Myofibrillar instability exacerbated by acute exercise in filaminopathy

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Abstract
Filamin C (FLNC) mutations in humans cause myofibrillar myopathy (MFM) and cardiomyopathy, characterized by protein aggregation and myofibrillar degeneration. We generated the first patient-mimicking knock-in mouse harbouring the most common disease-causing filamin C mutation (p.W2710X). These heterozygous mice developed muscle weakness and myofibrillar instability, with formation of filamin C- and Xin-positive lesions streaming between Z-discs. These lesions, which are distinct from the classical MFM protein aggregates by their morphology and filamentous appearance, were greatly increased in number upon acute physical exercise in the mice. This pathology suggests that mutant filamin influences the mechanical stability of myofibrillar Z-discs, explaining the muscle weakness in mice and humans. Re-evaluation of biopsies from MFM-filaminopathy patients with different FLNC mutations revealed a similar, previously unreported lesion pathology, in addition to the classical protein aggregates, and suggested that structures previously interpreted as aggregates may be in part sarcomeric lesions. We postulate that these lesions define preclinical disease stages, preceding the formation of protein aggregates.

Introduction
Filamin C-related protein aggregate myopathies belong to the myofibrillar myopathies (MFMs), a numerically significant subgroup of hereditary and sporadic protein aggregate myopathies with marked clinical and genetic heterogeneity due to mutations in desmin, αB-crystallin, BAG-3, FHL1, myotilin, plectin and ZASP (1,2). Filamins are large actin cross-linking proteins interacting with a plethora of ligands of great functional diversity, indicating
both structural and signalling roles (3–6). Mutations in the ubiquitously expressed variants FLNA and FLNB cause a wide variety of congenital malformations, affecting brain, bone and other organs (7–9). Heterozygous mutations of the human FLNC gene (located on chromosome 7q32; OMIM 102565) encoding the isoform mainly expressed in striated muscles cause late-onset, autosomal-dominant and sporadic myopathies, and cardiomyopathies (10–16). Filamin C-associated myopathies comprise three classical disease manifestations: (i) protein aggregation myopathy affecting skeletal and cardiac muscles with initial proximal weakness, (ii) distal myopathies due to haploinsufficiency or altered actin-binding capacity without protein aggregation pathology and (iii) isolated hypertrophic cardiomyopathy without symptoms of skeletal myopathy (11,16). We described the first pathological mutation in filamin C (p.W2710X) in families from ethnically diverse populations with a protein aggregation myopathy phenotype, implying that codon 2710 is a mutational hotspot (10,13,17). This mutation causes a deletion of the carboxy-terminal 16 amino acids, which abolishes the intrinsic dimer formation of filamin C, making the mutant protein more prone to proteolysis and aggregation in striated muscles (10,18). The aggregation pathology is characterized by sarcoplasmic and subsarcolemmal filamin C- and desmin-positive protein aggregates in conjunction with degenerative myofibrillar changes (1,2).

Muscle biopsies from affected patients reflect only late stages of the disease process and are only available in small amounts. Moreover, biopsy material from preclinical, early and intermediate disease stages is usually not accessible. With the purpose of being able to perform pathophysiological and therapeutic intervention studies, we generated the first knock-in mouse model for human MFM-filaminopathy. In our mice, the expression of mutant W2710X filamin C (W2711X in mice) is controlled by endogenous gene regulation sites, thus providing a patient-mimicking genetic disease model. The subsequent analysis unravelled a novel principle of pathogenesis, in which the mutant filamin C interferes with the mechanical stability and stress resistance of myofibrillar Z-discs, subsequently leading to progressive muscle damage.

**Results**

**Heterozygous W2711X filamin C knock-in mice: a patient-mimicking pathogenetic model for human W2710X filaminopathy**

Heterozygous W2711X filamin C knock-in mice were generated using a gene-targeting strategy, which replaced the triplet ‘TGG’ by ‘TGA’, encoding a stop codon in exon 48, mimicking the most common MFM-filaminopathy mutation in man (10). Since mouse FLNc has an extra amino acid in the amino-terminal actin-binding domain, the codon 2711 in mice corresponds to codon 2710 in the human ortholog. After homologous recombination and removal of the neomycin selection cassette by Flp-mediated excision, the original gene structure is preserved and only one FRT site in intron 46 and two loxP sites in exon 46 and in the 3’ untranslated region (UTR) encoding part of exon 48, respectively, remain. Southern blotting, polymerase chain reaction (PCR) genotyping and sequencing of genomic DNA confirmed correct targeting (Fig. 1).

Mating of heterozygous filamin C knock-in mice (10 independent crossings; n = 73 newborn mice) resulted in 26% wild type (WT) (n = 19), 47% heterozygote (n = 34) and 27% homozygous mice (n = 20) indicating Mendelian inheritance, and suggesting no prenatal or perinatal death of W2711X knock-in mice. Since the heterozygous genotype mirrors that of filaminopathy patients, and direct comparison of the human and mouse filaminopathy pathology was a primary goal, we here focus on these mice and refer to them as ‘mutant’ mice, and compare them with WT littermates.

**Expression levels of wild-type versus mutant filamin C mRNA and protein**

To determine expression levels of WT and W2711X mutant Flnc mRNAs in our mice, we performed real-time reverse transcriptase-polymerase chain reaction (RT-PCR) (Fig. 2A) and semi-quantitative multiplex RT-PCR (Supplementary Material, Fig. S1C) using RNA purified from different muscles and primer pairs amplifying both WT and mutant Flnc CDNAs. Both indicated no significant differences in total Flnc mRNA levels in mutant animals. Furthermore, experiments with a primer pair that enables discrimination of both genotypes due to the presence of the loxP site in exon 48 in mutant animals indicated that both alleles are expressed at similar levels in mutant animals (Fig. 2B). To exclude that the lack of intact filamin C was compensated by increased expression of filamin A and/or B, mRNA expression levels of Flna and Flnb were analysed. However, no significant differences between the two genotypes were detected (Supplementary Material, Fig. S1A and B). Concomitantly, Flnc was confirmed as the main isoform in skeletal muscle (Supplementary Material, Fig. S1D).

Subsequently, we analysed filamin C protein expression in our mice with two different anti-filamin C antisera. One pan-filamin C antiserum recognizing both WT and mutant variants showed a highly similar protein level in both genotypes (further confirmed by proteomic analysis, Supplementary Material, Table S1). A second antiserum raised against a peptide representing the carboxy-terminal 16 amino acids of filamin C that are lacking in the mutant variant (that does not recognize filamins A and B and mutant filamin C, Supplementary Material, Fig. S7) showed that the WT filamin C level in the mutant animals was approximately half of that of the WT mice (Fig. 2C). This indicates that approximately half of the total filamin C in mutant animals represents mutant filamin C. Comparison of protein expression levels of the MFM aggregate markers BAG3, desmin, Hsp70 and Xlnb in WT and mutant animals by quantitative western blotting did not reveal significant differences (Fig. 2D).

**Progressive muscle weakness in mutant mice**

Analysis of the body weight of mutant and WT mice revealed no significant differences at any age between 2 and 12 months (Fig. 3A). No dysmorphology such as kyphosis or focal muscle atrophy was observed in mutant mice at any age (data not shown). Animals were monitored repeatedly for motor function every 2 months up to 12 months by hanging-wire test to monitor sustained limb strength (maximal time: 5 min; Fig. 3B) and direct grip strength measurements of the forelimbs and all four paws (Fig. 3C). Remarkably, hanging-wire tests showed a decreased latency to fall in mutant mice at all ages, becoming significant from the age of 10 months. However, statistically significant changes in the forelimbs and four paw grip strength measurements between the WT and mutant mice were only noted at the age of 4 months and at the ages of 4 and 8 months in the 4-paw (significant interaction P < 0.0001) and forelimb grip strength testing (P < 0.05), respectively. Altogether, older mutant mice demonstrated moderate yet significant signs of muscle weakness.
Muscle pathology in mutant mice

Staining of transverse cryosections from soleus (Supplementary Material, Fig. S2), quadriceps and gastrocnemius muscles (not shown) of WT and mutant mice (3 and 8 months of age, 4 mice per genotype) with haematoxylin and eosin (HE), modified Gomori trichrome, succinate dehydrogenase (SDH) and cytochrome oxidase (COX) essentially showed no obvious differences between WT and mutant animals. However, analysis of longitudinal sections of both genotypes by electron microscopy (EM) revealed many pathological alterations in the mutant mice that remained undetected by routine histochemical staining procedures. Already in 3-month-old mutant mice, fibres from soleus muscles displayed Z-disc streaming, i.e. sarcomeric disruptions characterized by electron-dense material bridging adjacent Z-discs (Fig. 4A), resembling the sarcomeric ‘lesions’ typically seen after eccentric exercise (19,20). In other areas, myofibrillar degeneration with disassembled Z-discs (Fig. 4B, asterisks) associated with fused, enlarged mitochondria was seen (Fig. 4B, arrowheads). Myofibrils at different stages of degeneration were observed, ranging from mild Z-disc pathology (Fig. 4C) over disassembly of the thin-thick filament systems (Fig. 4D), to complete disintegration of the sarcomere with scattered remnants of electron-dense material (Fig. 4E). This suggests a progressive and profound remodelling of sarcomeric structures. Furthermore, areas with subsarcolemmal accumulations of autophagic vacuoles indicating increased proteolytic activity were observed (Fig. 4F and G).

Though sedentary aged mutant animals did not develop muscle dystrophic changes, skeletal muscle specimens from 16-month-old mutant mice occasionally displayed degenerating fibres (Supplementary Material, Fig. S3A) not seen in aged-matched WT mice. Note that the presence of ‘tubular aggregates’ seen in our 16-month-old male mutant mice (Supplementary Material, Fig. S3B) is a well-documented phenomenon in WT male mice (21,22) and is not linked to the pathology under investigation.

Myofibrillar lesion formation in mutant mice and filaminopathy patients

Although we did not find typical signs of MFM-related protein aggregation by histochemical staining, immunohistochemistry revealed focal Z-disc streaming in longitudinal sections,
particularly in solei of mutant mice. Z-disc streaming was characterized by intensified lamin C staining bridging two or more neighbouring Z-discs (Fig. 5Ai), resembling previously described sarcomeric lesions after eccentric exercise (23). They correspond to sarcomeric lesions seen by EM (Fig. 4A). Occasionally, these structures spanned very large areas, across many myofibrils and covering almost the entire fibre width (Fig. 5Aii). To differentiate between the extent of those structures, we refer to them here as microlesions (spanning up to five sarcomeres) and macrolesions (Fig. 3).

Figure 3. Body weight and clinical phenotyping. (A) Body weight of mice carrying the heterozygous recombinant W2711X allele (Mut) and their WT littermates at different ages between 2 and 12 months. No significant differences in body weight are observed between WT and mutant mice at any age. (B) Hanging-wire tests performed at different ages show a statistically significant decreased latency to fall in mutant mice starting at the age of 10 months (10 months, P = 0.0177; 12 months, P = 0.0038). (C) Forelimbs and four paw grip strength measurements of WT and mutant mice aged between 2 and 12 months. Statistically significant changes were only noted at the age of 4 months (P < 0.001) and at the ages of 4 (P = 0.0362) and 8 (P = 0.0241) months in the 4-paw and forelimb grip strength testing, respectively (WT: n = 5; Mut: n = 7; error bars represent standard deviation).

Figure 2. Expression of filamin C mRNA and protein levels in soleus muscles of WT and mutant (Mut) mice. (A) Real-time PCR (with Gapdh as internal normalization control) indicates no significant differences in total Flnc mRNA levels between WT and mutant mice. (B) RT-PCR analysis shows that both the WT and recombinant W2711X knock-in allele (KI) are expressed in mutant animals (amplicons derived from the recombined locus are longer since they include the loxP site in exon 48). (C) Analysis of filamin C protein expression in WT and mutant mice muscles using an antiserum recognizing both filamin C variants (Flnc total) and an antiserum specific for WT filamin C (Flnc WT). Total filamin C levels are not altered compared with WT animals, whereas WT filamin C levels are approximately half of the total level, indicating that the other half represents mutant filamin C. (D) Comparison of the protein expression levels of the MFM aggregate markers BAG3, desmin, Hsp70 and XinB in WT (red line) and mutant animals. None of the analysed proteins is expressed at significantly altered levels in mutant animals. GAPDH was used as normalization control.
more than five sarcomeres and across multiple myofibrils). Macrolesions were frequently seen in close association with blood vessels (Supplementary Material, Fig. S4). In transverse sections, lesions were seen as high-intensity filamin C-positive areas of variable size (Fig. 5Aii). Furthermore, COX and SDH stains of transverse serial sections suggested the absence of mitochondria in those regions (Supplementary Material, Fig. S5). Even though such lesions were not found in every fibre when examining a section, they were, however, seen in each preparation.

We subsequently looked for the presence of such micro- and macrolesions in longitudinal sections of the diagnostic muscle biopsies of human filaminopathy patients carrying three different mutations in filamin C [W2710X, V930_T933del and a recently identified mutation in domain 24, see Patient 6 in (24)]. Indeed, we observed micro- and macrolesions in all three samples (Fig. 5; Supplementary Material, Fig. S6), indicating that the lesion pathology is a common phenomenon in human and mouse filamin C-associated pathology. However, MFM-related protein aggregates were only observed in patient (Fig. 5B and C; Supplementary Material, Fig. S6) and not in mutant mouse skeletal muscle tissue. Sarcomeric lesions can be distinguished from amorphous and rounded aggregates via their filamentous appearance and their association with the myofibrils in a striated manner (see Supplementary Material, Fig. S8). Interestingly, in
patients, both structures were positive for the sarcomeric lesion-markers filamin C, desmin, Xin (Fig. 5C; Supplementary Material, Fig. S7) and myotilin (Supplementary Material, Fig. S7). Regarding desmin, it is noteworthy that its immunoreactivity was accentuated at the periphery of individual aggregates in the W2710X patient (Fig. 5C).

Acute strenuous exercise exacerbates the formation of lesions in limb muscles and diaphragm of mutant mice

We then examined the effects of a single bout of acute strenuous treadmill exercise on the formation of lesions in soleus muscles of mutant and WT mice. For quantification of lesion formation, transverse sections were stained to visualize the effect across

Figure 5. Soleus muscles of the mutant mouse show sarcomeric lesions similar to those found in human MFM-filaminopathy. (A) (i) Filamin C staining of mutant mouse muscle fibres reveals small filamin C-positive ‘microlesions’ (µ), spanning 1–5 sarcomeres, (ii) ‘macrolesions’ (M) spanning larger areas are also seen and (iii) both structures are identifiable in transverse sections. (B) Muscle fibres of a human W2710X filaminopathy patient contain similar micro- and macrolesions flanked by large filamin C-positive protein aggregates (Ag) appearing rounded and amorphous, whereas lesions have a striated appearance. Boxed areas in (B) are shown enlarged below the upper figures. Lesions (µ, M) and protein aggregates (Ag) are positive for filamin C and Xin, known markers of sarcomeric lesions. (C) Aggregates (Ag) in W2710X patient muscle fibres contain filamin C, Xin and desmin. Note that desmin is accentuated in the periphery of the aggregates. Scale bars: 10 µm.
the whole muscle. Large areas positive for the lesion marker Xin, corresponding to macrolesions, were present in muscles of exercised mutant animals but not in exercised WT control muscles (Fig. 6A). Micro- and macrolesions were quantified (Fig. 6B). Even though some microlesions were occasionally found in non-exercised WT solei, their number was significantly higher in the non-exercised mutant mice. Moreover, large macrolesions seen already in sedentary mutant mice were not observed in sedentary WT mice. The chosen acute strenuous exercise protocol had a dramatic effect on the occurrence of both lesion types. Whereas running WT mice showed a moderate increase in the number of microlesions, the effect in mutant mice was considerably more pronounced. In parallel, also the number of exercise-induced macrolesions was dramatically increased in mutant mice (Fig. 6). Altogether, these results show that soleus muscles of mutant mice are more prone to myofibrillar instability and damage than WT mice, and that this is exacerbated during acute intensive exercise.

As the macrolesions were generally located near the sarcolemma and could be mistaken for the well-known MFM-related protein aggregates, we further characterized their pattern of immunoreactivity with established lesion and sarcolemma marker.

**Figure 6.** High prevalence of sarcomeric lesions in the soleus muscles of mutant animals, exacerbated by treadmill exercise. (A) Examples of transverse sections through soleus muscles after exercise; individual fibres demarcated by staining for the membrane protein caveolin 3 (Cav3, red), lesions detected by the lesion marker Xin (green). Note the high prevalence of fibres with Xin-positive areas in the mutant. (B) Prevalence of micro- and macrolesions in the WT and mutant animals before and after exercise, expressed as the number of lesion-positive fibres per section. Even before exercise, significantly higher numbers of both micro- and macrolesions are seen in mutant muscles compared with WT muscles. After exercise, the number of both structures increased in both genotypes, with a significantly higher number of macrolesions in the mutant animals. (C) Xin-positive macrolesions as seen in longitudinal sections are most commonly associated with the fibre membrane. (D) Membrane marker ankyrin-G (Ank G) is seen weaker or absent in positions where macrolesions meet the membrane. (E) Membrane-bound macrolesions detected in transverse sections are positive for BAG3 and HSP27 but negative for desmin. Scale bars: (A) 200 µm and (C–E) 10 µm.
proteins. Like filamin C, the lesion marker Xin strongly stained macrolesions (Fig. 6C). The well-known costameric filamin C-binding partners ankyrin-G (Fig. 6D) and caveolin-3 (data not shown) were markedly reduced or even absent in sarcolemmal areas adjacent to macrolesions. Transverse sections through macrolesions also showed immunoreactivity with the autophagy marker BAG3 and the small heat shock protein HSP27 (Fig. 6E), both established protein aggregate components. This suggests that structures typically identified as aggregates in such sections may in fact be macrolesions. Although morphologically distinct, both macrolesions and protein aggregates show increased immunoreactivities for the same markers. Surprisingly, the classical MFM-marker desmin was not enriched in the macrolesions in the mouse (Fig. 6E), even though both lesions and aggregates in the patient were desmin positive either in the centre or the periphery of these structures (Fig. 5C; Supplementary Material, Fig. S7).

Since respiratory insufficiency is one of the most severe complications in MFM-filaminopathy patients, we also analysed the appearance of lesions in the diaphragm of exercised mice. While staining for filamin C did not reveal any lesions in the diaphragms of WT mice (Fig. 7B and D), numerous lesions were seen in the fibres of mutant animals (Fig. 7A and C), indicating that those muscles are especially vulnerable to the strain induced by exercise.

Effects of acute exercise on physical activity and respiratory exchange ratios in mutant mice

The drinking and feeding behaviour, metabolic performance and home-cage activity in X + Y as well as Z planes of mutant and WT mice were monitored before and after treadmill exercise using a fully integrated, automated intra-home-cage monitoring system. Both mutant and WT mice showed a significant reduction in total locomotor activity after exercise compared with corresponding dark-phase activity levels measured before exercise; however, the effect was stronger in mutant mice (Fig. 8A). Even more pronounced, after acute exercise rearing activity (Z plane activity counts) was significantly reduced only in mutant mice ($P = 0.008$), while that of WT mice remained largely unchanged (Fig. 8B). This may reflect an increased post-exhaustion effect in mutant mice.

The respiratory exchange ratio (RER) is defined as the ratio of CO$_2$ produced and O$_2$ consumed. Values approaching 0.70 are indicative of fat being the predominant source of energy, whereas those around 1.00 refer to a more carbohydrate-based metabolism. The RER of all mice tested followed a circadian pattern with higher values during activity (dark phases) than at rest (light phases) (Fig. 8C). However, compared with WT mice, RER values of mutant mice were slightly lower at baseline conditions (Fig. 8C). Food consumption of mutant animals was also slightly reduced (Supplementary Material, Fig. S9A). After acute exercise, mean dark-phase RER values were significantly reduced in mutant mice (Fig. 8D and E), which is due to a lower mean CO$_2$ production, while O$_2$ consumption remained rather constant (Supplementary Material, Fig. S9B and C). These data are congruent with reduced food consumption during the dark phase after acute exercise (Supplementary Material, Fig. S9A), as well as the diminished post-exercise activity levels observed in both genotypes (Fig. 8A). However, the altered RER values in mutant animals could also be aggravated by insufficient respiratory biomechanics inflicted by exercise-induced multiple lesion formation in the fibres of the diaphragm (Fig. 7).
FLNC mutations cause a late-onset protein aggregate myopathy and cardiomyopathy leading to progressive muscle weakness, often causing premature death (10–12,17,25). The lack of cell and animal models for the disease and of patient biopsy material from preclinical disease stages has impeded deciphering the molecular pathogenesis behind the onset and progression of the disease. To overcome these limitations, we generated an MFM-filaminopathy mouse model, harbouring the mouse ortholog of the most prevalent human p.W2710X filamin C mutation (10).

Our heterozygous W2711X Flnc knock-in mice are the first patient-mimicking animal model for this disease. To ensure that these mice indeed reflect the situation in patients, we confirmed that the mutant allele is expressed without elimination of mutant mRNA by nonsense-mediated decay. WT and mutant filamin C proteins were expressed in similar quantities, with the total filamin C amount comparable with that of WT mice. The latter finding was further corroborated by proteomic analysis. This rules out that our gene-targeting strategy leads to haploinsufficiency, or otherwise reduced levels of filamin C, as has been...
reported in other filamin C-related mouse (26) or fish models (27,28).

Histological analyses of skeletal muscle from our sedentary heterozygous mice did not show any overt alterations up to the age of 8 months. However, at the ultrastructural level, abnormalities such as enlarged mitochondria and autophagic vacuoles were already observed in 3-month-old mutant mice. Furthermore, even though no MFM-associated protein aggregates were seen, different stages of myofibrillar degeneration starting at Z-discs, and myofibrillar lesions were observed. These lesions are areas of focal disruption resembling those implicated in post-exercise remodelling (29,30). They appear as electron-dense material ‘streaming’ between adjacent Z-discs and have also been described as Z-disc streaming (19,20,29,31). Lesions border on a Z-disc and can span as little as a single sarcomere or extend across multiple sarcomeres and include several laterally neighbouring myofibrils. They display strongly enhanced staining for filamin C, its binding partners Xin, myotilin, accinulin, and actin, as well as desmin (23,30,32–34). Even though these structures are also found in healthy, unexercised individuals (35), their prevalence increases after eccentric exercise (20,36–39). Here, we show that this prevalence is significantly higher in our mutant mice, even when those were sedentary. Note that in our mice (in contrast to the patients), macrolesions were negative for desmin. It has been documented, however, that young lesions are desmin negative, only becoming desmin positive later, after remodelling has been initiated (23,32), and sampling was performed very soon after the exercise experiments in our mice.

We validated the lesion pathology in human MFM-laminopathies by analysing longitudinal sections from diagnostic muscle biopsy specimens from patients carrying different MFM-causing FLNC mutations. This revealed—in addition to the characteristic amorphous protein aggregates—a large number of micro- and macrolesions. Indeed, structures previously characterized as aggregates or early mini-aggregates in transverse muscle sections are at least in part macrolesions and microlesions, respectively (24). Since macrolesions in our mice appeared negative for histological mitochondrial stains, a characteristic of MFM-associated ‘rubbed-out areas’ (40,41), we cannot exclude that in patients some of these areas correspond to macrolesions or aggregates. Thus, our data in mice and men demonstrate that lesions are a typical sign of MFM-laminopathies (and possibly other MFM’s) in early and late stages of the disease. In the routine diagnostic work-up of human muscle biopsies, the standard interpretation of transverse sections; however, differentiation between lesions and aggregates is not possible by that approach, especially as the protein markers commonly used for detection are present in both structures.

Intra-home-cage monitoring including indirect calorimetry suggested that the respiration of mutant animals is affected post-exercise. Formation of sarcomeric lesions is considered to cause post-exercise muscle weakness, as they represent disruptions of the force-generating myofibrils (39,42). Therefore, the lesion pathology observed in diaphragm and skeletal muscles of our mutant mice may explain both respiratory insufficiency and muscle weakness experienced by MFM-laminopathy patients. In contrast to lesions, protein aggregates in patients are cytosolic and largely found flanking intact myofibrils. We, therefore, propose that the lesion pathology, rather than the presence of cytoplasmic aggregates, is the leading cause of muscle weakness in these patients. Assuming that the occurrence of lesions is a normal process in striated muscles, their increased occurrence in our mutant mice and MFM-laminopathy patients indicates either a decreased stability of myofibrils or a delay in repair processes.

The presence of lesions and aggregates in single fibres also provides a connection between both pathologies. The presence of lesions even in sedentary mutant mice suggests that they may form in patients long before the presentation of a clinical phenotype and could be utilized as a preclinical predictor of myopathy. It has been previously postulated that macrolesions seen after eccentric exercise may escalate progressively from microlesions (43). Based on our findings in mice and men, it is tempting to speculate that macrolesions eventually give rise to amorphous protein aggregates in MFM-laminopathy patients. Diagnostic specimens of patients showing massive aggregation pathology are only taken after the disease has progressed to a symptomatic phenotype, usually at an age that can hardly be recapitulated in mice. Indeed, large protein aggregates typical for MFM-laminopathy patients were not observed in our mutant mice. We, therefore, suggest that protein aggregation represents a late stage of the disease and is a consequence of the increasing formation of lesions and/or progressive difficulties to cope with the high demand for repair. Currently, the most accepted assumption is that during early life, damaged filamin C (WT as well as mutant) is continuously removed from muscle fibres, mostly by chaperone-assisted selective autophagy (CASA) and the ubiquitin-proteasome system (44,45). The high level of mutant filamin C in our W2711X mice, however, indicates that even in early life these processes are not sufficiently active to efficiently remove misfolded, mutant filamin C. In addition, it suggests that laminopathy patients express equal large quantities of mutant filamin C in their muscles. Considering the absence of protein aggregates in the mice and the many unaffected Z-disc- and sarcolemmal protein-binding sites in mutant filamin C, we assume that it is localized together with the normal protein at Z-discs and sarcolemma. In contrast to overexpressed mutant filamin C in cultured cells (17,18), it does not seem to spontaneously aggregate. Since we cannot specifically immunolocalize the mutant protein, we cannot rule out that it remains soluble until its degradation. In any case, its expression does not lead to clinically pronounced muscle weakness in the first decades of patient’s life. The reason for the development of aggregates only in later life remains to be determined, but is probably due to overstrain of the ubiquitin proteasome and autophagy pathways that show reduced activity in later life (reviewed in (46,47)).

The Z-disc pathology and lesion formation in mutant animals can be explained on the basis of the proposed functions of filamin C. At Z-discs, it interacts with multiple partners such as myotilin, accinulin, myopodin and FATZ/calsarcin/myozenin (34,48–53). These interactions and its upregulation early during myocyte differentiation imply a role for filamin C in myofibril assembly and maintenance (26,48,54). The human W2711X mutant filamin C lacks the last 16 amino acids of Ig-like domain 24 and is unable to dimerize in vitro (10,18,55). This probably interferes with its cross-linking and stabilizing function at Z-discs, thereby causing the observed Z-disc pathology. At the sarcolemma, filamin C interacts with γ- and δ-sarcoglycans, ankyrin-G and ponsin (54,56,57). We previously demonstrated reduced binding of the truncated domain to both sarcoglycans (18), implying that it can no longer associate with the dystrophin-associated glycoprotein complex. This might destabilize this complex and increase the prevalence of local membrane damage. Indeed, we found reduced ankyrin-G and caveolin 3-staining at the sarcolemma flanking macrolesions. Notably, these macrolesions frequently occurred in the vicinity of blood vessels, adding local mechanical pressure to the flanking muscle fibres, further indicating their increased mechanical susceptibility to damage. Taken together, the expression of mutant filamin C induces a dual pathology at
the level of Z-discs and the sarcolemma, presumably due to its impaired stabilizing function.

Beyond novel insight into the molecular pathogenesis of filaminopathies, our work has implications for counselling of affected patients. Acute strenuous exercise leads to a dramatic increase in myofibrillar lesion pathology. Translated into the human disease context, our findings strongly imply that MFM-filaminopathy patients are more susceptible to acute exercise-induced myofibrillar damage, which is likely to contribute to the progressive muscle weakness. However, the important question if chronic, low-intensity exercise is beneficial or harmful will have to be addressed in future studies. Furthermore, in this work, we did not examine the cardiac phenotype of our mice, something that should be addressed in the future as a potential way to study the cardiomyopathy element in filaminopathy.

Taken together, our work has unravelled novel insight into the pathomechanisms underlying MFM-filaminopathy: (i) mutant filamin C interferes with mechanical stability and strain resistance of myofibrillar Z-discs; (ii) this reduced stability is the basis of the formation of micro- and macrolesions, which we regard as preclinical disease stages preceding development of the characteristic protein aggregates; and (iii) the lesion pathology (rather than the formation of protein aggregates) may be the major contributing factor to muscle weakness experienced by patients. Based on these findings, we propose that acute strenuous exercise may significantly enhance progressive myofibrillar damage and thus should be avoided in MFM-filaminopathy patients.

Materials and Methods

Generation of the Flnc W2711X knock-in mouse model

The knock-in strategy was designed and carried out by genOway (Lyon, France). The Flnc gene-targeting vector was constructed from genomic C57Bl/6 mouse strain DNA. The p.W2711X point mutation was inserted into Flnc exon 48, while a Neo cassette flanked by Flp recombinase target (FRT) sites was inserted in intron 46 (Fig. 1B).

The linearized targeting vector was transfected into C57Bl/6 embryonic stem (ES) cells. G418-resistant clones were isolated, amplified and genotyped by PCR and Southern blot analysis. PCR analysis was performed using primers 72 772 and 0069-Neo (for primer sequences see Supplementary Material, Table S2) that specifically amplifies the targeted locus. The products of a second PCR (using primers 72 797 and 72 798) were sequenced to validate the presence of the mutation. The targeted locus was confirmed by Southern blot analysis using internal and external probes on both 5' and 3' ends. Fourteen clones were identified as correctly targeted.

Several clones were microinjected into C57Bl/6J-Tyr2-2/J blastocysts and gave rise to 13 male chimeras with a significant ES cell contribution. These mice were bred to C57Bl/6 mice expressing Flp recombinase to remove the Neo cassette. Genotyping primers (72 777, 72 778) were designed to discern between the different alleles by PCR, and selected animals were further validated by Southern blot analysis using a 5'-external probe.

Mice were housed in isolated ventilated cages (IVC) equipped with spruce granulate embedding and a nest under specific pathogen-free (SOPF) conditions at 22 ± 2°C, 50–70% air humidity, 70 air exchanges per hour and a light-dark cycle of 12/12 h with free access to water and food. Littermates were separated at weaning by sex and housed at maximal five animals per cage. Health monitoring was done as recommended by the Federation of European Laboratory Animal Science Associations (FELASA). Mice were handled in accordance with the German Animal Welfare Act and the German Regulation for the protection of animals used for experimental purposes or other scientific purposes. For experiments, mice were euthanized by a lethal dose of isoflurane followed by cervical dislocation.

Genotyping of mice

Tail DNA was obtained using DNeasy Blood & Tissue Kit (Qiagen) according to instructions of the manufacturer. Primers 72 777 and 72 778 were used for identifying the Flnc W2711X and WT alleles (Fig. 1; Supplementary Material, Table S2).

Expression at mRNA level

RNA was isolated from the soleus muscles of 2-month-old WT and mutant animals using TRIzol reagent (Life Technologies) or the RNeasy Fibrous Tissue Mini Kit in combination with TissueLyser LT (Qiagen, Hilden, Germany). First-strand cDNA was synthesized using the Superscript First-strand Synthesis System (Life Technologies) or Omniscript RT Kit (Qiagen) with random hexamers, according to the manufacturers. Multiplex, semi-quantitative RT-PCR (25–30 cycles) of lamin and Gapdh as endogenous reference was performed using 5X FIREPol Master Mix (Solis BioDyne, Tartu, Estonia). For quantification, all reactions were performed in triplicate, using three different cDNA samples prepared from different individuals. Bands were quantified using ImageJ and normalized to the endogenous reference, before means were calculated and plotted.

Real-time PCR was performed using Applied Biosystems 7500 Real-Time PCR system and Assay on Demand reagents according to the recommendations of the manufacturer (Applied Biosystems). The expression levels obtained were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (Gapdh). Assay-on-Demand references for Flnc and Gapdh were Mm00471824_m1 and Mm99999915_g1, respectively.

Infrared western blot analysis and protein quantification

Muscle samples were weighed and snap frozen in liquid nitrogen. Samples were mechanically disrupted using a TissueLyser LT (Qiagen) at 50 Hz and dissolved in 15 µl urea buffer [2 M thiourea, 7 M urea, 5 mM EDTA, 1 mM DTT and protease inhibitors (Sigma, P8340) in 100 mM Tris pH 8.6] per 1 mg of muscle sample by homogenization for 3 min at 50 Hz. Protein concentrations were determined using the 2D Quant Kit (GE Healthcare Life Sciences, Freiburg, Germany). SDS sample buffer with a concentration of 5x was added to a final concentration of 2x, and samples were incubated for 5 min at 55°C. Proteins were separated by SDS-PAGE and transferred onto a polyvinylidenefluorid (PVDF) membrane. Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Bad Homburg, Germany). Primary antibodies diluted in Tris-buffered saline with 0.05% Tween 20 (TBST) were applied overnight at 4°C. Subsequently, membranes were washed in TBST and incubated with IRDye-680- or -800-conjugated secondary antibodies (LI-COR Biosciences). Samples were analysed using a LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences). Bands were quantified using the Odyssey Infrared Imaging Software v. 1.2 (LI-COR Biosciences) and normalized to GAPDH.

Antibodies

Antibodies used in this study and their dilutions are given in Supplementary Material, Table S3. A novel rabbit antiserum was

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raised against a peptide representing the C-terminal 16 amino acids of mouse filamin C that are deleted in mutant mice (Biogenes, Berlin, Germany). Specificity of affinity purified antibodies for WT filamin C was confirmed by testing its reactivity on recombinant filamins A and B, and WT and mutant filamin C fragments (Supplementary Material, Fig. S10).

Clinical phenotyping/grip strength measurement and hanging-wire test

Both tests were performed by standard protocols as described previously (58).

Acute exercise

Acute exercise was performed by subjecting 8-month-old WT and mutant male mice to a forced run until exhaustion using a computerized, electronically controlled treadmill device (TSE Treadmill ‘Kombi’ with air puff, TSE Systems, Bad Homburg, Germany). Before the trial, mice were trained at 3 consecutive days at a maximal speed of 0.1 m/s for 5 min. During the trial, a starting speed of 0.1 m/s was increased by 0.02 m/s every 2 min until mice got exhausted. Subsequently, mice were kept for 24 h in a Phenomaster system (see below), before they were euthanized and muscles were dissected for further studies.

Automated phenotyping

Automated phenotyping of mice followed a previously described (59) and validated (60) approach. For details, see Supplementary Material, Methods section.

Four male WT and four mutant male mice (8 months old) were individually observed under a 12 h light–dark cycle before (68 h; three dark phases and two light phases) and directly after (24 h) acute treadmill exercise. Data were collected automatically in 1 min registration–sample intervals, and pooled in 20 min bins, or in complete dark phases. Data from the second dark phase were used as pre-exercise reference conditions. A two-way ANOVA for repeated measurements was applied, with ‘genotype’ as inter-individual factor and ‘parameter across time’ (e.g. X + Y activity, Z-plane ‘rearing’ activity, vCO2, etc.) as intra-individual factor. An uncorrected Fisher’s least significance difference test was applied as multiple comparisons test. Prism software v6 (GraphPad Software, Inc.) was used for all statistics. A critical value for significance of P < 0.05 was used throughout the study. All data represent means ± SEM.

For lesion quantification, muscles from exercised mice were collected directly after the second automated phenotyping. For comparison, muscles from four unexercised 8-month-old male WT and mutant mice were sampled.

Histology, immunochemistry and light microscopy

Muscles were dissected and frozen in liquid nitrogen-cooled isopentane. Cryostat sections (5 μm thick) were prepared and stained with HE, modified Gomori trichrome, NADH (reduced nicotinamide-adenine dinucleotide) tetrazolium reductase and SDH as described (61).

For immunostaining, cryostat sections were air dried for 30 min, fixed with acetone (−20°C) for 10 min, air dried for 30 min and incubated with blocking medium (3% foetal bovine serum (FBS), 1% goat serum, 0.1% sodium azide in phosphate-buffered saline (PBS)) for 1 h at RT. Sections were incubated overnight at 4°C with primary antibodies diluted in 1% blocking medium in PBS, and after washing, with the appropriate FITC (fluorescein isothiocyanate)-, Alexa fluor 555- or Alexa fluor 647-conjugated secondary antibodies (Southern Biotech, Birmingham, AL, USA; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Molecular Probes/Life Technologies, Darmstadt, Germany). Slides were mounted with Mowiol mounting medium. Images were acquired using an LSM710 or LSM780 confocal laser scanning microscope or a Cell Observer SD spinning disc microscope (Carl Zeiss GmbH, Oberkochen, Germany).

Electron microscopy

A standard EM protocol was used in this study. Briefly, soleus muscle samples from WT and mutant mice (n = 2 per genotype) were fixed in 4% paraformaldehyde, 1% saturated picric acid and 0.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 overnight at 4°C. After rinsing in PBS, sections were treated with 0.5% OsO4, washed and counterstained with uranyl acetate, dehydrated via ethanol series and embedded in Durcupan resin (Fluka, Switzerland). Ultrathin sections were prepared (Ultracut S; Leica, Germany) and examined with a Zeiss LEO906E or Zeiss LEO 910 electron microscope (Carl Zeiss GmbH).

Statistical analysis

Data analyses and statistical evaluations were performed using Excel 2010 (Microsoft), Student’s t-test and Mann–Whitney U (Wilcoxon rank-sum) test, using the Mann–Whitney U test calculator at www.socscistatistics.com/tests/mannwhitney. For the clinical phenotyping, data were subjected to two factorial ANOVA for repeated measurements, with the inter-individual ‘genotype’ and one or more factors depending on the test used (GraphPad).

Preparation of all figures was done using Corel Draw Graphics Suite 12 or X7.

Supplementary Material

Supplementary Material is available at HMG online.

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