FLNC myofibrillar myopathy results from impaired autophagy and protein insufficiency

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Abstract

Myofibrillar myopathy is a progressive muscle disease characterized by the disintegration of muscle fibers and formation of protein aggregates. Causative mutations have been identified in nine genes encoding Z-disk proteins, including the actin binding protein filamin C (FLNC). To investigate the mechanism of disease in FLNC\textsuperscript{W2710X} myopathy we overexpressed fluorescently tagged FLNC or FLNC\textsuperscript{W2710X} in zebrafish. Expression of FLNC\textsuperscript{W2710X} causes formation of protein aggregates but surprisingly, our studies reveal that the mutant protein localizes correctly to the Z-disk and is capable of rescuing the fiber disintegration phenotype that results from FLNC knockdown. This demonstrates that the functions necessary for muscle integrity are not impaired, and suggests that it is the formation of protein aggregates and subsequent sequestration of FLNC away from the Z-disk that results in myofibrillar disintegration. Similar to those found in patients, the aggregates in FLNC\textsuperscript{W2710X} expressing fish contain the co-chaperone BAG3. FLNC is a target of the BAG3-mediated chaperone assisted selective autophagy (CASA) pathway and therefore we investigated its role, and the role of autophagy in general, in clearing protein aggregates. We reveal that despite BAG3 recruitment to the aggregates they are not degraded via CASA. Additionally, recruitment of BAG3 is sufficient to block alternative autophagy pathways which would otherwise clear the aggregates. This blockage can be relieved by reducing BAG3 levels or by stimulating autophagy. This study therefore identifies both BAG3 reduction and autophagy promotion as potential therapies for FLNC\textsuperscript{W2710X} myofibrillar myopathy, and identifies protein insufficiency due to sequestration, compounded by impaired autophagy, as the cause.
Introduction

Myofibrillar myopathies are progressive muscle diseases characterized by disintegration of muscle fibers and abnormal protein accumulation. Myofibrillar myopathy patients present with progressive, relatively late onset, muscle weakness often accompanied by cardiomyopathy and/or respiratory complications. Causative mutations for myofibrillar myopathies have been identified in nine genes; desmin (1), αB-crystallin (2), myotilin (3), Z-band alternatively spliced PDZ motif-containing protein (ZASP; (4)), filamin C (FLNC; (5)), bcl-2-associated athanogene 3 (BAG3; (6)), four and a half LIM domain 1 (FHL1; (7)), titin (8), and actin (9). All of these genes encode proteins found at the Z-disk, a key structure involved in the transmission of tension and contractile forces along the muscle fiber.

Given that protein aggregate formation is consistent between myofibrillar myopathies regardless of the genetic basis (10), considerable effort has been put into their characterization. Using laser microdissection and proteomic analysis a range of sarcomeric and extra-sarcomeric proteins have been shown to be included within them, with the most abundant protein being the product of the gene mutated (11-13). Whilst the aggregates are a characteristic of the disease their characterization has not led to an understanding of their contribution to muscle weakness. We therefore investigated the mechanism of disease in myofibrillar myopathy caused by a mutation in the Z-disk protein FLNC.

FLNC belongs to the Filamin family of actin-binding proteins and is expressed predominantly in skeletal and cardiac muscle (14). The majority of FLNC localizes to
the Z-disk, which is important in the maintenance of structural integrity of the sarcomere. A small fraction of FLNC localizes to the sarcolemma (14), where it interacts with the transmembrane receptor β1-integrin (15) and components of the dystrophin-glycoprotein complex (14). FLNC therefore connects components of the sarcolemma to the extracellular matrix, providing both a structural linkage and a mechanism for signal transduction.

The Filamin proteins, including FLNC, are highly conserved in their structure, containing two actin binding domains in the N-terminal region followed by 24 immunoglobulin (Ig) repeats (16). The first identified and most prevalent myofibrillar myopathy causing FLNC mutation is FLNC\(^{W2710X}\) which results in truncation of the last 16 amino acids of the 24\(^{th}\) Ig repeat that is important for dimerization (5). Since this initial discovery four additional myofibrillar myopathy causing FLNC mutations have been identified all demonstrating autosomal dominant inheritance and ages of onset ranging from 24 to 60 (17-19). More recently, mutations in FLNC have been identified in distal myopathy, resulting in fiber disintegration but not protein aggregation (20, 21). The presentation of fiber disintegration in both FLNC-related myofibrillar and distal myopathies is suggestive of an overlap in the mechanism of disease.

The effect of the W2710X mutation has been thoroughly examined \textit{in vitro}. Biochemical studies revealed that a partial loss of the dimerization domain of FLNC\(^{W2710X}\) disturbs its overall secondary structure, and although this has no effect on its actin binding capacity (20), FLNC\(^{W2710X}\) is incapable of dimerizing and has an increased propensity to aggregate. Indeed, transient transfection of mini-FLNC\(^{W2710X}\)
(actin binding domains fused to domains 23 and 24) in cells results in the formation of α-actinin negative protein aggregates, similar to those seen in patients (22). Interestingly, using differentially tagged mini-FLNC\textsuperscript{wt} and mini-FLNC\textsuperscript{W2710X} Lowe et al. (2007) showed that the aggregates are composed purely of mutant protein and that mini-FLNC\textsuperscript{W2710X} does not disrupt dimerization of FLNC\textsuperscript{wt} (22). It has therefore been proposed that the pathology observed in FLNC\textsuperscript{W2710X}-related myofibrillar myopathy is due to a toxic gain of function whereby the FLNC\textsuperscript{W2710X} aggregates sequester FLNC binding partners therefore resulting in muscle weakness (22, 23).

Recent studies have also assessed the involvement of protein quality control mechanisms in explaining the pathobiology of myofibrillar myopathies. Extensive immunofluorescence analyses on muscle biopsies from FLNC-related myofibrillar myopathy patients have revealed abnormal expression and localization of proteins involved in the ubiquitin-proteasomal and autophagic degradation pathways (24), including BAG3, a key component of the chaperone assisted selective autophagy (CASA) pathway (25). These results suggest impaired protein quality control systems may contribute to aggregate accumulation in FLNC-related myofibrillar myopathy.

We have previously shown that loss of both zebrafish FLNC homologues results in myofiber disintegration and formation of Myosin enriched aggregates (26). However, studies examining the composition of aggregates in FLNC\textsuperscript{W2710X} myofibrillar myopathy failed to detect Myosin (13) suggesting that the aggregates seen in the loss of function model differ from those seen in FLNC\textsuperscript{W2710X} patients. We show that loss of FLNC results in the formation of large filamentous aggregates but not the typical
granular aggregates seen in myofibrillar myopathy. Instead, overexpression of FLNC\textsuperscript{W2710X} results in the formation of numerous smaller protein aggregates.

Contrary to previous reports, FLNC\textsuperscript{W2710X} was found to localize correctly to the Z-disk of the sarcomere and prevent myofiber disintegration. We did, however, find significant differences in the dynamics of FLNC\textsuperscript{W2710X} suggesting that interactions with some of its binding partners at the Z-disk are altered, potentially contributing to the muscle weakness. We propose that the formation of aggregates and subsequent FLNC depletion results in myofiber disintegration and muscle weakness in FLNC-related myofibrillar myopathy.

Having identified the aggregates as the primary cause of muscle weakness we examined the role of BAG3 and CASA in promoting their clearance. We found that although the BAG3-mediated CASA pathway is impaired and insufficient in clearing the FLNC\textsuperscript{W2710X} aggregates, treatment with autophagy stimulating drugs significantly reduces protein aggregation providing a potential therapeutic approach for FLNC-related myofibrillar myopathy.
Results

Expression of FLNC<sup>W2710X</sup> results in protein aggregation

To determine if presence of mutant FLNC results in protein aggregation we expressed fluorescently tagged variants of human full-length wildtype FLNC (FLNC<sup>wt</sup>-eGFP) or myofibrillar myopathy causing FLNC<sup>W2710X</sup> (FLNC<sup>W2710X</sup>-eGFP) under the muscle specific promoter actcb1. Live imaging of embryos expressing FLNC<sup>wt</sup>-eGFP (Fig. 1A) or FLNC<sup>W2710X</sup>-eGFP (Fig. 1B) at 48-, 72-, 96- and 120-hours post fertilization (hpf) revealed strong myoseptal and weaker sarcomeric localization of the tagged protein. Expression of FLNC<sup>W2710X</sup>-eGFP additionally resulted in the formation of protein aggregates at all stages investigated (Fig. 1B).

FLNC<sup>W2710X</sup> correctly localizes to the Z-disk

To determine the localization of the tagged forms of FLNC we performed immunohistochemistry on 48-hpf embryos expressing FLNC<sup>wt</sup>-eGFP (Fig. 2A, C) or FLNC<sup>W2710X</sup>-eGFP (Fig. 2B, D) using antibodies against eGFP and α-actinin or myosin. Both, FLNC<sup>wt</sup>-eGFP and FLNC<sup>W2710X</sup>-eGFP localized to the Z-disk of the sarcomere as shown by the overlap between actinin and the tagged protein (Fig. 2A, B), and the complementary staining of myosin and eGFP (Fig. 2C, D). Therefore, despite the formation of aggregates, FLNC<sup>W2710X</sup>-eGFP correctly localizes to the Z-disk highlighting that partial loss of the dimerization domain of FLNC does not affect its sarcomeric localization.

Loss of FLNC results in fiber disintegration and formation of filamentous aggregates

We have previously shown that the loss of both zebrafish FLNC homologues results
in myofibrillar disintegration and formation of Myosin containing aggregates (26) (Fig. 3B). Given that the aggregates seen in FLNC-myofibrillar myopathy patients have been reported to lack Myosin (13), and the differences with the aggregates observed in FLNC\(^{W2710X}\)-eGFP fish, we examined the aggregates seen in \(flnca\) and \(flncb\) morphant embryos in more detail. Electron microscopy experiments revealed accumulation of filamentous material at the myosepta and mitochondrial infiltration in areas devoid of myofibrils (Fig. 3C). There was no evidence of granular protein aggregates suggesting that the aggregates seen following loss of FLNC differ from the typical FLNC-related myofibrillar myopathy aggregates, and are potentially the remnants of disintegrated myofibers.

**FLNC\(^{W2710X}\) is functional and can rescue the loss of function**

Previous *in vitro* studies have shown that FLNC\(^{W2710X}\)-eGFP is unable to dimerize rendering it non-functional (5, 22). To test this hypothesis *in vivo* we injected either FLNC\(^{wt}\)-eGFP or FLNC\(^{W2710X}\)-eGFP together with morpholinos targeting both zebrafish FLNC homologues (\(flnca+flncb\) MO), and compared the frequency of broken slow muscle fibers in these fish, to those injected with morpholinos alone. As in our previous studies (26), knockdown of \(flnca\) and \(flncb\) results in catastrophic fiber disintegration with an average of 60% of fibers affected per somite (\(n=12\) fish; total intact fibers: 175; total broken fibers: 260; Fig. 4A, D). Of the slow muscle fibers expressing FLNC\(^{wt}\)-eGFP or FLNC\(^{W2710X}\)-eGFP in embryos injected with \(flnca+flncb\) morpholino 100% of the GFP positive fibers were intact (Fig. 4B-D; \(p<0.0001\); FLNC\(^{wt}\)-eGFP \(n=13\) fish; total intact fibers: 35; total disintegrated fibers: 0; Fig. 4B, D; FLNC\(^{W2710X}\)-eGFP \(n=7\) fish; total intact fibers: 22; total disintegrated fibers: 0; Fig. 4B, D). Alignment of the morpholino target sites with both FLNC\(^{wt}\)-eGFP and
FLNC\textsuperscript{W2710X}-eGFP rescue constructs revealed a lack of binding sites in the later demonstrating that the rescue seen in transgene expressing fibers is not due to sequestration of the morpholinos by the rescue construct. The ability of FLNC\textsuperscript{W2710X}-eGFP to rescue the loss of FLNC fiber disintegration phenotype demonstrates, contrary to previous reports, that mutant FLNC\textsuperscript{W2710X} is capable of performing the functions required for the maintanance of myofiber integrity.

To determine if FLNC\textsuperscript{wt} is necessary for the formation of FLNC\textsuperscript{W2710X} aggregates we examined protein aggregation in embryos injected with FLNC\textsuperscript{W2710X}-eGFP and both \textit{flnc} targeting morpholinos. Despite the loss of endogenous Flnc aggregates were still evident in FLNC\textsuperscript{W2710X} expressing cells (Supplementary Fig. 1) demonstrating that FLNC\textsuperscript{wt} is not required for their formation.

**FLNC\textsuperscript{W2710X} demonstrates reduced dynamics at the Z-disk**

Whilst the W2710X mutation does not affect FLNC localization or abolish the functions required for Z-disk integrity we wished to examine the dynamics of FLNC\textsuperscript{W2710X} within the Z-disk to determine the impact of the mutation. We therefore performed fluorescent recovery after photobleaching experiments (FRAP) on five days post fertilisation (dpf) embryos expressing either FLNC\textsuperscript{wt}-eGFP (Fig. 5A) or FLNC\textsuperscript{W2710X}-eGFP (Fig. 5B). An initial examination of the recovery profiles demonstrated slower recovery of the mutant FLNC compared to wildtype, evident by differences in the slope of the curves (Fig. 5C). To investigate this further, we analysed the data using two phase non-linear correlation, which determined the slow and fast half lives of FLNC recovery. In line with diffusion being the primary determinant of the fast half life, we found no significant difference between the fast
half life of FLNC\textsuperscript{wt}-eGFP and FLNC\textsuperscript{W2710X}-eGFP. However, we found a significant increase in the slow half-life of FLNC\textsuperscript{W2710X}-eGFP compared to FLNC\textsuperscript{wt}-eGFP (Fig. 5D) demonstrating that the ability of FLNC\textsuperscript{W2710X} to interact with its binding partners at the Z-disk may be impaired. Despite the slower dynamics of FLNC\textsuperscript{W2710X} we found no differences in the mobile fraction compared to FLNC\textsuperscript{wt} (Fig. 5E).

**FLNC\textsuperscript{W2710X} expressing fibers display ultrastructural defects**

Given the impaired interaction of FLNC\textsuperscript{W2710X} with its binding partners at the Z-disk we performed correlative light and electron microscopy (CLEM) on 48-hpf embryos expressing FLNC\textsuperscript{wt}-eGFP (Fig. 6E-F) or FLNC\textsuperscript{W2710X}-eGFP (Fig. 6A-B) to examine the ultrastructure. Our analysis revealed misalignment of myofibrils in FLNC\textsuperscript{W2710X}-eGFP (Fig. 6B) expressing cells whereas FLNC\textsuperscript{wt}-eGFP expressing fibers (Fig. 6F) display normal sarcomeric organization. Additionally, FLNC\textsuperscript{W2710X}-eGFP expressing cells were found to contain thickened Z-disks (Fig. 6C) and regions of myofibrillar disarray (Fig. 6D), both of which were absent in FLNC\textsuperscript{wt}-eGFP (Fig. 6G) expressing cells.

**BAG3 is contained within FLNC\textsuperscript{W2710X}-eGFP aggregates**

As BAG3 has been shown to be incorporated into the protein aggregates in FLNC\textsuperscript{W2710X} myofibrillar myopathy we examined its localization in our model. We co-injected mCherry tagged human BAG3 (BAG3-mCherry) with FLNC\textsuperscript{W2710X}-eGFP and imaged the embryos at 48-hpf. Our studies indicate that most FLNC\textsuperscript{W2710X} aggregates contained BAG3 (Fig. 7B). To confirm that was not merely due to overexpression of BAG3 we co-injected the FLNC\textsuperscript{W2710X}-eGFP construct with mCherry tagged zebrafish actinin3b, a protein expected to be absent in the aggregates.
Indeed, actinin3b was not contained within the aggregates (Fig. 7D) confirming that the localization of BAG3 seen within the FLNC\textsuperscript{W2710X} aggregates is not due to overexpression.

**FLNC\textsuperscript{W2710X} blocks BAG3 mediated clearance of protein aggregates**

Having found that BAG3 is localized within the aggregates we wished to determine the potential role of the BAG3 dependent CASA pathway in clearing them. We treated 32-hpf FLNC\textsuperscript{WT}-eGFP (Fig. 8A) or FLNC\textsuperscript{W2710X}-eGFP (Fig. 8B) injected embryos with the autophagy inhibitors chloroquine or ammonium chloride (NH\textsubscript{4}Cl) and counted the number of aggregates. Inhibition of autophagy had no effect on FLNC\textsuperscript{WT}-eGFP localization or on FLNC\textsuperscript{W2710X}-eGFP aggregation, highlighting that autophagy is not actively involved in clearing the aggregates (FLNC\textsuperscript{WT}-eGFP: untreated, n=120 fibers in 21 embryos; chloroquine, n=101 fibers in 15 embryos; NH\textsubscript{4}Cl, n=119 fibers in 13 embryos p>0.05. FLNC\textsuperscript{W2710X}-eGFP: untreated, n=266 fibers in 31 embryos; chloroquine, n=142 fibers in 18 embryos; NH\textsubscript{4}Cl, n=239 fibers in 22 embryos, p>0.05, Fig. 8C). To determine the role of BAG3 in aggregate clearance we coinjected two bag3 targeting morpholinos that have been previously validated (27), with FLNC\textsuperscript{WT}-eGFP (Fig. 8A) or FLNC\textsuperscript{W2710X}-eGFP (Fig. 8B) and assessed protein aggregation. Loss of Bag3 did not promote the aggregation of FLNC\textsuperscript{WT}-eGFP (bag3 MO, n=90 fibers in 19 embryos; bag3 MO + chloroquine, n=156 fibers in 13 embryos; bag3 MO + NH\textsubscript{4}Cl, n=217 fibers in 22 embryos, p>0.05; Fig. 8C). Reduction of Bag3 in FLNC\textsuperscript{W2710X}-eGFP expressing cells however, resulted in a significant decrease in the number of aggregates per cell. Inhibition of autophagy, using either chloroquine or ammonium chloride, in BAG3 depleted FLNC\textsuperscript{W2710X}-eGFP expressing cells prevented this reduction in aggregate number (bag3 MO,
n=351 fibers in 29 embryos; bag3 MO + chloroquine, n=321 fibers in 24 embryos; bag3 MO + NH4Cl, n=187 fibers in 22 embryos; Fig. 8C). These results demonstrate that rather than promoting the clearance of aggregates, BAG3 prevents the autophagic degradation of FLNC\textsuperscript{W2710X} aggregates.

To directly examine autophagy induction, rather than its effect on aggregate number we examined Lc3 accumulation in untreated and chloroquine treated bag3 morphant embryos (Supplementary Fig. 2). The reduced accumulation of Lc3 in chloroquine treated bag3 morphant embryos compared to wildtype demonstrates they have reduced autophagic capacity. Despite the reduced autophagic activity in bag3 morphant embryos, the increased autophagy dependent clearance of the aggregates, as demonstrated by chloroquine and NH4Cl inhibition, further supports an inhibitory role for Bag3 in aggregate degradation.

**Stimulation of autophagy reduces protein aggregates**

Despite an impairment of the BAG3-mediated CASA pathway we tested drugs that upregulate autophagy as a strategy to promote the clearance of aggregates. Embryos expressing FLNC\textsuperscript{wt}-eGFP (Fig. 9A) or FLNC\textsuperscript{W2710X}-eGFP (Fig. 9B) were exposed to the autophagy stimulating drugs rapamycin, carbamazepine, or spermidine, and the number of aggregates examined. We found that stimulation of autophagy had no significant effect on FLNC\textsuperscript{wt}-eGFP expressing cells (untreated, n=120 fibers in 21 embryos; rapamycin, n=52 fibers in 9 embryos; carbamazepine, n=66 fibers in 12 embryos; spermidine, n=96 fibers in 18 embryos; p>0.05; Fig. 9C). Treatment of FLNC\textsuperscript{W2710X}-eGFP expressing embryos with each of the three drugs on the other hand, resulted in a significant reduction in the number of aggregates per cell, with
rapamycin and carbamazepine treated cells comparable to FLNC\textsuperscript{WT}-eGFP expressing cells (untreated, \(n=266\) fibers in 31 embryos; rapamycin, \(n=144\) fibers in 16 embryos; carbamazepine, \(n=314\) fibers in 25 embryos; spermidine, \(n=147\) fibers in 16 embryos; \(p<0.0001\); Fig. 9C). These results demonstrate that although autophagy is not normally active in removing aggregates in FLNC\textsuperscript{W2710X}-eGFP expressing cells, once activated it is effective at reducing the protein aggregates. As such, pharmacological upregulation of autophagy may serve as a therapy for FLNC-related myofibrillar myopathy.

Given that \textit{bag3} depletion and autophagy stimulation both reduced protein aggregation we wished to examine if a combination of the two would result in a more pronounced reduction in the number of aggregates. Since rapamycin reduced aggregates to a level not significantly different to FLNC\textsuperscript{WT}-eGFP expressing fish we examined Bag3 reduction and spermidine treatment in combination. This resulted in a significant decrease in protein aggregation compared to either treatment alone (\(n=207\) fibers in 19 embryos, \(p<0.05\); Supplementary Fig. 3). This indicates a combination of autophagy stimulation and BAG3 depletion as a potentially powerful therapeutic strategy for FLNC-related MFM.
Discussion

To investigate the mechanism of disease in FLNC$^{W2710X}$ myofibrillar myopathy we examined zebrafish with reduced FLNC expression or ectopic expression of FLNC$^{W2710X}$ in the muscle. Examination of a FLNC loss of function model using electron microscopy revealed that the myosin enriched aggregates observed by immunofluorescence staining are filamentous and appear to be the remnants of disintegrated myofibers. This suggests that myofibrillar disintegration, but not the formation of granular aggregates, results from loss of FLNC function. To examine this question further we tested the function of FLNC$^{W2710X}$. Expression of FLNC$^{W2710X}$ in the muscle is sufficient to cause the formation of protein aggregates but, contrary to previous studies, FLNC$^{W2710X}$ also localizes correctly to the Z-disk despite the loss of the last 16 amino acids of the dimerization domain. An examination of the dynamics of FLNC$^{W2710X}$ however, revealed a slower recovery rate of mutant FLNC compared to FLNC$^{wt}$ following photobleaching. This suggests that the W2710X mutation alters the interaction of FLNC with some of its binding partners at the Z-disk. Although the interaction of FLNC$^{W2710X}$ with actin has been shown to be undisturbed (20), it is possible that its binding to other Z-disk proteins, such as myotilin, myozienin, myopodin and titin, may be affected.

Surprisingly, we also show that FLNC$^{W2710X}$ is capable of rescuing the fiber disintegration phenotype caused by loss of FLNC, demonstrating that its ability to maintain fiber integrity is retained. Therefore, whilst differences in the ability of FLNC$^{W2710X}$ to interact with its binding partners may contribute to myofibrillar myopathy pathology, they are not sufficient to account for the structural failure of
muscle fibers and subsequent muscle weakness. Instead, we propose that as the aggregates are formed FLNC$^{W2710X}$ gets sequestered within them resulting in insufficient FLNC at the Z-disk. FLNC is a dosage sensitive protein and its decreased availability would be detrimental to muscle function causing muscle fibers to disintegrate resulting in muscle weakness (21, 26, 29). This also explains the similar pathology observed in FLNC-related myofibrillar myopathy and distal filaminopathies, with the later characterized by fiber disintegration but not the formation of protein aggregates. In both cases a reduction in FLNC function either due to sequestration into aggregates combined with disrupted protein-protein interactions, as seen in myofibrillar myopathy, or alterations in its ability to interact with binding partners as seen in distal filaminopathy (20), results in fiber disintegration and corresponding muscle weakness.

The mechanism we propose for FLNC$^{W2710X}$ myofibrillar myopathy is similar to the mechanism we recently reported for BAG3$^{P209L}$ myofibrillar myopathy (27). In both cases the formation of protein aggregates results in a reduction in the availability of the protein resulting in insufficiency and a loss of function phenotype. Given the similarity in mechanism between these two genetically different forms of myofibrillar myopathy and the highly consistent pathological presentation we suggest this mechanism could be further extended to other causes of myofibrillar myopathy. Consistent with this idea the most abundant component of the myofibrillar myopathy aggregates is the mutated protein (30), suggesting that sequestration of the mutant protein is a common feature. The proposed mechanism is further supported by experiments in which expression of myofibrillar myopathy causing mutant Desmin (31-33), αB-Crystallin (34, 35), Myotilin (36), ZASP (37), BAG3 (6, 38) and FHL-1
(39) in cell culture systems and animal models results in protein aggregation but not fiber disintegration whereas animals deficient in DES (40), CRYAB (41), ZASP (42), or BAG3 (43) exhibit increased susceptibility to damage, muscular dystrophy, and fiber disintegration phenotypes.

Whilst protein insufficiency due to sequestration and subsequent myofibrillar disintegration may be a common mechanism in myofibrillar myopathy, mutation specific changes also contribute to the phenotypic presentation. The disruption to myofibrillar patterning in FLNC\(^{W2710X}\) expressing cells and reduced protein dynamics of FLNC\(^{W2710X}\), identified by immuno-CLEM and FRAP respectively, suggest that whilst the mutant protein retains the functions necessary for fiber integrity the mutation does disrupt other aspects of FLNC function that potentially contribute to muscle weakness prior to complete myofibrillar disintegration.

Uniquely amongst the proteins known to be mutated in myofibrillar myopathy FLNC has been identified as a target of BAG3-mediated CASA (25). We therefore examined the role of CASA in the clearance of FLNC\(^{W2710X}\) aggregates. We found that, consistent with the immunofluorescence studies from patient biopsies (24), BAG3 is contained within the FLNC\(^{W2710X}\) aggregates. Remarkably, despite the decrease in autophagy in \(bag3\) morphant embryos, there was a 40% reduction in the number of FLNC\(^{W2710X}\) protein aggregates following \(bag3\) knockdown, demonstrating that the presence of BAG3 was preventing degradation of the aggregates rather than promoting their clearance. Inhibition of autophagy in the Bag3 depleted cells prevented the reduction in aggregate number, demonstrating the clearance of aggregates following removal of Bag3 was autophagy dependent. Together this data
suggests that BAG3 is recruited to the mutant FLNC protein aggregates but their
degradation via CASA and alternative autophagy pathways is blocked. Interestingly,
it has been shown that in ageing cells there is a switch from BAG1 regulated
proteasomal degradation of proteins to BAG3 mediated autophagic degradation of
proteins (44). Aged cells therefore rely more heavily on BAG3 mediated autophagy to
clear aggregates and blocking of this pathway in FLNC\textsuperscript{W2710X} myofibrillar myopathy
may contribute to the late onset of the disease.

In addition to its role in CASA BAG3 is known to have anti-apoptotic functions (45).
BAG3 is selectively upregulated in various forms of cancer (46-49) and as such there
is on-going research to identify compounds that reduce BAG3 levels to make the
cancers susceptible to apoptosis (50). Our results, showing that decreasing BAG3
levels results in fewer FLNC\textsuperscript{W2710X} aggregates, suggests that these compounds may
also prove to be effective in reducing the severity of FLNC\textsuperscript{W2710X} myofibrillar
myopathy.

Given that our data has revealed a block in CASA and alternative autophagy
pathways in FLNC\textsuperscript{W2710X} expressing cells we investigated the potential for autophagy
promoting compounds to enhance aggregate clearance. Treatment with rapamycin, or
Carbamazepine results in the dramatic reduction of FLNC\textsuperscript{W2710X} aggregates to levels
comparable to FLNC\textsuperscript{wt}-eGFP expressing cells. This demonstrates that the
pharmacological upregulation of autophagy may be a suitable treatment for
myofibrillar myopathy. Furthermore, autophagy stimulation and Bag3 depletion
together resulted in a more pronounced reduction in aggregate frequency than either
strategy alone indicating that a combination of the two treatments may serve as a
In conclusion we have examined the mechanism of disease in FLNC^{W2710X} myofibrillar myopathy. Our studies reveal that the W2710X mutation does not remove the functions of FLNC required for the maintenance of Z-disk integrity. Instead, we propose that the formation of protein aggregates by FLNC^{W2710X}, which is exacerbated by a block in BAG3-mediated chaperone assisted selective autophagy, results in FLNC sequestration and ultimately insufficiency. This is further compounded by reduced protein-protein interactions affecting the sarcomeric function of the mutant protein. A reduction in protein aggregates following reduction of BAG3 and treatment with autophagy promoting compounds suggests these may be effective therapies for FLNC^{W2710X} myofibrillar myopathy, with the later potentially beneficial for the entire family of myofibrillar myopathies.
Materials and Methods

Fish maintenance

Fish maintenance and handling was carried out as per the standard operating procedures approved by the Monash Animal Services Animal Ethics Committee. All fish were maintained in the Fish Core facility at Monash University under breeding colony license MARP/2015/004/BC. All experiments were carried out on embryos of TU/TL background. Fish were anesthetized using Tricaine methanesulfonate (3-amino benzoic acid ethylester, Sigma) at a final concentration of 0.16% in E3 embryo medium (5 mM NaCl, 0.17mM KCl, 0.33 mM CaCl, 0.33 mM MgSO4 in water).

Overexpression constructs

The full length FLNC\textsuperscript{wt}-eGFP and FLNC\textsuperscript{W2710X}-eGFP constructs were provided by Dr. Peter van der Ven and Prof. Dieter Fürst (24) and subsequently cloned under the actc1b promoter (51) to generate the final expression construct. The constructs were injected at 100ng/µl into one cell stage embryos. The final mCherry tagged actinin3b expression vector was generated using LR Gateway recombination with the actc1b promoter in the 5’ entry cassette, full length zebrafish actinin3b in a modified form of middle entry cassette (52) and mCherry in the 3’ cassette. Both, the actinin3b-mCherry and previously published BAG3-mcherry constructs (53) were injected at 50ng/µl into one cell stage embryos.

Immunohistochemistry and confocal microscopy

To determine localization of the tagged proteins within the sarcomere
immunohistochemistry was performed according to previously described protocols (26). 4% paraformaldehyde (4% PFA) was used as a fixative for all antibody labels. The primary antibodies used in this study were anti-eGFP (Invitrogen, A-11122, 1:150), anti-Actinin (Sigma, A7811, 1:100), anti-Myosin (DSHB, A4.1025, 1:10). Embryos were mounted in 1% low melting point agarose and imaged using the Zeiss LSM 710 confocal microscope. The maximum intensity projections were obtained using Fiji (http://fiji.sc; (54))

**Morpholino microinjections**

All antisense morpholino oligonucleotides were obtained from Gene Tools and coinjected with Cascade Blue labeled dextran (Molecular Probes) at 0.01 mg/ml. The morpholinos presented in this study have previously been described and validated (26, 27): flnca MO (CATGGTGGGACTGCTGCTTTTATAC, 0.25µM), flncb MO (GAGTTTTCTAATGGCCCTTACCTGC, 0.5µM), a bag3 translation-blocking morpholino (TTGAGCCATTACAGCCGCTACAGAC, 0.5µM) and a bag3 splice site-targeting morpholino (GATAAATAACACACACACACCTACCTACAGAC, 0.5µM).

**Fluorescence recovery after photobleaching**

To examine protein dynamics FRAP experiments were performed on five-day-old embryos expressing FLNCwt-eGFP (n=13) or FLNCW2710X-eGFP (n=14) anesthetized in Tricaine and mounted in 1% low melting point agarose. Using a 20X 1.0 numerical aperture water dipping objective and a 488nm laser on a LSM 710 confocal microscope (Zeiss), a region of the muscle fiber was bleached with four iterations of 100% laser and imaged every 0.5 seconds for 200 seconds. Four images were taken prior to bleaching and averaged to obtain the pre-bleach value. The ImageJ package
Fiji was used to determine fluorescence intensity of bleached and unbleached areas at each time point. The data was obtained in triplicate and the analysis was carried out as per (55). Graphpad Prism was used to perform the two-phase non-linear regression analysis to determine the slow and fast half-lives and mobile fraction.

**Drug treatments**

Autophagy was examined by incubating 32-hpf embryos in chloroquine (Sigma; final concentration 100µM in E3 embryo medium), ammonium chloride (Sigma; final concentration 100mM in E3 embryo medium, (56)); rapamycin (Sigma; final concentration 10µM in E3 embryo medium), carbamazepine (Sigma; final concentration 0.5mM in E3 embryo medium) or spermidine (Sigma; final concentration 5mM in E3 embryo medium) for 16 h and imaged live using a Zeiss LSM 710 confocal microscope. Drug treatments were performed in triplicate and all images were blinded using Fiji before counting aggregates, to prevent any bias. The total number of fish examined for each drug treatment is detailed in the results section. A two-way ANOVA statistical test was used to test for significant changes in protein aggregation post-drug treatment.

**Western blot analysis**

Protein lysates were obtained as per (57) and quantified using the Bradford assay. 50µg of each sample, along with reducing agent (Invitrogen) and protein loading dye (Life Technologies), was heated at 70°C for 10 min, separated by SDS-PAGE on NuPAGE 4-12% Bis-Tris gels, and transferred onto PVDF membrane (Millipore). Following transfer, the membrane was blocked with 5% skimmed milk in PBST and subsequently probed with anti-Lc3 (Novus Biologicals, NB100-2220, 1/2000) and
anti-α-tubulin (Sigma, T6704, 1/2000), washed and incubated with HRP-conjugated secondary antibody (1:10,000, Southern Biotech). Immunoblots were developed using ECL prime (GE healthcare) and imaged using a chemiluminescence detector (Vilber Lourmat). The blot images were quantified using Image Studio (Licor) and a two-way ANOVA statistical test was used to test for significant changes in Lc3 accumulation.

**Conventional EM sample preparation**

24-hpf zebrafish embryos were fixed according to standard procedures in 2.5% glutaraldehyde, 2% paraformaldehyde in sodium cacodylate buffer, post-fixed in 1% OsO4, 1.5% K3Fe(III)(CN)6, and block-stained in 0.5% uranylacetate. The embryos were dehydrated in ethanol and were flat embedded in Epon 812. Ultrathin sections of 70nm were cut on a Leica Ultracut UCT7 and stained with uranylacetate and lead citrate. EM imaging was done on a Hitachi H-7500 TEM and a FEI Tecnai G2 Spirit TEM.

**Immuno Correlative Light Electron Microscopy (CLEM) sample preparation**

Fixation and embedding of 2-day zebrafish embryos was performed according to the Tokuyasu method (58, 59). Ultrathin cryo-sections were cut on a Leica FCS-UCT7. The combined fluorescence- and immuno-labeling was carried out as described in Oorschot et al. (59) using goat anti-GFP/Biotin and rabbit anti-Biotin (Rockland), goat anti-rabbit Alexa 488 (Molecular Probes), and 10nm ProteinA-gold (Department of Cell Biology, University Medical Center, Utrecht). Fluorescence Imaging was carried out using a Leica AF6000LX wide field microscope running the LAS AF software (Leica MicroSystems, Manheim, Germany). Imaging for low magnification EM overviews was done on a FEI Nova NanoSEM 450 at 30kV using the bright field
mode of the STEM3 detector and the MAPS software. Higher resolution EM images were taken at 80kV on a FEI Tecnai G2 Spirit TEM. For image analysis and correlation Fiji software was used.
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Conflict of Interest

The authors declare no conflicts of interest.
References


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Figure Legends

Figure 1. Expression of FLNC<sup>W2710X</sup>-eGFP results in the formation of protein aggregates. Sarcomeric and cytoplasmic localization of FLNC<sup>Wt</sup>-eGFP (A) or FLNC<sup>W2710X</sup>-eGFP (B) at 48-, 72-, 96- and 120-hpf. FLNC<sup>W2710X</sup>-eGFP additionally results in the formation of protein aggregates (arrowheads).
Figure 2. FLNC\textsuperscript{W2710X} -eGFP localizes to the Z-disk of the sarcomere. Perfect overlap of Actinin and GFP in 48-hpf FLNC\textsuperscript{wt} -eGFP (A) or FLNC\textsuperscript{W2710X} -eGFP (B) expressing embryos confirms Z-disk localization of tagged forms of FLNC. Complementary staining pattern of myosin and GFP in 48-hpf FLNC\textsuperscript{wt} -eGFP (C) or FLNC\textsuperscript{W2710X} -eGFP (D) expressing embryos demonstrates that both tagged forms of FLNC localize to the Z-disk.
Figure 3. Loss of FLNC results in fiber disintegration and the formation of filamentous aggregates. (A) Myosin antibody staining in 26-hpf uninjected (uninj) embryos showing muscle fibers spanning the length of the somite. (B) Embryos injected with morpholinos targeting both FLNC homologues (flnca+flncb MO) display catastrophic fiber disintegration and formation of Myosin enriched aggregates at both somitic boundaries, as seen by Myosin antibody labelling. (C) Transmission electron micrograph of slow muscle fibers from flnca+flncb MO embryos reveals presence of filamentous aggregates at myoseptal boundaries (arrows), and mitochondrial infiltration (arrowhead) in areas devoid of myofibrils.
Figure 4. FLNC$^{W2710X}$ is functional and capable of rescuing the loss of Flnc myofibrillar disintegration phenotype. (A) Myosin antibody label on embryos injected with both $flnc$ targeting morpholinos ($flnca+flncb$ MO) display severe myofibrillar disintegration. Fibers expressing FLNC$^{wt}$-eGFP (B) or FLNC$^{W2710X}$-eGFP (C) coninjected with $flnca$ and $flncb$ morpholinos are intact (arrowhead), despite failure of neighbouring slow muscle fibers, as seen by Myosin antibody staining. (D) In an affected somite of a $flnca+flncb$ MO embryo, 60% of slow muscle fibers undergo myofibrillar disintegration. In an affected somite 100% of FLNC$^{wt}$-eGFP or FLNC$^{W2710X}$-eGFP expressing slow muscle fibers are intact.
Figure 5. FLNC<sup>W2710X</sup> is less dynamic than FLNC<sup>wt</sup>. Time lapse images documenting fluorescence of FLNC<sup>wt</sup>-eGFP (A) or FLNC<sup>W2710X</sup>-eGFP (B) before bleaching (pre-bleach), immediately after (0 s), 5 s, and 100 s after bleaching in 5-day-old zebrafish embryos. (C) Mean fluorescence recovery profiles ± SE of FLNC<sup>wt</sup>-eGFP or FLNC<sup>W2710X</sup>-eGFP at the various time points. (D) Two-phase non-linear correlation analysis reveals no significant difference in the fast half-lives of FLNC<sup>wt</sup>-eGFP and FLNC<sup>W2710X</sup>-eGFP. A significant difference between the slow half lives of the tagged variants of FLNC is observed. (E) Mobile fraction of FLNC<sup>wt</sup>-eGFP or FLNC<sup>W2710X</sup>-eGFP as determined by the two-phase non-linear correlation analysis.
Figure 6. FLNC$^{W2710X}$-eGFP expressing fibers display ultrastructural defects. Immuno-correlative light and electron microscopy on FLNC$^{W2710X}$-eGFP expressing cells at 48-hpf. The nuclei positions (asterisk) in the fluorescent image (A) and
scanning transmission electron micrograph (B) are used to identify the transgene expressing cell. FLNC\textsuperscript{W2710X}-eGFP expressing cells contain misaligned myofibrils (B), thickened Z-disks (solid arrowhead) (C), disintegrating Z-disks (arrow) and areas of myofibrillar disarray (open arrowhead) (D). Immuno-correlative light and electron microscopy on FLNC\textsuperscript{wt}-eGFP expressing cells. The nuclei positions (asterisk) in the fluorescent image (E) and scanning transmission electron micrograph (F) are used to identify the transgene expressing cell. FLNC\textsuperscript{wt}-eGFP expressing cells have normal myofibrillar patterning (F) and normal sarcomere structure (G).
Figure 7. The CASA protein BAG3 is incorporated in FLNC$^{W2710X}$ aggregates. Live imaging of 48hpf embryos coinjected with BAG3-mCherry and FLNC$^{WT}$-eGFP (A) or FLNC$^{W2710X}$-eGFP (B) reveals inclusion of BAG3 in FLNC$^{W2710X}$ aggregates (arrowheads). Live imaging of 48hpf embryos coinjected with actinin3b-mCherry and FLNC$^{WT}$-eGFP (C) or FLNC$^{W2710X}$-eGFP (D) reveals absence of actinin3b in FLNC$^{W2710X}$ aggregates (arrowheads).
Figure 8. Impaired CASA results in increased protein aggregation. Confocal images of 48-hpf embryos expressing FLNC<sup>wt</sup>-eGFP (A) or FLNC<sup>W2710X</sup>-eGFP (B) treated with the autophagy inhibitors chloroquine (CQ) or ammonium chloride (NH<sub>4</sub>Cl) or coinjected with bag3 targeting morpholinos, with or without CQ and NH<sub>4</sub>Cl treatment. (C) Neither treatment with CQ or NH<sub>4</sub>Cl nor knockdown of BAG3 had any
effect on FLNC\textsuperscript{wt}-eGFP aggregation. Treatment with CQ or NH\textsubscript{4}Cl also had no
significant effect on the number of FLNC\textsuperscript{W2710X}-eGFP aggregates. Knockdown of
BAG3 however, resulted in a significant decrease in protein aggregation, which is
reversed with the treatment of CQ or NH\textsubscript{4}Cl. Error bars indicate standard error. *
significantly different from FLNC\textsuperscript{wt}-eGFP;  # significantly different from
FLNC\textsuperscript{W2710X}-eGFP.
Figure 9. Pharmacological stimulation of autophagy promotes the clearance of aggregates. Confocal images of 48-hpf embryos expressing FLNC\textsuperscript{wt}-eGFP (A) or FLNC\textsuperscript{W2710X}-eGFP (B) treated with the autophagy stimulators rapamycin (Rap), carbamazepine (CAZ) and spermidine (Sper). (C) Treatment of 32-hpf embryos expressing FLNC\textsuperscript{wt}-eGFP with Rap, CAZ and Sper has no effect on the formation of aggregates. Treatment of 32-hpf embryos expressing FLNC\textsuperscript{W2710X}-eGFP with Rap, CAZ and Sper however results in a significant decrease in the number of aggregates per cell with the number of aggregates seen following Rap and CAZ treatment comparable to that of FLNC\textsuperscript{wt}-eGFP. Error bars indicate standard error. * significantly different from FLNC\textsuperscript{wt}-eGFP; # significantly different from
FLNC\textsuperscript{W2710X}-eGFP.
**Abbreviations:**

BAG3 - bcl-2-associated athanogene 3  
CASA - chaperone assisted selective autophagy  
CLEM - correlative light and electron microscopy  
dpf - days post fertilisation  
FHL1 - four and a half LIM domain 1  
FLNC - filamin C  
FRAP - fluorescent recovery after photobleaching experiments  
hpf – hours post fertilisation  
ZASP - Z-band alternatively spliced PDZ motif-containing protein
FLNC myofibrillar myopathy results from impaired autophagy and protein insufficiency

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Supplementary Figure 1: Flnc is not required for aggregate formation.
At 48 hours post fertilization aggregates are evident in embryos co-injected with FLNC$^{W2710X}$-eGFP and flnca+flncb morpholinos (A; arrowhead) but not in control embryos injected with FLNC$^w$-eGFP and flnca+flncb morpholinos (B).
**Supplementary Figure 2**: Bag3 reduction results in decreased autophagic activity. (A) Western blot for Lc3 and α-tubulin (loading control) on protein lysates obtained from wildtype embryos or bag3 MO embryos treated with chloroquine (CQ) and/or rapamycin (Rap). (B) Lc3 levels corrected for protein loading and normalised to the levels in the respective untreated group. The increase in Lc3 following CQ treatment indicates autophagic activity, which is reduced in bag3 MO injected embryos. Error bars indicate standard deviation. ns = not significant, * p<0.05, ** p<0.01, ***p<0.001
Supplementary Figure 3: Autophagy induction and reduced bag3 results in pronounced aggregate clearance. While stimulation of autophagy with spermidine treatment (blue) and knockdown of bag3 (red) both promote aggregate reduction a combination (green) of the two results in a more pronounced aggregate reduction compared to either treatment alone. Error bars indicate standard error. * p<0.05, ** p<0.01, ***p<0.001