TITLE: (150 characters or less - 138)

Transcriptional up-regulation of BAG3, a Chaperone Assisted Selective Autophagy factor, in animal models of KY-deficient hereditary myopathy

AUTHORS:

Elliot J Jokl, Gideon L Hughes, Tobias Cracknell, Mary E Pownall & Gonzalo Blanco

Department of Biology, University of York, Wentworth Way, United Kingdom, YO10 5DD

Corresponding author e-mail address: gonzalo.blanco@york.ac.uk

ORCIDs:

Elliot J Jokl: 0000-0003-2556-3777

Mary E Pownall: 0000-0003-2329-5844

Gonzalo Blanco: 0000-0003-3827-3111

RUNNING TITLE: (32 characters or less - 27)

CASA in KY-deficient models

KEY TERMS: (3-6 - 5)

Kyphoscoliosis peptidase; Chaperone Assisted Selective Autophagy; BAG3; FLNC; myopathy

SUMMARY STATEMENT: (15-30 words - 24)

This paper shows that increased transcriptional activation of tension mediated autophagy factor BAG3 is a consistent molecular hallmark of KY deficiency in animal models.

ABSTRACT: (250 words max - 236)

The importance of *kyphoscoliosis peptidase* (KY) in skeletal muscle physiology has recently been emphasised by the identification of novel human myopathies associated with KY deficiency. Neither the pathogenic mechanism of KY deficiency nor a specific role for KY in muscle function have been established. However, aberrant localisation of FLNC in muscle fibers has been shown in humans and mice with loss of function mutations in the *KY* gene. FLNC turnover has been proposed to be controlled by Chaperone Assisted Selective Autophagy (CASA), a client-specific and tension-induced pathway that is required for muscle maintenance. Here, we have generated new C2C12 myoblast and zebrafish models of KY-deficiency by CRISPR/Cas9 mutagenesis. To obtain insights into the pathogenic mechanism caused by KY deficiency, expression of the co-chaperone BAG3 and other CASA factors was analyzed in the cellular, zebrafish and *ky/ky* mouse models.

Ky-deficient C2C12 derived clones show trends of higher transcription of CASA factors in differentiated myotubes. The *ky*-deficient zebrafish model (ky^{yo1}/ky^{yo1}) lacks overt signs of pathology but shows significantly increased *bag3* and *flnca/b* expression in embryos and adult muscle. Additionally, ky^{yo1}/ky^{yo1} embryos challenged by swimming in viscous media show an inability to further increase expression of these factors in contrast to WT controls. The *ky/ky* mouse shows elevated expression of *Bag3* in the non-pathological EDL and evidence of impaired BAG3 turnover in the pathological soleus. Thus, upregulation of CASA factors appears to be an early and primary molecular hallmark of KY deficiency.

INTRODUCTION

The first model of KY-deficiency was the *ky/ky* mouse, which emerged spontaneously as a model of recessive, hereditary kyphoscoliosis in an inbred mouse strain over 40 years ago (Dickinson and Meikle, 1973; Mason and Palfrey, 1984). Subsequent extensive histopathological analyses showed that postural, tonically active muscles undergo a cycle of

degeneration and regeneration whereas fast-twitch muscles remain relatively unaffected (Bridges et al., 1992). Postural muscles show ultrastructural evidence of sarcomeric damage, including Z-disc thickening and elevated levels of autophagic vacuoles (Bridges et al., 1992). The *ky* mutation is a GC deletion near the beginning of the coding sequence that results in a premature STOP codon (Blanco et al., 2001); thus, *ky/ky* mice lack any KY protein (Baker et al., 2010). Yeast 2-hybrid/biochemical interactions and co-localization studies in adult fibres led to the proposal of a Z-disc network involving KY, FLNC, ZAK and other proteins (Baker et al., 2010; Beatham et al., 2004). Crucially, the *ky/ky* soleus shows aberrant localisation of the cytoskeletal crosslinkers FLNC (Beatham et al., 2004) and XIN (Beatham et al., 2006), which suggests a failure of muscle to maintain its structural integrity under consistent high tension in the absence of KY. Additionally, the muscles of *ky/ky* mice do not undergo compensatory hypertrophy in response to muscle overloading via surgical ablation (Blanco et al., 2001), indicating that KY is necessary to translate increased mechanical tension into muscle growth.

The *ky* gene encodes a transglutaminase-like protein located at the Z-disc of skeletal muscle (Baker et al., 2010). KY is highly conserved across vertebrates and shares a near identical protein sequence among mammals. Though the putative catalytic residues are conserved, to date no direct evidence of enzymatic activity over any endogenous substrate has been found. Moreover, alternative alignments suggest that this domain has been co-opted for protein-protein interactions (Anantharaman et al., 2001).

Mutations in the *ky* gene associated with myopathy have been recently reported in human patients (Hedberg-Oldfors et al., 2016; Straussberg et al., 2016; Yogev et al., 2017). Common pathological hallmarks in mice and humans include muscle atrophy of the soleus and the presence of FLNC aggregates. Deficient turnover of FLNC may thus be core to the

"*ky*" pathogenic mechanism in humans and mice. FLNC has been established as a client of the Chaperone Assisted Selective Autophagy (CASA) pathway (Ulbricht et al., 2013b). CASA is a recently proposed tension-induced autophagy mechanism (Arndt et al., 2010) that is reliant on the Z-disc co-chaperone BAG3. BAG3 binds to chaperones HSC70 and HSPB8 and facilitates solubilisation of damaged FLNC from the cytoskeleton. FLNC is then ubiquitinated by the E3 ubiquitin ligase CHIP/STUB1 and further complexed with tethering factors for autolysosomal degradation. In addition to mediating the degradation of FLNC, under mechanical tension BAG3 interacts with components of the Hippo signalling network to induce *Flnc* transcription. Thus, BAG3 interacts with inhibitors of the YAP/TAZ transcription factors. Once released of its inhibitors, YAP/TAZ translocate to the nucleus and upregulate the synthesis of native FLNC (Ulbricht et al., 2013a). Hence, BAG3 facilitates degradation and synthesis of FLNC, leading to the suggestion that tension bearing cells adapt FLNC turnover rates to the level of tension (Ulbricht et al., 2013a).

Given that FLNC is a known KY interaction partner (Beatham et al., 2004) it is plausible that its abnormal distribution in KY-deficient mice and humans reflects an alteration of the CASA pathway. Here, we investigate factors involved in CASA in newly generated myotube (C2C12) and zebrafish models of KY-deficiency and in *ky/ky* mice. Our results indicate that changes in transcriptional activation of CASA components are an early hallmark of KY deficiency in these models.

Results

Elevated expression of CASA components in C2C12 derived Ky-knockout myotubes

Bag3 and filamin expression positively correlates with increasing tension in smooth muscle cells (Ulbricht et al., 2013a). To test whether KY is involved in a similar pathway in skeletal muscle cells, we used CRISPR/Cas9 technology to generate C2C12 derived clones with

disruptive mutations in *Ky* (Fig S1, see Materials and Methods for details). Quantitative RT-PCR assays showed a significant reduction of *ky* transcript in clones D, I, K after differentiation into myotubes using either *Hprt* (housekeeping gene) or *Myh7* (differentiation marker; see Fig S2) as controls for normalisation, showing that these differences cannot be accounted for by any varying levels of differentiation between WT and mutant clones (Fig S1E). This is consistent with previous evidence of non-sense mediated decay for this transcript found in the mouse *ky* mutant (Blanco et al., 2001). These *Ky*-deficient clones were then selected for further analyses.

Given that *Flnc* is only expressed in differentiated cells (Dalkilic et al., 2006) and *ky* is also highly expressed in these conditions (Baker et al., 2010) analyses of CASA factors was focused on myotubes. We tested the expression of *Bag3, Hspb8, Chip* and the main CASA client *Flnc*. For qRT-PCR assays, genes that are consistently expressed throughout differentiation were normalised to *Hprt*. However, differentiation in culture, particularly the length of time required for myotube formation, is influenced by a number of factors (e.g., number of passages, flask size, coating). To minimize the effect of the differentiation level as a confounding factor in our measurements of target genes relative expression, we used an indicator of the state of differentiation, *Myh7,* as internal normalization marker for *Flnc*. qRT-PCR results revealed a consistent trend towards elevated expression of CASA components, with all mutant lines showing higher means for all components, reaching significance for *Flnc* (clone D)*, Hspb8* (clone D) and *Bag3* (clone I) (Fig 1). However, the inconsistency of the significance of these trends between clones prevents these observations from being conclusive.

Mutagenesis of ky in zebrafish via CRISPR/Cas9 genome editing

A zebrafish ortholog of *ky* (Ensembl ENSDARG00000074036) is located on chromosome 13. A tBLASTn query of the zebrafish nucleotide database with the mouse KY protein retrieved only the annotated zebrafish *ky*, indicating that no other putative ortholog exists in the zebrafish genome. Consistent with this, our results using HMMER (Finn et al., 2011), a homology search software designed to detect remote homologs, to query the mouse genome with the putative zebrafish *ky* sequence returned murine KY as the top result in the mouse protein database. Expression of the *ky* ortholog in zebrafish was analysed in the embryo and adult tissues by endpoint RT-PCR. Expression was detected in adult skeletal muscle, but not in other adult tissues or early embryonic stages (Fig 2A). qRT-PCR detected expression of *ky* from 3dpf and higher levels of expression in adult skeletal muscle (Fig 2B). These results are consistent with the muscle specific expression previously reported for the mouse *ky* gene (Blanco et al., 2001).

Gene targeting of zebrafish *ky* was carried out using a gRNA directed against exon 2 (Fig 2C) that was generated by an annealed-extended oligo template method (Nakayama et al., 2013). The gRNA was co-injected with purified Cas9 protein into fertilised embryos at the 1-8 cell stages. The efficiency of gene targeting was assayed at 24hpf using heteroduplex analyses of PCR amplicons from individual embryos, confirming the presence of sequence variants. Chimeric F0 zebrafish were outcrossed to wild type fish to produce F1 offspring carrying defined heterozygous mutations in *ky.* Heterozygous fish carrying specific mutations were selected for incrosses to produce homozygous mutant fish. A disruptive mutation (5bp deletion) and a non disruptive one (3bp deletion), both occurring at codons 141/142 within exon 2 (Fig 2D), were used to generate homozygous zebrafish lines for further analyses.

qRT-PCR showed a significant 10-fold reduction in *ky* transcript levels in 5bp deletion fish muscle at 3 months compared to WT (Fig 2D), indicating degradation of mutant transcript by

non-sense mediated decay. No significant difference was observed in the transcript levels of 3bp deletion and WT (Fig 2D). The disruptive allele was given the ZFIN designation ky^{yo1}.

Upregulation of some CASA components in ky^{yo1}/ky^{yo1} zebrafish

Development of muscle in ky^{yo1}/ky^{yo1} zebrafish embryos is normal, consistent with the late-embryonic onset of *ky* expression, and adults are viable and fertile. No morphological differences were observed in ky^{yo1}/ky^{yo1} juvenile fish (2 - 4 months old), with length, height at anterior of anal fin (HAA) and tail area (area between HAA line and anterior boundary of the tail fin) no different to that of WT fish (Fig 3A). Skeletal muscle is morphologically normal on H&E stained cross sections (Fig 3B). Given that *ky/ky* mice show a progressive shift towards expression of slower fibre types (Marechal et al., 1996), we analyzed the expression of type I fibres in ky^{yo1}/ky^{yo1} juvenile fish muscles by immunofluorescence. Fibre typing showed that slow muscle remained exclusively in the lateral region of skeletal muscle both in WT and ky^{yo1}/ky^{yo1} fish (Fig 3B). We conclude that the absence of KY in zebrafish does not have the same impact on muscle morphology or fibre type as seen in ky mutant mice (Blanco et al., 2001) or in patients with ky associated myopathy (Hedberg-Oldfors et al., 2016). However, an analysis into the molecular effects of KY deficiency provided some evidence of functional conservation. We showed that C2C12 derived myotubes expressed higher levels of CASA factors in the absence of KY (see Fig 1), therefore we tested whether the zebrafish orthologs of *bag3* and *flnc* (*flnca* and *flncb*) were similarly regulated by KY. In Figure 4, we show transcriptional up-regulation of *bag3* and *flncb* in ky^{yo1}/ky^{yo1} zebrafish. qRT-PCR analysis of ky^{yo1}/ky^{yo1} mutant zebrafish compared to wildtype siblings reveals a significant increase of *bag3* (at 3 months), and *flncb* (at 7dpf and 9 months).

Embryos lacking ky show impaired mechanotransduction

To increase mechanical tension, 3dpf embryos were moved into E3 media supplemented with 1% methylcellulose (ME) which increases viscosity and makes it more difficult for the fish to swim. Compared to unmodified E3 media, WT embryos show a significant increase in *bag3* and *flnca* expression when raised in viscous media, demonstrating a robust induction of a stress response involving CASA components using this method (Fig 5A). ky^{yo1}/ky^{yo1} embryos show a significantly higher baseline of *bag3, flnca* and *flncb* expression than WT when swimming in normal media and no further upregulation when raised in viscous media (Fig 5A). These findings suggest that the KY-deficiency induces a constitutive stress response in zebrafish larvae and reveals an inability of ky^{yo1}/ky^{yo1} muscle to further adapt cellular stress responses to the challenge of additional mechanical load. Examination of transverse muscle sections shows no difference in fast/slow muscle distribution between treatment groups, with slow muscle remaining as a peripheral cell layer (Fig 5B). This indicates that transcriptional changes cannot be accounted for by a shift in muscle fibre type. Additionally, birefringence analysis shows no evidence of gross muscle damage or deformation in any treatment group (Fig 5C), meaning that these transcriptional changes, and the inability of ky^{yo1}/ky^{yo1} embryos to further upregulate CASA components, are not secondary to gross structural damage.

BAG3 turnover in the ky/ky mouse

Since the *ky/ky* mutant mouse model demonstrates overt pathology with marked similarity to the human myopathy, we sought to examine whether similar evidence of CASA disruption was apparent in this model. The EDL and soleus muscles were selected for these analyses. The EDL is relatively non-pathological compared to the severely dystrophic soleus (Bridges et al., 1992), allowing a distinction between primary, baseline effects of KY-deficiency in the EDL and secondary effects observed at high tension and in the context of muscle pathology in the soleus.

Initial experiments were unable to detect a difference in BAG3 protein levels between WT and *ky/ky* muscle lysates extracted using RIPA buffer (data not shown). This extraction was only capable of extracting soluble proteins, and thus did not allow the detection of elevated amounts of insoluble or cytoskeleton-associated proteins. Since impairments to CASA are likely to result in elevated levels of these latter proteins, the total muscle extract sample was processed to obtain a cytoskeletal fraction (see Methods for details). In this analysis, elevated levels of cytoskeleton-associated BAG3 were observed in the soleus muscle of *ky/ky* mice compared to WT (Fig 6 A-B). Consistent with the initial observations using RIPA buffer, there was no significant increase in the amount of soluble BAG3 in the soleus. This suggests that an increased proportion of BAG3 protein is associated with the cytoskeleton in the *ky/ky* soleus compared to WT. The non-atrophic EDL showed no indication of elevated soluble or cytoskeletal BAG3 (Fig 6 A-B).

Examination of *Bag3* mRNA levels shows a significant increase in the EDL, but not in the soleus (Fig 6C). This indicates that the elevated amount of cytoskeletal BAG3 protein in the soleus may not be accounted for by elevated mRNA levels, but is instead indicative of reduced or inefficient turnover. In contrast, the comparable BAG3 protein levels between *ky/ky* and WT EDL (Fig 6A) combined with elevated mRNA in *ky/ky* EDL (Fig 6C) indicates that BAG3 turnover is elevated in the mutant EDL tissue.

Increased immunoreactivity for BAG3 and FLNC in the ky/ky soleus

Immunofluorescence experiments were performed to visualise the levels and localisation of BAG3 and FLNC in *ky/ky* and WT soleus longitudinal sections. Both BAG3 and FLNC showed increased reactivity in a subset of fibers in the *ky/ky* mutant. Those fibres showing particularly high levels of BAG3 also showed similarly elevated FLNC (Fig 7A). The proteins remained primarily co-localised in a striated pattern, presumably the Z-disc. This is

consistent with an increased proportion of cytoskeletal-associated BAG3 (Fig 6A). qRT-PCR was performed to explore whether this increased immunoreactivity represented an increase in *Flnc* transcription. No significant increase in *Flnc* transcript was detected between WT and *ky/ky* tissues (Fig 7B). The unchanged *Flnc* transcript levels in contrast to the increased reactivity of FLNC antibodies on *ky/ky* soleus sections could be explained by a lower turnover of FLNC protein in the *ky/ky* soleus. However, this could not be confirmed on western blots with the same flnc antibodies (RR90) due to inconsistent performance of these antibodies on this application.

DISCUSSION

Disruptive mutations in the *Ky* gene result in overt muscle pathology in mice (Bridges et al., 1992) and humans (Hedberg-Oldfors et al., 2016; Straussberg et al., 2016; Yogev et al., 2017). Impaired FLNC turnover is a common reported molecular phenotype in mice and human, which potentially implicate CASA in the pathogenic mechanism. In this study we have described the generation of novel C2C12 derived myoblast and zebrafish models of *Ky*-deficiency. Significant *Bag3* upregulation was observed in the ky homozygous zebrafish at 3 months and in the 5dpf embryos and in the soleus of the *ky/ky* mice . Evidence of CASA upregulation was also apparent in the EDL muscle of the *ky/ky* mice, with elevated transcription of *Bag3* with no increase in protein levels indicating elevated BAG3 turnover.

The disruption to *ky* expression in zebrafish alone was not sufficient to induce overt signs of pathology in adult or embryonic tissue, either under normal conditions or after methylcellulose challenge. Given that KY-deficient pathology in mice and humans occurs primarily in muscles which experience consistent levels of high tension, this disparity may be at least partly explained by the fact that zebrafish muscles do not experience the same gravitational stresses. However, unchallenged ky^{yo1}/ky^{yo1} embryos showed a transcriptional

profile comparable to methylcellulose treated WT controls, suggesting the constitutive transcriptional upregulation of CASA components when deficient in *ky* even in the absence of sustained tension or indicators of structural damage. This phenotype is particularly remarkable considering that the onset of *ky* expression occurs only approximately 48 hours prior to this analysis. We cannot rule out that other tests (e.g., electron microscopy, expression of embryonic myosin isoform in adult muscle, as observed in human patients (Hedberg-Oldfors et al., 2016)) might reveal myopathic phenotypes in the embryonic zebrafish model. Likewise, experiments challenging adult *ky^{yo1}/ky^{yo1}* zebrafish muscle may also reveal myopathic phenotypes.

This lack of overt myopathy in the *ky^{yo1}/ky^{yo1}* model is somewhat mirrored by the fact that the *ky/ky* mouse EDL, a fast twitch muscle which only experiences tension sporadically, is largely spared by the pathology. This suggests that upregulation of CASA components in the zebrafish and spared *ky/ky* mouse EDL and the trend of upregulation in the myoblasts models represent a primary effect of the absence of KY rather than a downstream consequence of myopathy. In contrast to the EDL muscle, the *ky/ky* mouse soleus experiences consistent high tension and displays the most overt pathology, including atrophy, dystrophic changes and sarcomeric damage (Bridges et al., 1992). The apparent accumulation of cytoskeletal BAG3 with no evident increase in *Bag3* transcription would suggest that in the absence of KY protein turnover in the tonically active soleus is impaired. Accumulation of ubiquitinated proteins in mouse *ky/ky* muscles (Figure S3) is also consistent with the notion of a role for KY in facilitating protein turnover. Our results do not distinguish whether the absence of KY affects CASA complex solubilisation or the CASA mechanism is simply overwhelmed by the overarching pathological damage. This phenotype is mirrored by the inability of *ky^{yo1}/ky^{yo1}* zebrafish embryos to further upregulate *bag3*, *flnca* and *flncb* in response to mechanical challenge in viscous media, indicating either that the constitutive

upregulation of these genes is already maximised, preventing the translation of increased mechanical challenge into this pathway, or that the mechanotransduction mechanism itself is impaired. A role for KY in mechanotransduction has already been proposed based on an inability of *ky/ky* mice to undergo compensatory hypertrophy in response to surgical overloading (Blanco et al., 2001).

Though transcriptional upregulation of CASA components appears to be a consistent early molecular hallmark across the models examined, exactly how this relates to the molecular function of KY and the pathogenic mechanism is unclear. The relationship between autophagy and muscle integrity is complex, with upregulation and downregulation of autophagy contributing to myopathy in different contexts (for a review on autophagy disruption impacting muscle integrity, see (Jokl and Blanco, 2016)). How much the CASA mechanism contributes overall to skeletal muscle macroautophagy remains uncharacterised, particularly given FLNC is the only client identified in this context. Examination of macroautophagy flow and signalling in the absence of KY would be highly informative in this regard. Elevated autophagy in *ky/ky* muscles may account for the reduced muscle to body mass ratios observed in *ky/ky* mice, and subsequently the susceptibility of tonically active muscles to structural damage and atrophy. Additionally, BAG3 is proposed to have a role in cytoskeletal stabilisation by facilitating correct localisation of CAPZ, an actin-capping protein ((Hishiya et al., 2010)). If KY has a function in this process, reduced structural stability in its absence may account for the induction of cell stress.

Our data are consistent with the hypothesis that upregulation of CASA component transcription is a primary compensatory mechanism in response to KY deficiency. This allows low-tension muscles to effectively manage endogenous levels of tension without the emergence of pathology, but is insufficient to meet the demands of high-tension, tonically

active muscle. However, further evidence is required to link KY directly to the CASA mechanism or to demonstrate some other function in skeletal muscle maintenance.

MATERIALS AND METHODS

Animal research ethics

All animal procedures have been carried with approval from the University of York Ethics committee and followed the UK Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012, performed by under project licenses PPL 70/6827 (mice) and PPL 60/4460 (zebrafish) within an approved establishment (licence 5002510).

Ky-deficient cell line generation

Px459 plasmid (pSpCas9(BB)-2A-Puro; Addgene; (Ran et al., 2013) was modified by the insertion of annealed, phosphorylated oligonucleotides (F: *cacctcatcgtgcactccgagaag* and R: *aaaccttctcggagtgcacgatga*) containing the *Ky* target sequence into the site created by AgeI digestion of the plasmid. C2C12 myoblasts were transfected with the modified Px459 plasmid using GenJet In Vitro DNA Transfection reagent for C2C12 cells following manufacturer's instructions (SL100489-C2C12). To test construct mutagenic capacity, puromycin resistant cells selected *en masse* were harvested, lysed, and the target region amplified by PCR (F: *ggggccatttgcagccta* and R: *cggagaggttcggattagcc*). PCR products were incubated at 37°C for 1hr with T7 Endonuclease I, with cleavage at the target site indicating successful mutagenesis. Individual clones were isolated by dilution following another round of construct transfection and puromycin selection. Clones were tested for mutagenesis by screening for heteroduplex formation in annealed WT and clone PCR amplicons run on 15% PAGE ((Zhu et al., 2014)). Alleles were then characterised by TA-cloning of PCR products using a TA Cloning kit (Thermo Scientific) and Sanger sequencing. Clones predicted to have two disruptive alleles (D, I and K) were carried forward for analysis and validated by qRT-PCR (Fig S1).

qRT-PCR

For myotubes clones were grown to confluency in 6 well plates before switching to differentiation media (2% serum, 100 U/ml Penicillin, 100µg/ml Streptomycin, 0.5µg/ml Fungizone (Gibco) in DMEM). Cells were allowed to differentiate for 10 days. Progress through differentiation was confirmed visually by light microscopy. Myotubes were washed three times in ice cold PBS before the addition of TRIZOL (Invitrogen) and harvesting by scraping, pooling 2 wells per biological replicate for a total of three biological replicates. For zebrafish and mice, tissues were snap frozen and ground under liquid nitrogen prior to addition of TRIZOL. *ky/ky* and control C3H/HeH mice were 7-8 week old males, but not all littermates. Zebrafish embryos were mechanically homogenised in a dounce homogeniser directly in TRIZOL. RNA was extracted using the manufacturer's protocol and converted to cDNA using ReadyScript cDNA synthesis mix (Sigma). qRT-PCR was performed on an ABI StepOnePlus qPCR machine using 2 x SYBR Green qPCR master mix (Applied Biosystems). Fold change was calculated using the DDCt method (Livak and Schmittgen, 2001) using either *hprt* or *myh7* (mice, C2C12s) or *ef1a* (zebrafish) as the control gene. Mouse primers used were: *Hprt* F:*gttggatacaggccagactttgtt* and R: *gattcaacttgcgctcatcttagg* (Cassel et al., 2008)*; Flnc* F: *ccttactcgcccttccgcatccat* and R: *ctcgggagctgtgtagtagatgtc* (Chevessier et al., 2015)*; Bag3* F: *atggacctgagcgatctca* and R: *cacggggatgggggatgta* (Rusmini et al., 2015); *Myh7* F: *accctcaggtggctccgaga* and R: *tgcagccccaaatgcagcca* (Anderson et al., 2015)*; Chip/Stub1* F: *cgcaaggacattgaggagca* and R: *tagtcctctacccagccgtt*; *Hspb8* F: *gcaatgaaatcatcagctggc* and R: *gggttgcagactttctccagt*; *Ky* F: *acagcatgtaccacaagagtgaa* and R: *tctcgatggtgattgtggcttt*. Zebrafish primers used were *ef1a* F: *ctggaggccagctcaaacat* and R: *atcaagaagagtagtaccgctagcat* (Tang et al., 2007); *bag3* F: *tgcccattcagattcaacag* and R: *ggctgctgtgtaggttgttg; flnca* F: *ccttcgtgggtcagaagaac* and R: *ggagttctaggaccgtggac (Solchenberger et al., 2015)*; *flncb* F:*ggccctacaaagtggacatc* and R:

cttcaaaccaggcccataag (Solchenberger et al., 2015); *ky* F: *tgaccctcatacatcccaagc* and R: *gagctcctgtctggggatca*.

Generation of the ky^yo1 zebrafish line

sgRNA targeting the *ky* orthologue synthesised *in vitro* using an annealed oligonucleotide template (as in (Nakayama et al., 2013)) with a MEGAshortscript SP6 transcription kit (Ambion) F: *taatacgactcactatagggagggggtttcacaatctcctctgttttagagctagaaatagcaa* and R: *aaaagcaccgactcggtgccactttttcaagttgataacggactagccttattttaacttgctatttctagctctaaaac*. London Wild-Type (LWT) zebrafish embryos were co-injected with 1ng recombinant Cas9 protein and 250pg sgRNA at the 1-16 cell stage into the yolk proximal to the cell body. The *ky* target region from embryos and fin clips was amplified by PCR (F: *agccaccaatcagaagaagca* and R: *gtgttagcacagagtgcacaa*) and screened via the high percentage PAGE assay as described above, and by Sanger sequencing. This same assay was performed for genotyping in the mutant lines. Off-target screening using *agpat3* - identified as the closest match to the *ky* target sequence by BLAST alignment - showed no evidence of mutagenesis in the same assays from PCR products amplified around the potential off-target site (F: *tggaccatgattcaactgccc* and R: *agctacactgttctgctccg*). F0 fish were outcrossed to WT LWTs to produce heterozygous fish, allowing determination of mutant alleles by subtraction of the WT allele from Sanger sequencing basecalls. Heterozygotes carrying the 3bp deletion or the 5bp deletion (*ky^yo1* allele) were incrossed to produce homozygous mutants. For the methylcellulose experiments, incrossing of homozygous mutant fish was performed to produce all *ky^yo1*/*ky^yo1* offspring with a parallel WT incross for the control cohort,

Western Blots

Mice used were 7-8 weeks old. Paired *ky/ky* and controls were gender-matched littermates. Protein fractions were isolated from freshly dissected muscle tissue using a Subcellular

Protein Fractionation Kit For Tissues (Thermo Scientific; 87790) following the manufacturer's protocol. Samples were combined with 4x NuPAGE LDS buffer (Invitrogen; NP0008) and 10x NuPAGE sample reducing agent (Invitrogen; NP004) and heated to 70°C for 10 minutes prior to loading onto a 10% Tris-Glycine gel. Transfer was performed using the iBlot gel transfer system (Invitrogen) and nitrocellulose stacks (Invitrogen; IB301002). Ponceau S staining was used to check for adequate transfer of proteins to the membrane prior to blocking for 1hr at RT in 5% dried skimmed milk in PBST. Membranes were incubated overnight at 4°C with BAG3 antibody (10599; Proteintech; 1:1000) prior to three ten minute washes in PBST and incubation with HRP-conjugated secondary antibody (anti-rabbit IgG; sc-2030; Santa Cruz Biotechnology, 1:10,000) or HRP-conjugated GAPDH (G9295; Sigma; 1:30,000) at RT for 1hr with shaking. Three further washes were performed to remove unbound secondary antibody. The membrane was incubated in Lumisensor HRP substrate (Genscript; L00221V60) for 1 minute prior to detection using X-ray film. Levels of protein were determined by band intensity relative to the loading control as calculated by densitometry using ImageJ software. Blots used with anti-ubiquitin antibodies (P4D1, Cell Signaling, 1:200) required boiling for 2-5 minutes for detection of ubiquitinated proteins. P4D1 mouse monoclonal antibody was then detected with a donkey anti-mouse IgG-HRP (Santa Cruz, sc-2314; 1:5000) as described above.

Methylcellulose challenge

Embryos were raised to 3dpf in normal E3 media (5mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2$, 0.33mM $MgSO_4$, 0.0001% Methylene Blue), dechorionated and split between unchallenged groups (remaining in normal media) and challenged groups (transferred to media enriched with 1% w/w methylcellulose). Embryos were left for 48 hours before harvesting for downstream analyses. Three biological replicates of 15 pooled embryos were used for each treatment.

Sectioning and histology

Muscle tissue was dissected from sacrificed fish or mice and snap-frozen in liquid N_2-cooled isopentane and stored at -80°C. 10 micron sections were generated using a cryostat. For Hematoxylin and Eosin (H&E) staining of tissue sections, the following protocol was observed: Fixation in acetone for 5 seconds, air drying, 1 minute incubation in Gill's Hematoxylin, washing with running water on the back of the slide until clear, one dip in eosin (no more than 10 seconds), twenty dips in 100% alcohol (for 6 alcohol changes), 5 dips in Histoclear, 30 second incubation in clean Histoclear, mounting under glass coverslips with DPX mounting media.

Immunofluorescence

Sections were cut at 12 microns. For mice, control and mutant sections were arranged side by side on the same to share the same antibody pools. Sections were permeablised and blocked using 3% BSA in PBS + 0.3% Triton X100 for 1hr. Sections were incubated for 2h at RT or overnight at 4^0C with primary antibodies against BAG3 (described previously, 1:200), FLNC (RR90; mouse IgA monoclonal, kind gift from Peter van der Ven; 1:20), S58 (mouse IgA monoclonal against slow myosin MYH7B, DSHB; 1:10), F59 (mouse IgG monoclonal against all fast isoforms, DSHB; 1:10) or ubiquitin (P4D1, a mouse IgG monoclonal, Cell Signaling; 1:200) in blocking buffer followed by three washes with PBS for 5 minutes. Slides were then incubated for 1hr incubation with appropriate combinations of compatible secondary at RT in the dark (e.g., anti Rabbit IgG TRITC; ab6718; 1:70 and anti Mouse IgA FITC; ab97234; Abcam; 1:70) before three washes with PBS for 5 minutes. Slides were mounted with Mowiol (Sigma) plus DAPI to stain nuclei. Images were obtained with exposure times of 20 ms (DAPI), 200 ms (FITC) and 300 ms (TRITC), using a Leica

DMIL LED microscope plus a Leica DFC 3000 G camera and the Leica LAS X software.

Statistical analysis

Statistical analysis of data was performed using Graphpad Prism 7 (Graphpad Software). Data were tested for normality using the Shapiro-Wilk test. If normality could be assumed, unpaired two-tailed Student's T-tests were used for single comparisons and ANOVA for multiple comparisons with Dunnett's multiple comparison test for post-hoc identification of significantly different means from control. Where normality could not be assumed, Kruskal-Wallace was used for multiple comparisons with Dunnett's multiple comparison post-hoc test.

ACKNOWLEDGEMENTS

COMPETING INTERESTS

The authors declare no competing interests

REFERENCES

Anantharaman, V., Koonin, E. V. and Aravind, L. (2001). Peptide-N-glycanases and DNA repair proteins, Xp-C/Rad4, are, respectively, active and inactivated enzymes sharing a common transglutaminase fold. *Hum. Mol. Genet.* **10**, 1627–1630.

Anderson, K. J., Russell, A. P. and Foletta, V. C. (2015). NDRG2 promotes myoblast proliferation and caspase 3/7 activities during differentiation, and attenuates hydrogen peroxide – But not palmitate-induced toxicity. *FEBS Open Bio* **5**, 668–681.

Arndt, V., Dick, N., Tawo, R., Dreiseidler, M., Wenzel, D., Hesse, M., Fürst, D. O., Saftig, P., Saint, R., Fleischmann, B. K., et al. (2010). Chaperone-assisted selective autophagy is essential for muscle maintenance. *Curr. Biol.* **20**, 143–148.

Baker, J., Riley, G., Romero, M. R., Haynes, A. R., Hilton, H., Simon, M., Hancock, J., Tateossian, H., Ripoll, V. M. and Blanco, G. (2010). Identification of a Z-band associated protein complex involving KY, FLNC and IGFN1. *Exp. Cell Res.* **316**, 1856–1870.

Beatham, J., Romero, R., Townsend, S. K. M., Hacker, T., van der Ven, P. F. M. and Blanco, G. (2004). Filamin C interacts with the muscular dystrophy KY protein and is abnormally distributed in mouse KY deficient muscle fibres. *Hum. Mol. Genet.* **13**, 2863–2874.

Beatham, J., Gehmlich, K., van der Ven, P. F. M., Sarparanta, J., Williams, D., Underhill, P., Geier, C., Fürst, D. O., Udd, B. and Blanco, G. (2006). Constitutive upregulations of titin-based signalling proteins in KY deficient muscles. *Neuromuscul. Disord.* **16**, 437–445.

Blanco, G., Coulton, G. R., Biggin, A., Grainge, C., Moss, J., Barrett, M., Berquin, A., Maréchal, G., Skynner, M., van Mier, P., et al. (2001). The kyphoscoliosis (ky) mouse is deficient in hypertrophic responses and is caused by a mutation in a novel muscle-specific protein. *Hum. Mol. Genet.* **10**, 9–16.

Bridges, L. R., Coulton, G. R., Howard, G., Moss, J. and Mason, R. M. (1992). The neuromuscular basis of hereditary kyphoscoliosis in the mouse. *Muscle Nerve* **15**, 172–179.

Cassel, S. L., Eisenbarth, S. C., Iyer, S. S., Sadler, J. J., Colegio, O. R., Tephly, L. A., Carter, A. B., Rothman, P. B., Flavell, R. A. and Sutterwala, F. S. (2008). The Nalp3 inflammasome is essential for the development of silicosis. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 9035–9040.

Chevessier, F., Schuld, J., Orfanos, Z., Plank, A.-C., Wolf, L., Maerkens, A., Unger, A., Schlötzer-Schrehardt, U., Kley, R. A., Von Hörsten, S., et al. (2015). Myofibrillar instability exacerbated by acute exercise in filaminopathy. *Hum. Mol. Genet.* **24**, 7207–7220.

Dalkilic, I., Schienda, J., Thompson, T. G. and Kunkel, L. M. (2006). Loss of FilaminC (FLNc) results in severe defects in myogenesis and myotube structure. *Mol. Cell. Biol.*

26, 6522–6534.

Dickinson, A. G. and Meikle, V. M. (1973). Genetic kyphoscoliosis in mice. *Lancet* **1**, 1186.

Finn, R. D., Clements, J. and Eddy, S. R. (2011). HMMER web server: interactive sequence similarity searching. *Nucleic Acids Res.* **39**, W29–W37.

Hedberg-Oldfors, C., Darin, N., Engman, M. O., Orfanos, Z., Thomsen, C., van der Ven, P. F. M. and Oldfors, A. (2016). A new early-onset neuromuscular disorder associated with kyphoscoliosis peptidase (KY) deficiency. *Eur. J. Hum. Genet.* **24**, 1771–1777.

Hishiya, A., Kitazawa, T. and Takayama, S. (2010). BAG3 and Hsc70 interact with actin capping protein CapZ to maintain myofibrillar integrity under mechanical stress. *Circ. Res.* **107**, 1220–1231.

Jokl, E. J. and Blanco, G. (2016). Disrupted autophagy undermines skeletal muscle adaptation and integrity. *Mamm. Genome* **27**, 525–537.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–408.

Marechal, G., Beckers-Bleukx, G., Berquin, A. and Coulton, G. (1996). Isoforms of Myosin in Growing Muscles of Ky (kyphoscoliotic) Mice. *Eur. J. Biochem.* **241**, 916–922.

Mason, R. M. and Palfrey, A. J. (1984). Intervertebral disc degeneration in adult mice with hereditary kyphoscoliosis. *J. Orthop. Res.* **2**, 333–338.

Nakayama, T., Fish, M. B., Fisher, M., Oomen-Hajagos, J., Thomsen, G. H. and Grainger, R. M. (2013). Simple and efficient CRISPR/Cas9-mediated targeted mutagenesis in Xenopus tropicalis. *Genesis* **51**, 835–843.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A. and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308.

Rusmini, P., Polanco, M. J., Cristofani, R., Cicardi, M. E., Meroni, M., Galbiati, M., Piccolella, M., Messi, E., Giorgetti, E., Lieberman, A. P., et al. (2015). Aberrant Autophagic Response in The Muscle of A Knock-in Mouse Model of Spinal and Bulbar Muscular Atrophy. *Sci. Rep.* **5**, 15174.

Solchenberger, B., Russell, C., Kremmer, E., Haass, C. and Schmid, B. (2015). Granulin Knock Out Zebrafish Lack Frontotemporal Lobar Degeneration and Neuronal Ceroid Lipofuscinosis Pathology. *PLoS One* **10**, e0118956.

Straussberg, R., Schottmann, G., Sadeh, M., Gill, E., Seifert, F., Halevy, A., Qassem, K., Rendu, J., van der Ven, P. F. M., Stenzel, W., et al. (2016). Kyphoscoliosis peptidase (KY) mutation causes a novel congenital myopathy with core targetoid defects. *Acta Neuropathol.* **132**, 475–478.

Tang, R., Dodd, A., Lai, D., McNabb, W. C. and Love, D. R. (2007). Validation of zebrafish (Danio rerio) reference genes for quantitative real-time RT-PCR normalization. *Acta Biochim. Biophys. Sin.* **39**, 384–390.

Ulbricht, A., Eppler, F. J., Tapia, V. E., van der Ven, P. F. M., Hampe, N., Hersch, N., Vakeel, P., Stadel, D., Haas, A., Saftig, P., et al. (2013a). Cellular mechanotransduction relies on tension-induced and chaperone-assisted autophagy.

Curr. Biol. **23**, 430–435.

Ulbricht, A., Arndt, V. and Höhfeld, J. (2013b). Chaperone-assisted proteostasis is essential for mechanotransduction in mammalian cells. *Commun. Integr. Biol.* **6**, e24925.

Yogev, Y., Perez, Y., Noyman, I., Madegem, A. A., Flusser, H., Shorer, Z., Cohen, E., Kachko, L., Michaelovsky, A., Birk, R., et al. (2017). Progressive hereditary spastic paraplegia caused by a homozygous KY mutation. *Eur. J. Hum. Genet.*

Zhu, X., Xu, Y., Yu, S., Lu, L., Ding, M., Cheng, J., Song, G., Gao, X., Yao, L., Fan, D., et al. (2014). An efficient genotyping method for genome-modified animals and human cells generated with CRISPR/Cas9 system. *Sci. Rep.* **4**, 6420.

FIGURE LEGENDS

Figure 1. Transcriptional upregulation of CASA in *Ky*-deficient differentiated myotubes.

Chip, Bag3, Hspb8 and *Flnc* qRT-PCR results show consistent upward trends in expression in *Ky*-deficient myotubes relative to WT, reaching significance for CASA components *Bag3, Hspb8 or Flnc* in two of the KY deficient clones. (One-way ANOVA/Dunnett's for *Bag3 and Hspb8*; Kruskal-Wallace/Dunnett's for *Chip* and *Flnc*, * = p<0.05; ** = p<0.01, n≥6). Error bars indicate SEM for *Bag3 and Hspb8* and median and interquartile range for *Chip* and *Flnc.*

Figure 2. Generation and validation of a *ky* knockout zebrafish line *ky^{yo1}/ky^{yo1}*.

A) RT-PCR of the identified *ky* ortholog shows no expression in the early embryonic stages or in adult fin and gut tissue. Robust expression is seen in skeletal muscle (white arrow), consistent with the expression profile reported in mice. Embryonic time points (hpf, hours post fertilization) and tissues, as indicated. E, positive control *ef1a*; K, *ky*.

B) Quantitative RT-PCR shows that the onset of *ky* expression is around 3dpf, with expression appearing to increase between 3 and 5dpf. Expression at these stages is much lower than in adult skeletal muscle, but this may partially be accounted for by the relative

contribution of muscle specific transcripts to the whole embryo. Error bars indicate standard deviation of three technical replicates.

C) Full exon intron structure of zebrafish *ky* (XM_001335276.5, verified by RT-PCR) at scale with the CRISPR/Cas9 target sequence highlighted in grey in the exon 2 sequence.

D) Quantitative RT-PCR of *ky* shows that fish homozygous for a 3bp deletion (i), modelled to result in the deletion of a single E residue, show no decrease in transcript expression but fish homozygous for a 5bp deletion (ii), modelled to result in a frameshift and premature stop codon, show a significant decrease in transcript (Student's T-test, $p<0.01$, n=3), indicating disruption at the mRNA level presumably by nonsense mediated decay. Error bars indicate SEM.

Figure 3 – Organism and tissue level characterisation of the *ky^{yo1}/ky^{yo1}* zebrafish shows no overt pathology.

A) Longitudinal growth study shows no difference in *ky^{yo1}/ky^{yo1}* zebrafish morphology. Three metrics were assessed in WT and *ky^{yo1}/ky^{yo1}* zebrafish at 2, 3 and 4 months post-fertilisation: length (blue) from the jaw to the anterior of the tail fin, height (green) at the anterior of the anal fin, and tail area (red) measured between the anterior of the anal fin and the anterior of the tail fin. No differences were seen between WT and *ky^{yo1}/ky^{yo1}* zebrafish.

B) Representative transverse sections of 3 month old zebrafish show muscle fibres in cross section. A small number of very small fibres and fibres with centralised nuclei can be seen with similar frequency in both WT and *ky^{yo1}/ky^{yo1}* fish (examples indicated by yellow arrows), indicating that these are not pathological changes.

C) Slow muscle distribution is preserved in the *ky^{yo1}/ky^{yo1}* zebrafish. Immunofluorescence against the slow muscle marker (s58) on transverse sections shows that slow muscle remains restricted laterally in the *ky^{yo1}/ky^{yo1}* zebrafish. Image representative of sections from 2 WT and 2 *ky^{yo1}/ky^{yo1}* fish at 6 months post fertilisation. Since sections are derived from

different positions on the anterior-posterior axis, slow muscle area and cell number cannot be directly compared.

Figure 4. Transcriptional analysis of CASA components in the *ky^{yo1}/ky^{yo1}* zebrafish line.

Quantitative RT-PCR of *bag3, flnca* and *flncb* at 7 days, 3 months and 9 months post-fertilisation shows a persistent trend of increased *bag3* transcription, though this only achieves significance at the 3 month stage (One-way ANOVA/Dunnett's, $p<0.01$, n=3). *flnca* shows no apparent differences, and *flncb* shows a significant increase at 7dpf and 9 months (One-way ANOVA/Dunnett's, $p<0.05$, n=3). Error bars indicate SEM.

Figure 5. Methylcellulose challenge does not increase transcriptional upregulation of CASA in *ky^{yo1}/ky^{yo1}* embryos.

A) Quantitative RT-PCR of *bag3, flnca* and *flncb* shows significantly increased transcription in 5dpf *ky^{yo1}/ky^{yo1}* embryos compared to WT controls when grown in normal (E3) media (One-way ANOVA/Dunnett's, * = $p<0.05$, ** = $p<0.01$, n=3). Swimming in media enriched with 1% methylcellulose from 3dpf to 5dpf induces significant increases in *bag3* and *flnca* transcription, and a trend towards increased *flncb* transcription, in WT embryos (One-way ANOVA/Dunnett's, * = $p<0.05$, ** = $p<0.01$, n=3). No significant changes in transcription are observed between challenged and unchallenged *ky^{yo1}/ky^{yo1}* embryos. Error bars indicate SEM.

B) Fibre-typing of embryonic zebrafish muscle shows no changes to slow (green) or fast (red) muscle distribution. Images are representative of 5 WT and 4 *ky^{yo1}/ky^{yo1}* embryo per treatment group (see figure S4 for full panel).

C) Representative images of birefringence analysis of whole zebrafish embryos, showing no gross muscle damage or deformation in any treatment group, indicating the transcriptional changes are not secondary to gross muscle damage.

Figure 6. Altered BAG3 turnover in the *ky/ky* mouse.

A) Representative WB images of BAG3 in cytoskeletal and soluble fractions of muscle protein extracted from the soleus and the EDL.

B) Quantification of BAG3 protein levels by densitometry shows significantly higher levels of cytoskeletal-associated BAG3 in the *ky/ky* soleus compared to WT sibling controls (paired Student's t-test, $p < 0.05$, n=8). No differences are observed at the protein level in the EDL. Error bars indicate SEM.

C) Quantitative RT-PCR of *Bag3* shows no significant difference in transcript level between WT and *ky/ky* soleus, indicating that the increase in protein may not be accounted for by elevated transcription. Significant upregulation of transcript levels is seen in the EDL (paired Student's t-test, $p < 0.05$, n=3). Since no commensurate increase in protein level is observed, this suggests BAG3 turnover may be elevated in the EDL. Error bars indicate SEM.

Figure 7. Increase in BAG3 and FLNC immunoreactivity in *ky/ky* soleus muscle.

A) Representative images of immunofluorescence against BAG3 and FLNC on longitudinal sections of WT and *ky/ky* soleus. Higher reactivity for BAG3 and FLNC is observed in the *ky/ky* soleus consistent with the increased cytoskeletal BAG3 observed on WB in the mutant (see figure 6). Amplified insets (squares) show that BAG3 and FLNC appear to remain primarily co-localised to striations, presumed to be the z-disc. Antibody pools were shared between WT and *ky/ky* sections and camera settings were maintained.

B) Quantitative RT-PCR of *Flnc* shows no significant increases comparing WT and *ky/ky* tissue between siblings (paired student's T-test, $p > 0.05$, n=3). This suggests the apparent

increase in FLNC protein may not be accounted for by increased transcription. Error bars indicate SEM.