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SHORT REPORT

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A novel in-frame deletion in *MYOT* causes an early adult onset distal myopathy

Valeria Guglielmi[1] | Elia Pancheri[1] | Elena Cannone[2] | Vincenzo Nigro[3] | Manuela Malatesta[1] | Andrea Vettori[4] | Alejandro Giorgetti[4] | Annalaura Torella[3] | Stefania Aurino[3] | Barbara Cisterna[1] | Giulia Marchetto[1] | Giuliano Tomelleri[1] | Paola Tonin[1] | Marco Schiavone[2] | Gaetano Vattemi[1]

[1]Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Verona, Italy

[2]Department of Molecular and Translational Medicine, Division of Biology and Genetics, University of Brescia, Brescia, Italy

[3]Department of Precision Medicine, University of Campania "Luigi Vanvitelli", Napoli, Italy

[4]Department of Biotechnology, University of Verona, Verona, Italy

Correspondence
Marco Schiavone, Department of Molecular and Translational Medicine, Division of Biology and Genetics, University of Brescia, Brescia, Italy.
Email: marco.schiavone@unibs.it

Gaetano Vattemi, Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Verona, Italy.
Email: gaetano.vattemi@univr.it

Abstract

Missense mutations in *MYOT* encoding the sarcomeric Z-disk protein myotilin cause three main myopathic phenotypes including proximal limb-girdle muscular dystrophy, spheroid body myopathy, and late-onset distal myopathy. We describe a family carrying a heterozygous *MYOT* deletion (Tyr4_His9del) that clinically was characterized by an early-adult onset distal muscle weakness and pathologically by a myofibrillar myopathy (MFM). Molecular modeling of the full-length myotilin protein revealed that the 4-YERPKH-9 amino acids are involved in local interactions within the N-terminal portion of myotilin. Injection of in vitro synthetized mutated human *MYOT* RNA or of plasmid carrying its cDNA sequence in zebrafish embryos led to muscle defects characterized by sarcomeric disorganization of muscle fibers and widening of the I-band, and severe motor impairments. We identify *MYOT* novel Tyr4_His9 deletion as the cause of an early-onset MFM with a distal myopathy phenotype and provide data supporting the importance of the amino acid sequence for the structural role of myotilin in the sarcomeric organization of myofibers.

KEYWORDS
distal myopathy, I-band, myofibrillar myopathy, myotilin, zebrafish model

1 | INTRODUCTION

The myotilin gene (*MYOT*) encodes a 57-kDa sarcomeric Z-disk protein, which is expressed in skeletal muscle, cardiac muscle, and peripheral nerves.[1–3] Mutations in *MYOT* have been originally associated with different disorders subdivided according to the clinical and histological findings into three main categories including limb-girdle muscular dystrophy type 1A (LGMD1A), myofibrillar myopathy (MFM), and spheroid body myopathy.[1–3] Subsequently, LGMD1A has been classified as a form of MFM.[4] These disorders are usually transmitted as an autosomal dominant trait, rarely a recessive pattern has been reported[5,6] and, to date, only missense mutations in *MYOT* have been described.[3,4]

2 | MATERIALS AND METHODS

Detailed methods are provided in the Supporting Information.

3 | RESULTS

A 38-year-old man (II-2) was referred to our Clinic for progressive difficulty in walking and climbing stairs started in his early 30s (Figure 1A). At neurological examination, he had bilateral foot drop (MRC grade 3/5 on the left, 2/5 on the right). Atrophy of tibialis anterior, medial gastrocnemius, and adductor muscles were evident. The patient did not recall respiratory symptoms and there was neither clinical nor instrumental evidence of cardiac disease. Serum creatine kinase was sixfold above the normal and needle electromyography showed myopathic changes with spontaneous activity. His 77-year-old mother (I-2) experienced gait disturbance by age 40 due to distal lower limb weakness which progressively involved proximal legs and later proximal upper limb muscles. At age 67, she was wheelchair-bound. The mother was not available for further studies. No other family members had neuromuscular diseases.

A muscle biopsy of the gastrocnemius performed in the proband showed increased fiber size variation, internal nuclei, fiber splitting, few necrotic fibers, degenerating fibers with vacuoles and severe fibrosis. Amorphous material eosinophilic on H&E and irregular intermyofibrillar network were observed (Figure S1A). The abnormal fibers showed areas of increased reactivity for desmin, αB-crystallin, and myotilin (Figure S1B). Ultrastructural analysis documented myofibrillar disruption, Z-disk streaming, granulofilamentous material, degenerating membranous organelles, vacuoles, and myeloid structures (Figure S1C). The diagnosis of MFM was made and the screening for causative mutations in *DES*, *CRYAB*, *MYOT*, *LBD3/ZASP*, *BAG3*, and *FLNC* was performed by Sanger sequencing; a few years later the proband was tested using a NGS panel containing 93 neuromuscular genes.[7,8] The full gene sequencing of myotilin revealed a novel in-frame deletion on chromosome 5 spanning from bases 137 870 662 to 137 870 679 (Hg38; Hg19:chr5:137206351-137 206 368). The deletion in exon 2 (NM_006790.2:c.11_28del; Figure 1B) removes 18 nucleotides in the coding sequence, causing the loss of six amino acids (p.Tyr4_His9del). This variant was not previously reported, and it is not present in any database. The mother of the proband carried the same variant which was not detected in the three unaffected relatives including his brother, maternal uncle, and maternal aunt (Figure 1A).

To find a putative role of Tyr4_His9del, we built a structural model of the full-length myotilin protein (Figure 1C). The six amino acids deleted are involved in local interactions within the N-terminal portion of the protein and includes, among others, the residue Arg6 previously reported to be mutated in two patients. Moreover, four out of the six residues are charged, that is, Glu5, Arg6, Lys8, and His9. Indeed, the deletion would significantly modify the electrostatic properties of the N-terminal portion of the protein. To assess the effect on protein levels, immunoblotting was done. Myotilin was detected as a single band of 55 kDa after SDS-PAGE (Figure S1D) and as several basic spots with the same MW (55 kDa) and different pI values (approximately 9.0) after 2D-PAGE (Figure S1E). The pattern and the expression levels of myotilin were similar in patient and control subjects.

To investigate the pathogenicity of the novel mutation, we injected zebrafish embryos at 1-cell stage with in vitro synthesized mRNA encoding human wt (wtMYO) and c.11_28del myotilin (mutMYO). Based on the morphology, embryos were classified in *normal* and *abnormal* (Figure 2A). We observed that at 24 h after the injection, the number of embryos displaying an abnormal morphology was significantly higher in mutMYO-injected embryos compared to embryos injected with either wtMYO ($p = 0.0425$) or vehicle ($p = 0.0122$). Conversely, the number of embryos with a normal morphology was lower in mutMYO-injected embryos compared to wtMYO-injected embryos ($p = 0.00425$) or injected with the vehicle ($p = 0.0136$; Figure 2B).

We determined whether mutMYO can induce muscle alterations in zebrafish during development. Embryos at 48 hpf were stained with fluorescent phalloidin, which labels actin filaments in all muscle fibers.

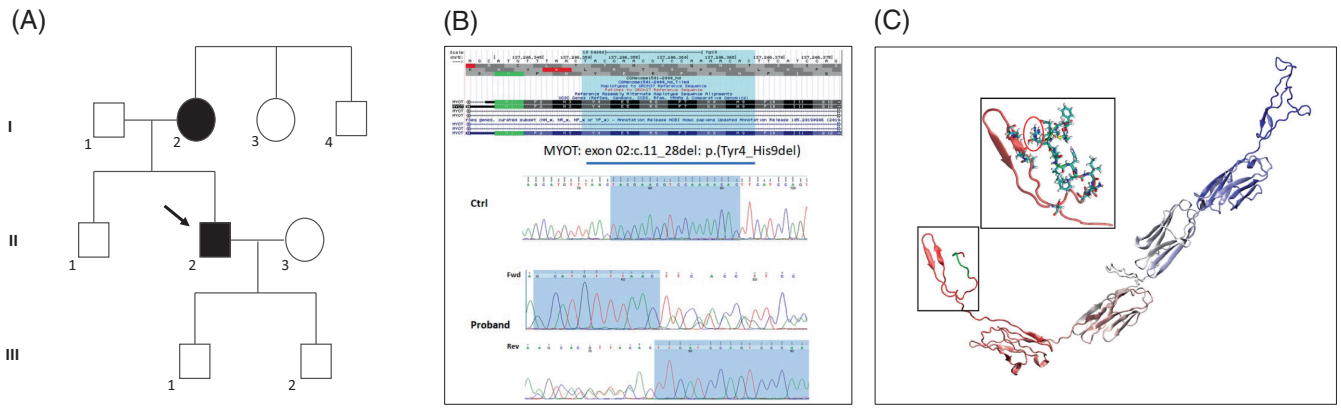

FIGURE 1 (A) Pedigree of the family. Arrow indicates the proband. Solid black symbols denote affected family member. (B) Sanger. UCSC graphic view of the N-terminal region of *MYOT* (upper panel). Electropherogram showing the heterozygous Tyr4_His9del in-frame deletion in exon 2 of *MYOT* (Lower panel). (C) Model of the human myotilin. The atom indexes are colored from red to blue depending on their sequence position. In green the p.Tyr4_His9del residues. The zoomed region illustrates the interaction between the residues involved in the deletion. The red circle shows the salt bridge formed by Arg6 and Glu5. [Colour figure can be viewed at wileyonlinelibrary.com]

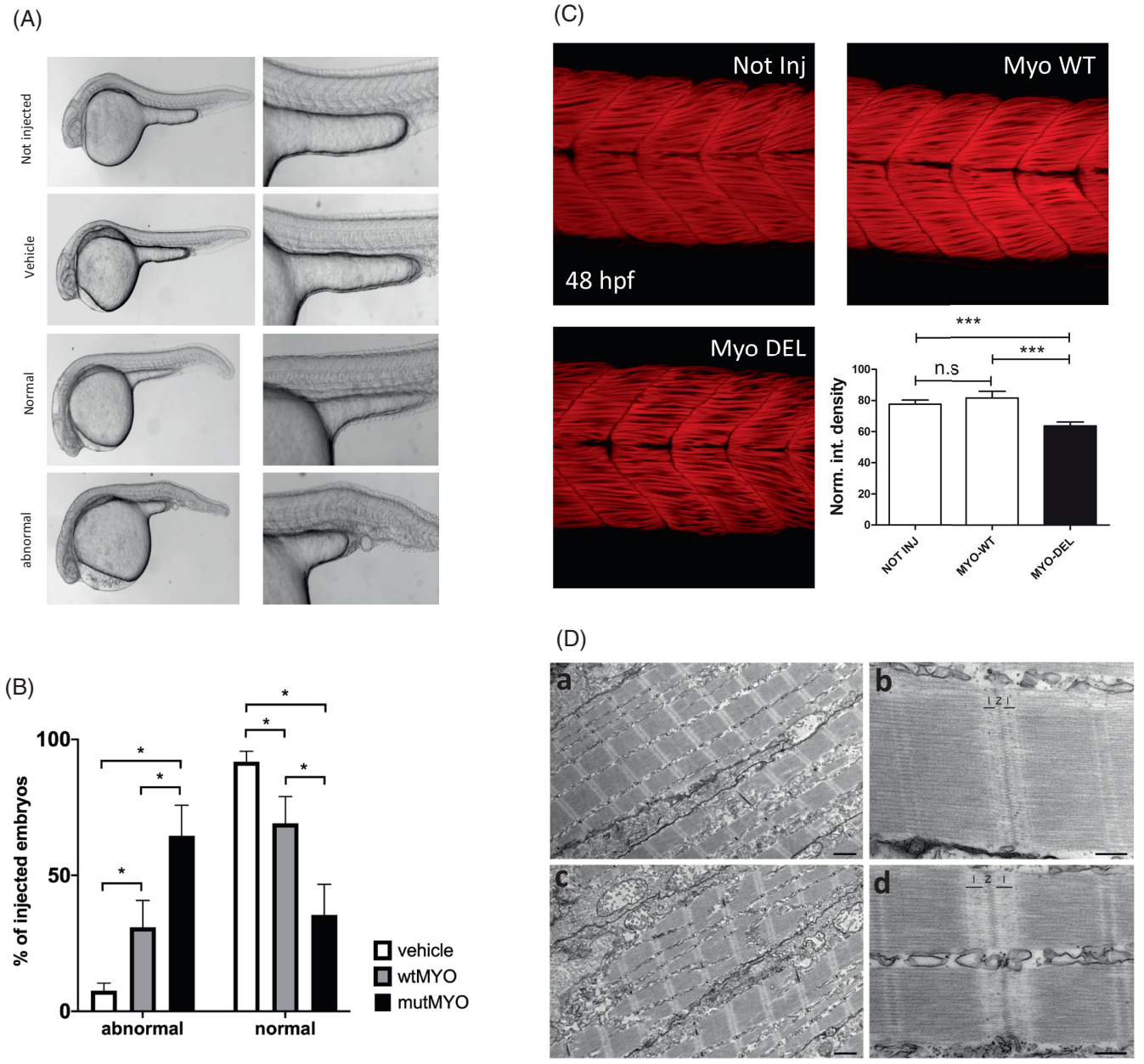

FIGURE 2 (A, B) Morphological features observed at the light microscope and quantitative analysis of the phenotypes of 24 hpf embryos injected with human wtMYO or mutMYO mRNA. (C) Representative lateral views of a single confocal projection (z-stack) of mutMYO, wtMYO, and control embryos at 48 hpf stained with phalloidin. Skeletal muscle fibers appear less consistent and compact in mutMYO-injected embryos compared to wtMYO-injected or not injected (control) larvae. (D) Transmission electron micrographs of skeletal muscles from embryos injected with wtMYO mRNA (a, b) and mutMYO mRNA (c, d). Note the irregular arrangement and the heterogeneous thickness of myofibres in mutant embryos (c) compared to controls (a). High magnification images (b, d) show in mutant embryos well-organized Z lines but I-bands wider than in controls. Bars: 1 µm (a, c); 200 nm (b, d). [Colour figure can be viewed at wileyonlinelibrary.com]

Unlike wtMYO and control fishes, that displayed a compact and well-organized muscular structure, mutMYO-injected embryos exhibited a disorganization of muscle fibers characterized by an irregular alignment and gaps between filaments (Figure 2C). The quantification of the phalloidin levels revealed a significant reduction of fluorescence in mutMYO embryos compared either with wtMYO and control non-injected larvae, suggesting an alteration of the muscular fibers in mutant embryos.

Ultrastructural examination of skeletal muscle showed that in mutMYO-injected zebrafish embryos the myofibrils were characterized by a heterogeneous thickness and an irregular pattern, and by I-bands of various width (Figure 2D). Quantitative evaluation of I-bands demonstrated a significant increase in width ($p < 0.001$) in mutant embryos (59.14 ± 13.71 nm) compared to controls (44.84 ± 8.64 nm). A strong dilation of T-tubules and swollen mitochondria with deranged cristae where also observed.

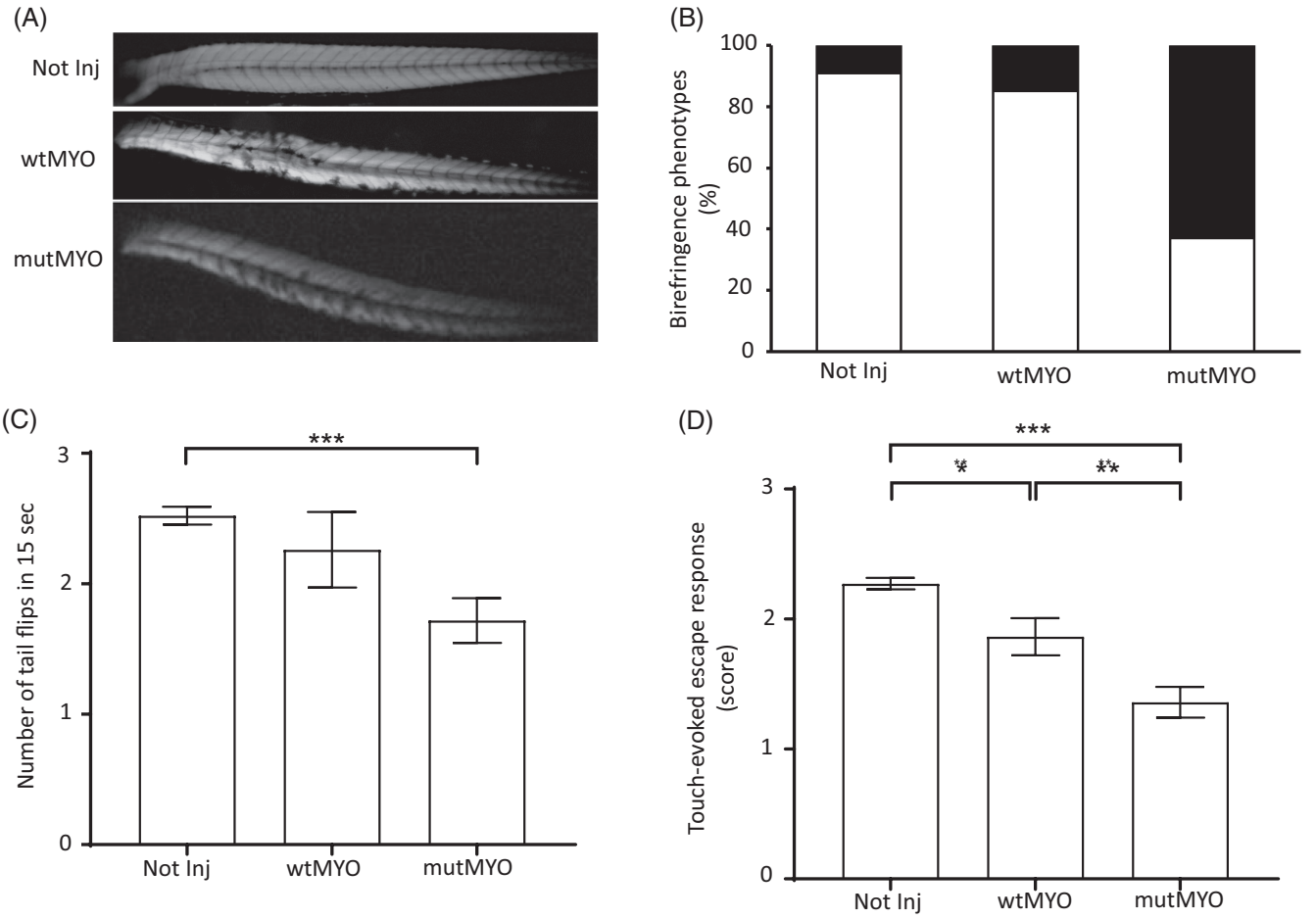

FIGURE 3 (A, B) Birefringence analysis of wildtype embryos injected with Tol2 plasmids harboring either human wild-type MYOT full cDNA (wtMYO) or human MYOT c.11_28del (mutMYO) was performed at 48 hpf. In (A), representative lateral images of single non-injected, wtMYO-, and mutMYO-injected embryos. Bar: 50 μm. The bar graph in (B) reports the birefringence phenotypes observed in not injected ($n = 200$), wtMYO ($n = 55$), and mutMYO ($n = 76$) embryos. Data are plotted as total percentage of fish showing wildtype (white closed bars) or myopathic (black closed bars) birefringence phenotypes. (C) Number of spontaneous coiling events (tail flips) performed in 15 s have been recorded at 24 hpf in non-injected ($n = 330$), wtMYO-injected ($n = 80$) and mutMYO-injected ($n = 85$) embryos and plotted in the bar graph. Values are reported as the average of the number of tail flips recorded for single embryos at 24 hpf ± SEM $^{***}p < 0.001$. (D) Touch-evoked escape response at 48 hpf was checked in non-injected ($n = 342$), wtMYO ($n = 86$), and mutMYO ($n = 78$). Arbitrary values (0–3) have been assigned to each larva based on the observed response. Data in the bar graph are reported as the average of escape values for single embryos ± SEM $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

To further corroborate these findings, zebrafish embryos were injected with Tol2 expression plasmids carrying the human cDNA sequences of either wildtype or c.11_28del myotilin gene. Muscle integrity was evaluated by birefringence analysis. At 48 hpf, about 65% of mutMYO-injected embryos showed a significant decrease in muscle birefringence (Figure 3A,B) whereas only 10% of non-injected wildtype embryos and 15% wtMYO-injected embryos displayed abnormal birefringence phenotypes (Figure 3B) suggesting that embryos with deleted myotilin undergo myofibrillar disorganization. Severe impairment of motor behavior was observed in mutMYO-injected embryos by measuring tail flips (Figure 3C) and touch-evoked escape response (Figure 3D). The number of spontaneous coiling events (tail flips) recorded at 24 hpf was significantly lower in mutMYO-injected embryos compared to non-injected wildtype embryos ($p < 0.001$; Figure 3C). The touch-evoked escape response, which measures the ability of embryos to escape and swim after experiencing a mechanical stimulus was significantly lower in mutMYO-injected embryos compared both to wtMYO-injected embryos ($p < 0.01$) and non-injected wildtype embryos ($p < 0.001$; Figure 3D). Notably, wtMYO-injected embryos showed a mild significant reduction of motility ($p < 0.05$) possibly due to the overexpression of the exogenous protein (Figure 3D).

4 | DISCUSSION

In the present study, we report a family carrying the first deletion Tyr4_His9del in *MYOT* in a heterozygous state. *MYOT* variant is clinically linked to an early-adult onset distal myopathy and pathologically associated to MFM. MFMs represent a group of neuromuscular

disorders with clinical and genetic heterogeneity.[3,9] Causative mutations have been identified in genes encoding Z-disk or Z-disk-associated proteins and usually show an autosomal dominant inheritance.[3,9] *MYOT* mutations-related myopathies have been associated with three main phenotypes including a proximal limb weakness that may later progress to distal muscles, a spheroid body myopathy with LGMD phenotype, dysphonia and respiratory failure, and a late-onset MFM presenting clinically in most patients with distal muscle weakness.[1,3] The proband of our family presented with foot drop in his 30s, earlier than the previously reported patients.[1,2] His mother had a relentlessly progression of proximal lower limb muscle weakness, with later involvement of shoulder girdle muscles, leading to the loss of ambulation in her 60s. Cardiac and respiratory muscles were spared in both patients.

Myotilin consists of an N-terminus that contains a serine-rich region responsible for its interaction with α-actinin and of two immunoglobulin-like domains at C-terminus that are necessary for protein homodimerization and the binding of actin and filamin C.[3] *MYOT* mutations so far reported are missense variations mainly located in exon 2 encoding the serine-rich domain and their pathogenic mechanism remains largely unknown.[3] We identified the first deletion, Tyr4_His9del, in myotilin protein that lacks six-amino acids in the extreme N-terminus. An amino acid substitution of arginine at position 6 was previously reported in two individual case reports.[5,10] These reports support the functional importance of the extreme N-terminus in myotilin although the mechanism by which this *MYOT* mutation induces the disease is not fully understood. The six-amino acid deletion (Tyr4_His9del) is located outside the functional domains involved in the interaction with actin and other actin-binding proteins as well as in the homodimerization.[3] Our bioinformatic analysis showed that these amino acid residues are engaged in local interactions within the N-terminal portion of myotilin suggesting that their deletion might critically alter the structural properties of the region. The N-terminus of myotilin is responsible for its interaction with other proteins besides α-actinin such as filamin C, FATZ-1, ZASP, and the ubiquitin E3 ligases MuRF1 and MuRF2,[3] thus we hypothesize that the small deletion might have a direct or indirect impact on myotilin localization and/or its binding partners in the Z-disc and that this could eventually lead to protein aggregation.

Our in vivo zebrafish model strengthens the evidence for the pathogenetic role of Tyr4_His9 deletion in muscle pathology. Zebrafish embryos injected with in vitro transcribed mRNA encoding mutated *MYOT* or with Tol2 expression plasmid harboring cDNA of human mutated *MYOT* develop phenotypical alterations as shown by histological and functional studies. Microscopic analysis of embryos stained with fluorescent phalloidin revealed that injection of mRNA encoding human mutant *MYOT* in zebrafish results in myofiber disorganization. The ultrastructural analysis further confirmed the loss of organized myofibrillar structure in the skeletal muscle of mutant *MYOT*-injected embryos that are consistent with alteration seen in human MFM muscles. TEM on longitudinal sections showed an enlargement of the I-band in mutant *MYOT*. Remarkably, myotilin localizes in the sarcomeric I-band, where it interacts with α-actinin and thereby contributes to I-band architecture, hence mutant *MYOT* could explain the wider I-band observed by electron microscopy in mutant embryos. The loss of myofibrillar organization is consistent with the significant motor impairment observed in zebrafish embryos injected with Tol2 plasmids carrying cDNA sequence of the human mutated *MYOT* as indicated by the lower number of spontaneous coiling events and touch-evoked escape response score compared to non-injected and wildtype *MYOT*-injected embryos. All together, these data indicate that the deletion of Tyr4_His9del leads to alterations in the normal myofibrillar organization in the muscle that perpetuate in the observed motility disfunctions.

In conclusion, we report the first deletion in *MYOT* causing an early onset MFM with distal myopathy phenotype and highlight the importance of the 4-YERPKH-9 amino acidic sequence of the myotilin protein in myofibrillar organization and the sarcome structure. We confirm the pathogenetic effect of the identified myotilin variant in zebrafish and show that this model organism can be useful to investigate the role of myotilin mutations in the pathogenesis of MFM.

AUTHOR CONTRIBUTIONS

Elia Pancheri, Manuela Malatesta, Barbara Cisterna, Paola Tonin, Giuliano Tomelleri, Gaetano Vattemi: Clinical work-up and muscle biopsy analysis. **Vincenzo Nigro, Annalaura Torella, Stefania Aurino:** Molecular genetic data. **Alejandro Giorgetti:** Structural modeling. **Valeria Guglielmi, Elena Cannone, Andrea Vettori, Giulia Marchetto, Marco Schiavone:** Zebrafish studies. **Valeria Guglielmi, Gaetano Vattemi, Marco Schiavone:** Wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/cge.14413.

DATA AVAILABILITY STATEMENT

All data relevant to the study are included in the article.

ETHICS STATEMENT

The study was conducted in accordance with the guidelines in the Declaration of Helsinki.

ORCID

Andrea Vettori https://orcid.org/0000-0003-4958-0619
Giulia Marchetto https://orcid.org/0000-0002-2447-7191
Gaetano Vattemi https://orcid.org/0000-0003-3412-8339

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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