, has been disputed. Experiments in a zebrafish model found that suppression of autophagy is not enough to induce the formation of aggregates. Instead, only the expression of mutated BAG3 protein produces the aggregates, which also sequesters the wild-type (WT) protein, other Z-disk proteins, and autophagy-associated proteins. These results suggest that there is a toxic gain of function that leads to BAG3 insufficiency because the protein is now bound within aggregates and therefore unavailable to function.²⁰ Indeed, the mutated protein stalled the HSP70 autophagy machinery because its activity as a nucleotide exchange factor is impaired. This finding leads to the formation of insoluble aggregates, which are composed of both the HSP70 system and other autophagic systems that accept the same clients,²¹ preventing degradation and leading to their accumulation.^{21,22}

Mice were generated with three different genotypes on C57Bl/6 background, carrying one [knock-in (KI)/ WT] or two copies of the P215L mutation analogous to the human P209L mutation (KI/KI), as well as mice with one copy of P215L and a knockout (KO) allele (KI/KO). A full characterization of heterozygote (KO/WT) mice was not completed because two other groups, both also using C57Bl/6 mice, independently reported that they did not develop a phenotype.^{23,24} Extensive phenotypic, histologic, and molecular characterization of the KI/WT, KI/KI, and KI/KO mice was performed. KI/KO crosses were of the greatest interest because the KI/KI and KI/WT had little or

no phenotype.²⁵ Further supporting the relevance of this cross are the results in zebrafish, which indicate that a reduction of protein quantity in addition to the P215L mutation may result in a muscle phenotype that is more severe than KI alone^{20,26} because the P209L mutation results in a lack of available and active BAG3.²¹ In addition, a reduction in BAG3 levels has been noted in patients.^{14,27} We hypothesized that reducing the protein quantity through a KI/KO cross would overcome the weak phenotype seen in KI/WT and KI/KI crosses in a manner that still reflected this dominant MF on a molecular level. Indeed, KI/KO mice develop myopathic changes that are not present in the other strains. The BAG3-P219L KI/KO mice are the first model of P209L-BAG3 MF with full-body expression of the mutant protein.

Materials and Methods

Generation of BAG3 P215L and KO Mice

Mice were generated by the Toronto Center for Phenogenomics using CRISPR-Cas9 on a C57Bl/6 background. The KI mice contain the change c.644_645CC>TG, which results in the P215L mutation. The KO mice have a 7-bp deletion, c.639_645delCATCCCC, causing a premature stop codon. The sequences were confirmed via Sanger sequencing using the primers 5'-GGTGTCTGGTGGCTATTTGG-3' (forward) and 5'-ATCTGCAGAACCTCTCAGCC-3' (reverse) (Supplemental Figure S1). The absence of protein was confirmed in the KO homozygote mice via Western blot using a polyclonal BAG3 antibody (LP11).²²

Western Blots

Mouse gastrocnemius muscles were dissected and snap frozen in liquid nitrogen. Muscles were finely ground using a mortar and pestle, suspended in radioimmunoprecipitation assay buffer, and then sonicated. The resulting extract was spun at 10,000 × g for 5 minutes at 4°C and the supernatant collected. Protein quantification was performed using the DC colorimetric assay (Bio-Rad, Hercules, CA). Aliquots of 5, 20, and 40 µg were separated onto a NuPAGE 4% to 12% Bis-Tris gel (Life Technologies, Carlsbad, CA) and transferred onto a nitrocellulose membrane (Bio-Rad). The resulting blots were incubated with the following primary antibodies: BAG3 (LP11),²² p62 (catalog number 610832; BD Biosciences, San Jose, CA), HSPB8 (catalog number 3C12-H11; StressMarq, Victoria, BC), αB-crystallin (catalog number ADI-SPA-222; Enzo); HSPB1 (catalog number 5D12-A12; StressMarq), glyceraldehyde-3-phosphate dehydrogenase (catalog number GT239; GeneTex, Irvine, CA), HSP70 (clone C92F3A-5; Enzo Life Sciences, Farmingdale, NY), sarcomeric α-actinin (clone EA-53; Abcam, Cambridge, MA). This was followed by incubating membranes with a horseradish peroxidase secondary antibody and visualized using chemiluminescence (Intas Science Imaging, Göttingen,

Germany). Bands were quantified using ImageJ software version 1.52 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij/>), and levels were normalized to glyceraldehyde-3-phosphate dehydrogenase and sarcomeric α -actinin.

Histologic Analysis

Cryostat sections of 10 μ m were fixed with 4% paraformaldehyde for 10 minutes at room temperature and stained using the following stains: hematoxylin and eosin (H&E), Gomori trichrome, NADH, and immunohistochemical staining. For immunostaining, antigens were initially demasked in serial sections using target retrieval solution (catalog number S1699; Dako Agilent, Santa Clara, CA) at 95°C for 45 minutes. Peroxidases were inactivated using 0.6% hydrogen peroxide for 15 minutes, followed by avidin and biotin blocking (catalog number SP-2001; Vector Laboratories, Burlingame CA) for 15 minutes. Blocking and permeabilization were performed with a -buffered saline solution that contained 5% goat serum and 0.25% Triton X-100 for 1.5 hours. Primary antibodies were incubated overnight at 4°C. Biotinylated secondary antibodies (Vector Laboratories) were incubated for 1 hour. Complex A + B (catalog number PK-6100; Vector Laboratories) was incubated for 1 hour, followed by diaminobenzidine solution (catalog number SK-4100; Vector Laboratories) for 5 minutes. Nuclei were stained briefly using hematoxylin and then mounted with xylene mounting medium (catalog number 245-691; Protocol, Waltham MA). Primary antibodies consisted of the following: BAG3 (LP11),²² desmin (catalog number M0760; Dako), and filamin C (catalog number NBP1-89300; Novus Biologicals, Centennial CO).

Counting of the RRFs, defined as fibers that have an irregularly thick or ragged contour, was performed using the Gomori trichrome-stained whole gastrocnemius sections. This counting was performed by an individual (R.R.) blind to the genotype associated with each section. The number of RRFs was quantified as a percentage of total fiber number.

Immunofluorescence

Cryostat cross-sections of 10 μ m were fixed with 4% paraformaldehyde for 10 minutes at room temperature. They were blocked and permeabilized for 1 hour in a -buffered saline solution that contained 5% normal donkey serum, 0.25% Triton X-100, and 0.1 mol/L glycine. Cells were incubated overnight at 4°C with anti-CD45 primary antibody. The secondary antibody, mixed with 488 Alexa Fluor wheat germ agglutinin, was applied for 1 hour at room temperature, followed by Hoescht staining for 5 minutes. Slides were mounted with ProLong™ Gold antifade reagent (Thermo Fisher, Waltham, MA). Fiber-type staining immunolabelling was performed as previously described²⁸ with the following primary antibody cocktail: anti-major histocompatibility complex (MHC) type I (BA-F8, 1:25), mouse IgG1 monoclonal anti-MHC type IIa (SC-71, 1:25), mouse IgM monoclonal anti-type 2 $\times$ MHC (6H1, 1:25), and a rabbit IgG polyclonal anti-laminin (catalog number L9393; Sigma-Aldrich, St. Louis, MO). The MHC monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, created by the Eunice Kennedy Shriver National Institute of Child Health and Human Development of the NIH and maintained at Department of Biology, The University of Iowa (Iowa City, IA; developed by Stefano Schiaffino).

Figure 1 Clinical phenotyping of wild-type (WT), knock-in (KI)/knockout (KO), KI/WT, and KI/KI strains. **A:** Measures of weight, strength, and endurance, which included rotarod, grip strength, and inverted grid, show no difference. **B:** Open field measures show significant difference between WT and KI/KO mice starting at 7 months of age. Data are expressed as means $\pm$ SEM. $n = 14$ WT; $n = 15$ KI/KO; $n = 15$ KI/WT; $n = 14$ KI/KI strains. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT (analysis of variance).

Electron Microscopy

Male mice at 7 months of age, three per genotype used, were perfused with phosphate-buffered saline followed by 4% glutaraldehyde in 0.2 mol/L cacodylate buffer. The gastrocnemius muscle was dissected out, cut into 1×2 -mm pieces, and left in the same glutaraldehyde mixture for 24 hours. Sections were postfixed in osmium tetroxide for 1 hour and embedded in epoxy resin. Sections were stained using uranyl acetate and lead citrate and then imaged using a FEI Tecnai G² Spirit Twin 120 kV Cryo-TEM at the Facility of Electron Microscopy Research at McGill University.

Behavioral Phenotyping

Motor behavior in mice was tested using rotarod, force transducers for grip strength, inverted grid, and open field. Fifteen male mice per genotype were used. Balance and

coordination were tested using the rotarod. Mice were subjected to 3 days of training, gradually increasing the maximum Rpm through the training sessions and returning mice to the rotarod after falling unless exhaustion had been reached. The actual testing was composed of five sessions, with the speed increasing from 5 to 40 Rpm during that time. The time at which the mice fell from the rod was recorded, and adequate rest was given between rounds. For inverted grid, mice were placed on the grid, which was then inverted over a padded surface for a maximum of 5 minutes. The time at which the mice fell was recorded. Grip strength testing was performed using forelimbs only, with the peak force at the point of failure recorded for five trials. All values for rotarod, grip strength, and inverted grid were normalized to body weight, and these tests were repeated at 3, 6, and 9 months of age. For testing of spontaneous locomotion in open field, mice were allowed to acclimate to the testing room 30 minutes before testing while still in their

Figure 2 Histologic analysis of gastrocnemius muscles of 4-month-old mice. Hematoxylin and eosin staining of wild-type (WT) (A), knock-in (KI)/WT (B), KI/KI (C), and KI/knock-out (KO) (D) animals showing centralized nuclei and splitting (arrow in D) and angular fibers (stars) in KI/KO mice. Gomori trichrome findings in KI/KO mice, including aggregates (E and F; asterisk and arrow) and ragged red fibers (G). H: NADH of KI/KO mice showing moth-eaten fibers. I–L: Immunohistochemistry showing aggregates of filamin C (FLNC), desmin, and BCL-2-associated athanogene 3 (BAG3) in KI/KO (asterisks); CD45 and wheat germ agglutinin (WGA) immunofluorescent labeling in WT (M) and KI/KO (N) animals, showing inflammation in KI/KO mice. Scale bars = 50 μ m (A–D). Original magnification: $\times 20$ (A–L); $\times 10$ (M and N).

home cages. They were then placed in a 50 × 50-cm field in a quiet, low-light room for 10 minutes and recorded using a ceiling-mounted camera and HVS Image Software version 2016.6 (HVS Image Software Ltd., Buckingham, UK). Testing was repeated at 5, 7, and 10 months of age. Individuals performing the testing were blind to genotype throughout the trials (R.R., M.-J.D.).

Statistical Analysis

Analyses that consisted of multiple genotypes were performed using two-way analysis of variance. If $P < 0.05$ was reached, post hoc testing (Dunnett multiple comparisons test) was performed to identify which group was different from WT.

Results

Reduction of Voluntary Movement in KI/KO Mice

KI/WT, KI/KI, and KI/KO mice were clinically assessed using rotarod, inverted grid, grip strength, and open field tests to assess their strength, endurance, and movement. Inverted grid, rotarod, and weighing were performed at 3, 6, and 9 months, and grip strength and open field were performed at 4, 7, and 10 months. No significant difference among KI/WT, KI/KI, and WT mice was seen for any measure (Figure 1A). The KI/KO mice did not have any coordination, endurance, or strength deficit as measured by rotarod, inverted grid, or grip strength tests up to 9 months of age, and there were no differences in weight. However, the mice did have a significant reduction in voluntary movement (Figure 1B). Open field measurements revealed a

significant decrease in movement, which became apparent at 7 months of age, moving a mean of 15.1 m compared with the 20.5 m by WT (total path and total entries $P < 0.05$) during a period of 10 minutes. This measurement decreased more at 10 months, moving 11.9 m compared with 17.85 m and moving 43% less of the time (total path $P < 0.01$, total entries and time moving $P < 0.001$). There was a decrease in overall movement and lower use of the field.

Myopathic Changes to the Muscle in P215L KI/KO Mice

The muscle of KI/KO mice has clear myopathic changes that are absent in KI/KI and KI/WT mice. A histologic examination of gastronomical muscle collected from 4-month-old male mice using H&E, Gomori trichrome, NADH, and immunohistochemical stains was conducted (Figure 2, A–C). H&E staining of cross-sections of the gastrocnemius of 4-month-old mice revealed centralized nuclei, splitting fibers, and angular fibers in areas near connective tissue in the KI/KO animals (Figure 2D). These abnormalities did not increase markedly with age up to 14 months (Supplemental Figure S2). The RRFs were visible on Gomori trichrome (Figure 2G) staining. The RRF count in KI/KO muscle is significantly increased (5.5%) compared with WT muscle (1.1%) ($P < 0.05$) (Supplemental Figure S3, A and B). Although the KI/KI mice also had a higher level of RRFs (2.09%) than the WT mice, this finding was not significant. NADH staining also found moth-eaten fibers (Figure 2H), indicating myofibrillar disruption and significantly higher disruption in KI/KO (0.54%) compared with WT (0.08%) mice ($P < 0.05$), with a nonsignificant increase in KI/KI mice (0.29%) (Supplemental Figure S3C). Aggregates were seen in immunohistochemical stainings of the Z-disk proteins BAG3,

Figure 3 Fiber cross-sectional area comparison of gastrocnemius among wild-type, knock-in (KI)/KI, and KI/knockout (KO) mice shows a significant reduction in the size of all fibers between wild-type and KI/KO mice and a reduction in the size of type 2 fibers in KI/KI mice. Data are expressed as frequency distribution (A) and means $\pm$ SD (B). ** $P < 0.01$, **** $P < 0.0001$ versus wildtype (analysis of variance). AU, arbitrary units.

desmin, and filamin C (Figure 2, I–L) as well as Gomori trichrome (Figure 2, E and F). This finding was largely absent in KI/KI mice (not shown). Within the KI/KO mice, there was a higher concentration of abnormal and aggregate-containing fibers near large areas of connective tissue, such as epineurium or myotendinous junctions, which are sites of high stress that have greater damage levels and repair after stretch injury.²⁹ The KI/KI and KI/WT muscle had little to no deviation from WT muscle and lacked the classic MFM pathologic findings of KI/KO muscle. The fiber cross-sectional area was reduced across all fiber types in KI/KO mice and in type 2A and 2B fibers in KI/KI mice (Figure 3 and Supplemental Figure S4). The reduction in KI/KO mice was by 19% in type 1, 22% in type 2B, and 25% in type 2A. In KI/KI mice, a 23% reduction in type 2B and 10% reduction in type 2A were observed. Otherwise, the KI/KI and KI/WT mice were histologically normal. In addition, H&E staining of muscle from 14-month-old KO/WT mice did not have abnormalities (Supplemental Figure S2) or a fiber cross-sectional area reduction in KO/WT and KI/WT mice (Supplemental Figure S5).

To investigate whether an inflammatory response accompanied these pathologic changes, immunofluorescent staining for CD45, a pan-leukocyte cell surface antigen, was performed (Figure 2, M and N). Inflammatory infiltrate was seen in the connective tissue near areas of abnormal and disrupted fibers in the KI/KO strain, although it did not extend into the muscle fibers themselves in most cases. CD45-positive staining was rare in WT and KI/KI muscle.

To further explore the myofibrillar disruption, transmission electron microscopy was performed on muscle tissue from the gastrocnemius of 7-month-old animals to assess ultrastructural changes to sarcomeres. Z-disk streaming and disruption, a classic feature of MFM, was seen in the tissue of KI/KO animals (Figure 4). Transmission electron microscopy also revealed the presence of megamitochondria, as well as other mitochondrial abnormalities, such as enlarged and mislocalized mitochondria, and subsarcolemmal accumulation, which corresponds to the increased RRFs. KI/KI animals were also assessed with transmission electron microscopy, which found some Z-disk disruption, although not as severely as in the KI/KO muscle and without the mitochondrial abnormalities.

Altered Levels of BAG3-Associated Proteins

Western blots revealed deviation between mutant strains and WT in levels of BAG3 interactors (Figure 5, A and C). BAG3 levels decreased to approximately half in the KI/KO and KO/WT mice compared with the WT and KI/KI mice. The KO/KO mice had no evidence of any remaining protein when tested with a polyclonal antibody (Figure 5B).

The following proteins, previously reported to be increased in patients with BAG3 MFMs, were also increased in the KI/KO mice: p62, HSPB8, and α B-crystallin.^{18,26} KI/KI mice also had an increase in p62 and α B-crystallin but not HSPB8.

Figure 4 Transmission electron micrographs (TEMs) of longitudinally sectioned gastrocnemius muscle of 7-month-old mice wild-type (WT) (A), knock-in (KI)/KI (B), and KI/knockout (KO) (C and D) mice. Z-disk streaming (arrows in B and C) is observed in KI/KI and KI/KO mice with focal disruption and loss (asterisk) in KI/KO mice. Mitochondrial abnormalities are also seen in KI/KO mice (D). Scale bars = 0.5 μ m (A–D). Original magnification, $\times 6800$ (A–D).

No increase was seen in HSPB1, a HSP known to have low affinity for BAG3.^{6,30} HSP70 levels also were not altered. These changes were not seen in KO/WT mice, which had levels of these proteins similar to that of WT mice except for HSPB8, which appeared to be lower in quantity.

Discussion

The BAG3 P215L/KO double mutant mouse is the first mouse model to recapitulate the skeletal muscle pathologic findings observed in patients with BAG3-P209L. Phenotypically, these mice do not have strength, coordination, or endurance deficits as measured by rotarod, grip strength, and inverted grid. However, they present with a significant reduction in voluntary movement with aging that appears at 7 months of age with further decline at 10 months. The decrease in voluntary movement seen may be an early clinical symptom of the muscle pathologic features, although heart, peripheral nerve, or even central nervous system pathologic findings cannot be ruled out as a cause because their investigation was beyond the scope of this study, which focuses on the skeletal muscle pathology. The absence of a strong

Figure 5 **A:** Western blot analysis of mouse muscle shows an increase in heat shock protein (HSP) B8, αB-crystallin, and p62 in knock-in (KI)/knockout (KO) animals, an increase in αB-crystallin and p62 in KI/KI animals, and a decrease in HSPB8 in KO/wild-type (WT) animals. **B:** BCL-2-associated athanogene 3 (BAG3) levels in KO, WT, KI/KI, KI/KO, and KO/WT mice. **C:** Protein levels normalized to loading controls (glyceraldehyde-3-phosphate dehydrogenase and actinin). Data are expressed as means ± SD (**C**).

phenotype likely reflects numerous physiologic differences between mice and humans, including much lower mechanical stress on the muscles of mice.³¹ These differences contribute to the often milder clinical phenotype seen in mice muscular dystrophy models. As such, additional mechanical stress may be needed to develop a phenotype that is more representative of what is seen clinically.

Despite only presenting with a reduction of movement with aging, histologically the KI/KO mice had many classic features of MFM, even before developing a clinical phenotype. These features include signs of muscle fiber damage, such as Z-disk streaming, myofibrillar disruption, inflammation, fiber atrophy, centralized nuclei, and Z-disk protein aggregates, which included filamin C, BAG3, and desmin.^{10,11} Like the other MFMs, these same histologic features are present in patients with BAG3^{P209L}.¹⁰ Muscle is continually exposed to mechanical stress and has a number of repair mechanisms, including the CASA system in which BAG3 is essential³² and is negatively affected by the human P209L mutation.²¹

As in human BAG3 MFM cases, there appears to be a mitochondrial phenotype present. Swollen, mislocalized, and

megamitochondria were seen on electron microscopy, and subsarcolemal clustering of mitochondria was noted in EM and in the form of RRFs on Gomori trichrome. Mitochondrial defects are a common feature of MFMs.^{2,3} Similar mitochondrial pathologic features, including megamitochondria, have been observed in a mouse model of the human R350P desminopathy.³³ Patients with BAG3^{P209L} have abnormalities in COX and NADH stainings,^{10,13,17} as well as clustered mitochondria as seen by electron microscopy.¹⁰ BAG3 is implicated in mitochondrial quality control through activation of mitophagy,³⁴ although it is unclear whether the presence of mitochondrial defects in the patients and mice is a primary or secondary effect of the myopathy.

Protein levels of various BAG3 interactors are affected in the KI/KO and KI/KI mice. A similar increase was not seen in KO/WT animals. In fact, they appeared to have lower levels of HSPB8. An increase was seen in p62 mice, αB-crystallin in KI/KI and KI/KO mice, and HSPB8 in KI/KO mice alone, all of which interact with BAG3. In contrast, HSPB1, a small HSP with little affinity for BAG3,^{6,30} is not affected. These increases may be attributable to increased stress response, stalling of the HSP70 machinery, and

aggregate formation during CASA or a combination thereof. Stalling of the HSP70 machinery has been seen in human cell lines that express the BAG3^{P209L} mutation,²¹ leading to accumulation of components of the CASA system and its clients.^{21,22} HSPB8 in particular is reliant on binding to BAG3 for stabilization; its levels are greatly decreased in BAG3 knock-down experiments *in vitro*,³⁰ explaining the lower levels in KO/WT mice and making the increased levels in KI/KO mice even more striking. Alterations to levels of these proteins and autophagic dysregulation have been seen in patient muscle²⁶ and heart,¹⁸ including an increase in small HSPs and p62. In addition, levels of HSP70 remain constant in mice and patient heart¹⁸ despite HSP70's essential role in the CASA complex.

Although the KI/KO mice present with a myopathic phenotype, the homozygous and heterozygous P215L strains have little or no deviations from the WT mice, despite carrying the dominant mutation. The KI/KI animals present with some mild alterations, including a reduction in fiber cross-sectional area, a nonsignificant increase in RRFs, moth-eaten fibers, and some Z-disk disruption, but have no quantifiable clinical phenotype and are missing many of the classic myopathic features that the KI/KO animals have. This lack of phenotype supports the hypothesis that BAG3 insufficiency in conjunction with the mutation is what leads to the MFM phenotype in humans.²⁰ KO/WT mice have repeatedly been found to have no skeletal muscle or molecular phenotype,^{23,24} with only a mild heart phenotype and no histologic abnormalities.³⁵ Human haploinsufficient patients also only have a heart phenotype.³⁶ We found that the KO/WT mice do not exhibit similar molecular or histologic changes to the KI/KO mice, excluding haploinsufficiency alone as the cause of the phenotype. Although this disease is dominant in humans, the KI/KO genotype still reflects the loss of available and functional BAG3 in patients. It leads to a greater loss of functional BAG3, exacerbating the pathologic phenotype. Therefore, this KI/KO mouse overcomes some of the difficulties associated with using mice to model a human myopathy through exacerbating the phenotype via reduction of protein levels.

Although the KI/KO model presents with a myopathic phenotype, it still presents a milder phenotype than humans, without measurable loss of strength or endurance. Because of the significantly lower levels of mechanical stress on mouse muscle and their short lifespans compared with humans, this model likely represents the early stages of the disease. Early pathologic features of BAG3 MFM has not been well studied in humans because of the rarity of the disease and the fact that most patients are more advanced in terms of muscle damage at the time of biopsy. The KI/KO BAG3 model may well be a powerful tool to study the mechanisms involved in disease progression, in particular, for the repeated muscle injury caused by physical stress during exercise, while also serving as a model for preclinical trials.

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Supplemental Data

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