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In vivo characterization of human myofibrillar myopathy genes in zebrafish

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ABSTRACT

Myofibrillar myopathies (MFM) are progressive diseases of human heart and skeletal muscle with a severe impact on life quality and expectancy of affected patients. Although recently several disease genes for myofibrillar myopathies could be identified, today most genetic causes and particularly the associated mechanisms and signaling events that lead from the mutation to the disease phenotype are still mostly unknown. To assess whether the zebrafish is a suitable model system to validate MFM candidate genes using targeted antisense-mediated knock-down strategies, we here specifically inactivated known human MFM disease genes and evaluated the resulting muscular and cardiac phenotypes functionally and structurally. Consistently, targeted ablation of MFM genes in zebrafish led to compromised skeletal muscle function mostly due to myofibrillar degeneration as well as severe heart failure. Similar to what was shown in MFM patients, MFM gene-deficient zebrafish showed pronounced gene-specific phenotypic and structural differences. In summary, our results indicate that the zebrafish is a suitable model to functionally and structurally evaluate novel MFM disease genes *in vivo*.

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1. Introduction

Myofibrillar myopathy (MFM) is a muscular dystrophy defined by the deposition of myofibrillar proteins into Desmin-positive protein aggregates and myofibrillar degeneration [1], severely affecting the quality of life of these patients. Mutations in a variety of sarcomeric and cytoskeletal proteins resulting in MFM such as αB-Crystallin, BAG3, Desmin, DNAJB6, FHL1, Filamin C, Myotilin, Plectin, VCP and ZASP [1,2,3,4,5] have been identified so far. Most forms of myofibrillar myopathy are inherited by monogenic point mutations with a dominant negative effect [3]. Today no causative or ameliorating therapy for this significant cohort of hereditary myopathies is available, as the molecular mechanisms that translate the different MFM gene mutations into the pathological features observed in MFM patients are not well understood [1,6]. Animal model systems are mostly lacking predominantly due to their early embryonic lethality [7], but are instrumental to gain novel insights into the molecular mechanisms that translate from the mutation to the pathology [6].

Due to their transparency, accessibility for genetic modification and particularly the evolutionary conserved genetic and molecular mechanisms that control muscle development and function in zebrafish and mammals, the zebrafish emerged as a vertebrate model system especially to study human striated muscle diseases such as cardiomyopathies or muscular dystrophies [8,9,10]. Here we study the *in vivo* role of MFM genes by inactivation studies in zebrafish to analyze MFM gene function on a molecular, cellular

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and structural level. Zebrafish reverse genetics using Morpholino-modified antisense oligonucleotide-mediated gene knock-down has been developed as an elegant and powerful approach to rapidly evaluate the impact of candidate genes on muscle development and function [8,11,12,13]. We demonstrate that targeted inactivation of MFM genes in zebrafish led to a severe myopathy accompanied by sarcomeric degeneration, however, without pathological protein aggregation. We introduce the zebrafish as a suitable model system to dissect the *in vivo* role of known and novel MFM-related genes that is crucial to decipher the pathomechanisms of MFM and finally to evaluate therapeutic options for patients suffering from MFM.

2. Materials and methods

2.1. Zebrafish strains

Care and breeding of zebrafish was carried out essentially as described [11]. Wild-type fish of the TE strain were used. The present study was performed after appropriate institutional approvals were secured, which conform to EU Directive 2010/63/EU.

2.2. Morpholino microinjections

Antisense oligonucleotides were directed against the translational start-sites or splice-sites of the respective genes. Each Morpholino was then injected in at least four different dosages into >100 wild-type embryos at the one-cell stage to empirically determine the optimal Morpholino dosage. The resulting phenotypes were analyzed at consecutive developmental stages, a standard control-oligonucleotide was injected at identical dosages. MO-BAG3 [14], MO-ZASP [15], MO-cmlc1 [16] and MO-plcγ1 [17] were described before.

2.3. RNA in situ hybridization, immunostaining, immunoblotting and birefringence analysis

Whole-mount RNA *in situ* hybridization was carried out as described [11]. Immunostaining of adult zebrafish cardiomyocytes was conducted using antibodies against BAG3 (Genetex #102396) and Desmin (Progen #10570) as described [18]. T12 antibody, recognizing an epitope of Titin close to the Z-disk, was described before [19]. Immunoblotting was conducted with antibodies against Pan-Cadherin (Abcam #ab16505) and Desmin (Sigma #D8281) [11]. Birefringence analysis were performed and analyzed as described [13].

Table 1
Summary of MFM gene expression data gained by either *in situ* hybridization or IF-staining.

Gene name	Heart	Skeletal muscle
αB-crystallin-a	•	•
αB-crystallin-b	•	•
desmin-a	•	•
desmin-b		•
BAG3	•	•
dnajb6-a		•
dnajb6-b		•
fhl1-a	•	•
fhl1-b	•	•
filamin C-a		•
filamin C-b		•
myotilin	•	•
plectin-a	•	•
plectin-b	•	•
VCP		•
ZASP	•	•

2.4. Electron microscopy

Zebrafish embryos were fixed (2.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3), post-fixed in 1% osmium tetroxide, dehydrated and embedded in Epon. Ultrathin sections (50 nm) were contrasted with uranyl acetate and lead citrate and examined with a Jeol 1400 or Zeiss EM 10 electron microscope.

2.5. Functional assessment and statistical analysis

Movement assays at 24 hpf and assessments of cardiac contractility and heart rate were carried out as described [8,13]. If not noted otherwise, data are presented as average ± S.D. and analyzed using Student's *t*-test, a value of $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. MFM disease genes are conserved between human and zebrafish

To investigate whether targeted gene inactivation in zebrafish is suitable for the functional and structural characterization of MFM disease genes, we first identified the zebrafish orthologs of the known human MFM genes. Consistently, the identified orthologs showed very high amino acid sequence homology to the human proteins, in particular within their functional domains. Overall, sequence identity ranged from 41% to 97% (Suppl. Fig. 1). As an example, the alignment for human Desmin and zebrafish Desmin-a shows an amino acid identity of 73% (Suppl. Fig. 2A). BAG3 homology on protein level between human and zebrafish BAG3 is only 41%, whereas functional domains share 77% sequence identity (Suppl. Fig. 2B), strongly suggesting the conservation of MFM protein functions across species. Gene duplication is often observed in the zebrafish, as a result of a genome duplication during teleost evolution [20]. Gene duplication was observed for *αB-crystallin*, *desmin*, *dnajb6*, *fhl1*, *filamin C* and *plectin* (Suppl. Fig. 1).

3.2. MFM genes are strongly expressed in skeletal and heart muscle cells in zebrafish

In mammals, MFM genes are predominantly expressed in heart and skeletal muscle cells [6]. We found for all investigated MFM orthologs increased expression in zebrafish skeletal- and/or heart muscle by mRNA and protein expression analysis as summarized in Table 1. In this context, expression was often pronounced during early muscle development, as demonstrated for *desmin-a* and *myotilin* (Fig. 1A–F). During development expression of *desmin-a* became first detectable in the developing somites starting from 16 h post fertilization (hpf) (Fig. 1A), and remained restricted to the heart and skeletal muscle throughout embryogenesis (Fig. 1B). The observed temporal and spatial expression pattern resembles the expression of *desmin* in mice and humans [21]. In addition to genes solely expressed in muscle tissue such as *desmin-a*, we observed expression of MFM-related genes in multiple other non-muscle tissues. For instance, we found in 18 hpf embryos *myotilin* mRNA ubiquitously expressed (Fig. 1D,E). Additionally, both, *desmin-a* and *myotilin* mRNAs showed strong cardiac expression at 24 and 48 hpf (Fig. 1C,F).

To further evaluate the subcellular localization of the MFM proteins in muscle cells, we performed immunofluorescence analyses in cultured adult zebrafish cardiomyocytes (Fig. 1 G–H). We found similar to the situation in mice and humans [12], zebrafish BAG3 protein specifically localized at sarcomeric Z-disks and the

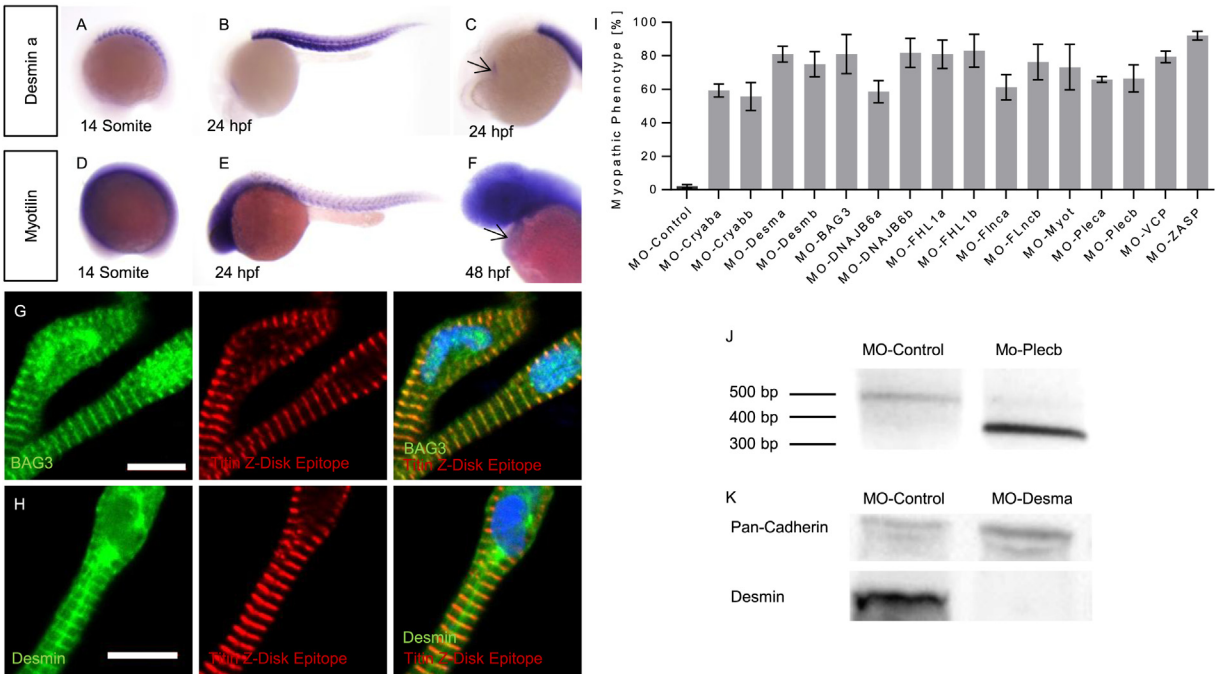

Fig. 1. MFM disease gene expression analysis and knock-down in zebrafish (A–E) Whole-mount antisense RNA *in situ* hybridization shows *desmin-a* and *myotilin* expression in skeletal muscle fibers at the 14 somite stage and 24 hpf. (C,F) At 24/48 hpf cardiac expression of *desmin a/myotilin* was observed (arrows). (G) As revealed by co-stainings with an antibody against Z-disk Titin (T12) or DAPI, respectively, BAG3 localizes to sarcomeric Z-disks and nuclei of zebrafish cardiomyocytes. (H) Desmin is localized at the sarcomeric Z-disk, scale bars = 10 μm. (I) Percentage of injected embryos showing a myopathic phenotype. (J) Injection of MO-Plecb blocks the splice donor site of exon 7 of Plectin b, leading to an abnormally short cDNA fragment due to skipping of exon 7. (K) Western-blot analysis shows the reduction of Desmin after injection of MO-Desma. Pan-Cadherin is used as loading control.

nuclei of zebrafish cardiomyocytes (Fig. 1G). Next, we analyzed the subcellular localization of zebrafish Desmin and found that Desmin was also distributed in a striated pattern and similar to mouse and human Desmin, localized to the Z-disk region. Additionally, Desmin was also detected outside the sarcomere, most likely representing densely packed Desmin networks in the cytoskeleton of the muscle cell (Fig. 1H). In summary, human and zebrafish MFM gene orthologs not only show very high amino acid identity but also conserved tissue expression as well as subcellular localization patterns, suggesting conserved biological functions between zebrafish and humans.

3.3. *Loss of MFM gene function leads to myopathy in zebrafish*

To investigate the structural and functional role of human MFM genes in zebrafish muscle *in vivo*, we next inactivated the identified zebrafish MFM orthologs by injecting Morpholino-modified antisense oligonucleotides into one-cell-stage wild-type embryos. All used Morpholinos were blasted against the zebrafish genomic sequence (Zv8; www.Ensembl.org) and are predicted to exhibit no cross-reaction with other expressed zebrafish genes (Suppl. Fig. 3, Table 2). Between 56% (MO-cryabb, 4 ng/embryo) and 92% (MO-ZASP, 8 ng/embryo) of injected wild-type embryos ($n > 120$) developed a homogenous myopathic phenotype. The efficiency for each individual gene inactivation study is summarized in Fig. 1I. Furthermore, to demonstrate the effectiveness of the knock-downs, we analyzed pre-mRNA splicing by RT-PCR or protein levels by Western-Blotting (Table 2). For instance, MO-Plecb is designed to block splicing at the exon 7–intron 7 boundary. Consistently, we detected alternative splicing resulting in the skipping of exon 7 (116 bp), a frame shift and premature termination of protein translation (Fig. 1J). Furthermore, as shown in Fig. 1K, injection of MO-Desma resulted in severely reduced Desmin protein levels, whereas normal protein levels were observed in control embryos.

Table 2
Monitoring of Morpholino knock-down efficacy.

Gene	Morpholino abbreviation	Splice assay	Western blot
αB-crystallin-a	MO-Cryaba	•	
αB-crystallin-b	MO-Cryabb	•	
desmin-a	MO-Desma	•	•
desmin-b	MO-Desmb	•	•
BAG3	MO-BAG3	•	
dnajb6-a	MO-DNAJB6a	•	
dnajb6-b	MO-DNAJB6b	•	
fhl1-a	MO-FHL1a	•	•
fhl1-b	MO-FHL1b	•	•
filamin C-a	MO-Flnca	•	
filamin C-b	MO-Flncb	•	
myotilin	MO-Myot	•	
plectin-a	MO-Pleca	•	
plectin-b	MO-Plecb	•	
VCP	MO-VCP	•	•
ZASP	MO-ZASP		

Next, to evaluate the impact on skeletal muscle structure and function after knock-down of the MFM orthologs in zebrafish, the individual morphant phenotypes were evaluated in detail. Inactivation of each MFM gene resulted in reduced motility of the morphants shown by voluntary movement measurements at 24 hpf (Fig. 2A–C). 73 ± 17% of MO-control injected individuals moved spontaneously in a 10 s timeframe (Fig. 2A). By contrast, 28 ± 9% of the Desmin-a and only 1 ± 0.5% of ZASP morphants showed spontaneous movement at 24 hpf (Fig. 2B–D). Other morphants such as Plectin-b (63 ± 9%) or αB-crystallin-b (57 ± 13%) deficient embryos were less affected at early stages of development (Fig. 2D). To analyze the progression of muscle function in these MFM gene deficient embryos, we additionally analyzed the "touch escape response" in the morphants at 48 and 72 hpf. Control individuals reacted rapidly, bending their whole body resulting in an instant

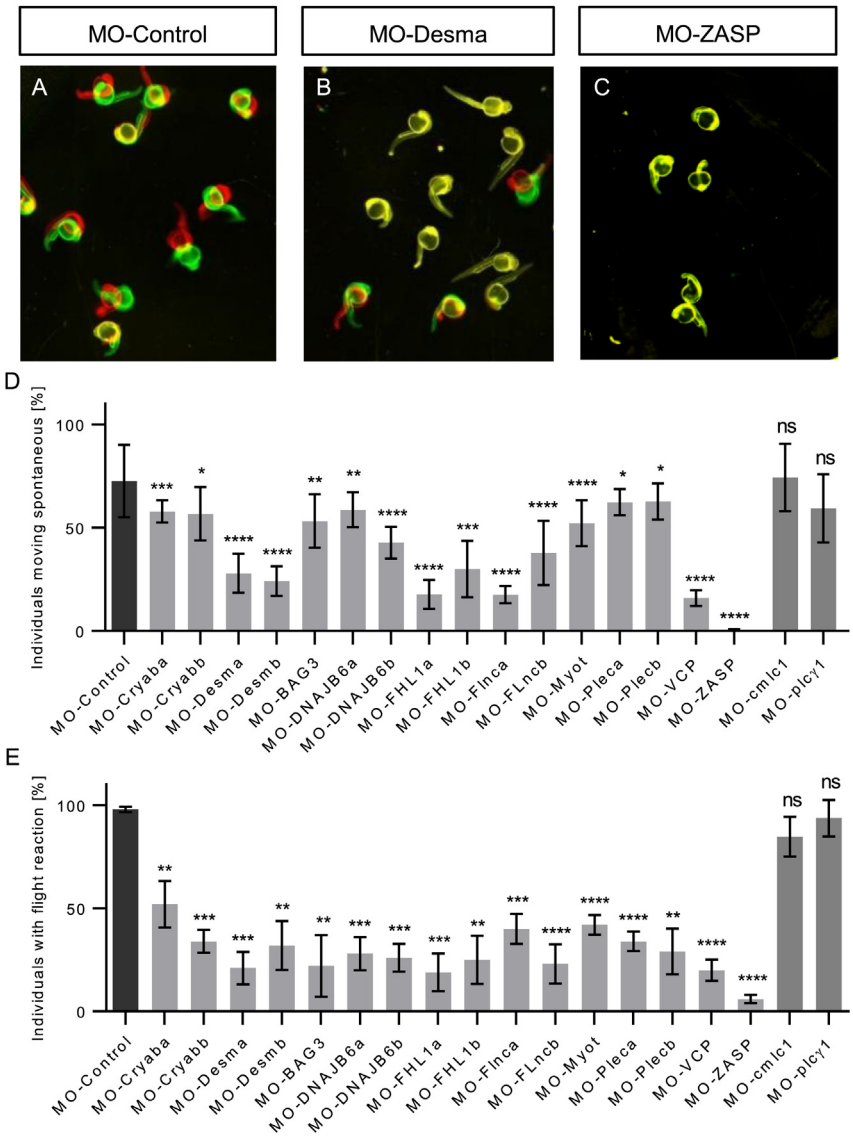

Fig. 2. Loss of MFM gene function results in a progressive motility deficiency (A–C) Spontaneous movement assay with false-colored superimposed overviews of 24 hpf control (A) and MFM morphants (B–C); Red pictures = 0 s; green pictures = 10 s. (D) Summary of voluntary movement at 24 hpf of MFM morphants. (E) Quantification of flight reaction to a touch stimulus of 72 hpf individuals. Darker gray bars indicate the effect of Morpholinos directed against non-MFM genes as controls. Asterisks indicate significance compared to the MO-control group.

and explosive escape reaction upon touch stimulation (Fig. 2E; Supplementary Movie 1). We found that all investigated MFM morphants displayed severe but variable skeletal muscle dysfunction and that tactile stimulation often only resulted in an insufficient flight reaction (Fig. 2E; Supplementary Movie 2). Furthermore, we never observed an improvement of motility during embryonic development, but regularly a significant deterioration of skeletal muscle function, suggesting a progression of the myopathy in MFM gene deficient embryos (Fig. 2D–E). A severe progression of the myopathy was particularly observed for αB-crystallin, BAG3, DNAJB6, FHL1, Filamin C and Plectin morphant embryos (Fig. 2E). For instance, in DNAJB6-a morphants spontaneous movement was reduced in only 41 ± 8% of the embryos at 24 hpf (Fig. 2D). By contrast, at 72 hpf, 72 ± 8% of the embryos failed to flight upon touch stimulation (Fig. 2E). Additionally, Plectin-a and Plectin-b morphants only exhibited a slight reduction of motility at 24 hpf (MO-Pleca 62 ± 6%; MO-Plecb 63 ± 9%), while at 72 hpf motility was significantly reduced compared to controls (MO-Pleca 34 ± 5%; MO-Plecb 29 ± 11%, p-values < 0.001).

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.149.

To finally exclude a systematic influence of Morpholino injection on motility of the zebrafish embryo, we also included different established Morpholinos that provoke a cardiac phenotype such as MO-cmlc1 (cardiac myosin light chain-1) [16] or MO-plcγ1 (phospholipase C, gamma 1) [17] in addition to the standard control Morpholino. No significant influence on motility was observed for cMlc1 or Pclγ1 morphants (4 ng/embryo, n > 120, in three independent experiments) at 24 and 72 hpf (darker gray bars Fig. 2D–E), implying that the effect of MFM gene inactivation on skeletal muscle function is specific.

In summary, all MFM gene deficient embryos showed significantly impaired motility, demonstrating the importance of these genes for the regular function of the musculature in zebrafish.

3.4. Targeted knock-down of MFM genes leads to sarcomeric disorganization in vivo

To investigate whether impaired motility of MFM morphants is caused by structural alterations of the muscle, we first analyzed muscle architecture of MFM gene morphants by birefringence measurements [10,13]. Highly organized muscle tissue polarizes light, and disorganization of the muscle tissue can be easily visualized by reduction in birefringence signal. Birefringence signal intensity from the skeletal muscle was severely reduced in MFM gene deficient embryos, whereas muscle structure was unaffected in embryos injected with control Morpholino or cardiac-specific Morpholinos (Suppl. Fig. 4B,X,Z). For instance, Desmin-a and DNAJB6-b morphants displayed a reduced birefringence signal from the skeletal muscle indicating a loss of organized muscle tissue in these specific areas (Fig. 3B–C). The quantification of the birefringence data revealed a significant reduction of signal intensity in Desmin-a morphants (MO-Desma: 67 ± 26%, p-value = 0.002), this is even more pronounced in other MFM gene deficient embryos as shown for DNAJB6-b (MO-DNAJB6b: 48 ± 15%, p-value < 0.0001) (Fig. 3D).

To further structurally characterize the myopathic phenotype of MFM gene deficient embryos, muscle ultrastructure was analyzed by transmission electron microscopy. Control myocytes were densely packed with sarcomeres composed of highly organized, precisely aligned thick and thin myofilaments, flanked by well-defined Z-disks (Fig. 3E,I). Common ultrastructural traits of all MFM gene morphants muscle were disorganized sarcomeres and rounded muscle cell nuclei (dotted arrows, Fig. 3F,H) in contrast to the elongated nuclei in control samples (dotted arrow, Fig. 3E). We were able to detect gene specific alterations at the ultrastructural level, such as vacuolizations in the muscle cells of Desmin-a and αB-crystallin-a morphants (asterisks, Fig. 3F,H). In addition, Desmin-a and αB-crystallin-b morphants showed a severe reduction in the number of cristae in mitochondria (arrowheads Fig. 3F, J), while the cristae in Plectin-b morphants appeared thicker and more electron dense compared to controls (Fig. 3K). Furthermore, a severe autophagic pathology was observed in DNAJB6-a deficient embryos (Fig. 3G), with large vesicles resembling an autophagic rimmed-vacuolar degenerative pathology [2]. Areas devoid of myofibrils were observed after targeted inactivation of Myotilin function, containing only stress fiber-like structures or premyofibrils with Z-bodies (arrows, Fig. 3L).

In summary, targeted inactivation of the known MFM genes resulted in severe myofibrillar disorganization and degeneration. In addition we found distinct gene-specific structural alterations that resemble pathologies also observed in human MFM patients, supporting the usability of the zebrafish model to investigate the *in vivo* role of novel MFM genes.

3.5. MFM gene inactivation leads to heart failure in zebrafish

In addition to myopathies, patients suffering from MFM gene mutations frequently develop heart muscle diseases [4,5]. Hence, to assess whether targeted inactivation of MFM gene function also leads to cardiac dysfunction in zebrafish, we evaluated heart

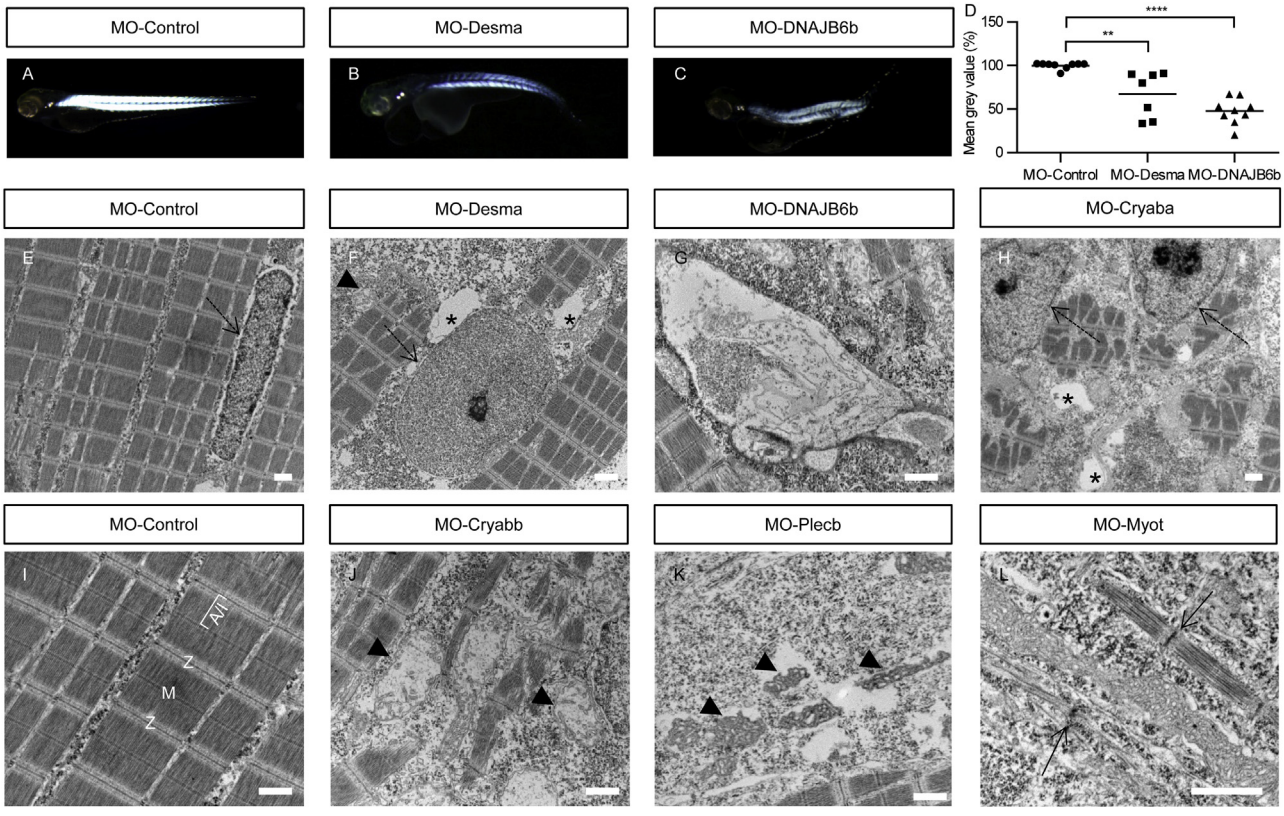

Fig. 3. Disturbed myofibrillar structure in MFM gene deficient embryos (A–C) Lateral views of a control zebrafish (A) at 72 hpf showing bright homogenous birefringence compared to reduced birefringence in Desmin-a (B) and DNAJB6-b (C) morphants. (D) Quantification of birefringence in Desmin-a and DNAJB6-b morphants. (E–L) Electron microscopy of MFM gene deficient and control embryos at 72 hpf. (E) Nuclei (dotted arrows) in the skeletal muscle are elongated in controls; (F,H) rounded nuclei in myopathic muscle. (I) Control injected embryos develop highly ordered myofibrils with ordered sarcomeric units, Z-disks, M-Bands and A/I regions. (F,H) Vacuolization in MO-Desma and MO-crayaba injected embryos (asterisk). (G) Autophagic pathology in Dnajb6-a morphants. (F,J,K) Mitochondrial alteration in Desmin-a, Plectin-b and αB-crystallin-a morphants (arrowheads). (L) Stress fiber-like structures in Myotilin morphants (arrows). Scale bars 1 μm.

morphology and contractile function. Most morphant hearts developed pericardial edemas within 72 hpf (Fig. 4A–D, Suppl. Fig. 4). As early as 48 hpf, heart rate (Fig. 4E–F) and fractional shortening of morphant heart chambers were regularly reduced (Fig. 4G–H). Exemplarily, heart function of αB-crystallin-a, -b and Myotilin deficient hearts is shown in Fig. 4. Starting from 48 hpf, αB-crystallin-a, -b and Myotilin-deficient embryos showed severely reduced heart rates (Fig. 4E) further declining during development (Fig. 4F). By 72 hpf, fractional shortening of morphant ventricles was significantly reduced to 12% in αB-crystallin-a, 24% in αB-crystallin-b and 8% in Myotilin-deficient embryos and decreased further during embryonic development (Fig. 4G–H), while it was as high as 47% in MO-control injected embryos. Remarkably, whereas at 48 hpf atrial fractional shortening in Myotilin morphants was only slightly reduced (46%), it was already decreased in αB-crystallin-b (35%) and dramatically reduced in αB-crystallin-a-deficient embryos (9%), demonstrating that although inactivation of the MFM genes consistently led to heart failure, gene specific differences in the manifestation of the pathology could be observed. In this context, an involvement of the cardiac conduction system with arrhythmias is a characteristic feature of human Desmin-related MFM (Desminopathy) [5]. Interestingly, 20% of Desmin-a-deficient embryos at 72 hpf frequently showed significant arrhythmias and intermittent sinus exit blocks (Suppl. movie 3).

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.149.

In summary, independent of the targeted MFM gene, the corresponding morphant hearts showed reduced systolic contractile function and beating frequency.

4. Discussion

Genetic animal models are crucial to characterize the molecular pathomechanisms of MFM, to evaluate novel candidate genes and to identify and test therapeutic options. To systematically evaluate whether the zebrafish is a suitable model organism to study human MFM genes, we inactivated these genes and evaluated the resulting phenotypes. Inactivation of all investigated MFM genes consistently led to pronounced myopathy with severely impaired motility and myofibrillar degeneration. Furthermore, for the majority of the morphants, we found next to impaired skeletal muscle function additional phenotypic characteristics such as diminished cardiac contractility or arrhythmias, which are also frequently found in MFM patients carrying mutations in the corresponding genes [1,4,5]. Importantly, several pathological alterations that were unique to the respective morphant phenotype were observed. In this context, it is important to note that ultrastructural analysis of MFM patient biopsies also revealed disease gene specific alterations [22]. In DNAJB6 deficient embryos we observed an autophagic pathology that resembles the pathology reported in a Finnish patient suffering from limb-girdle muscular dystrophy due to a mutation in the DNAJB6 gene [2]. Likewise, ultrastructural abnormalities observed in our study are often confirmed by findings in other MFM gene knockout animal models. For instance, vacuolization within skeletal myocytes in αB-crystallin deficient embryos is also observed in αB-crystallin knockout mice [23]. Furthermore, mitochondrial alterations observed in this study in distinct morphants such as Desmin and αB-crystallin deficient embryos, is one of the described pathological features of MFM [24]. Mitochondrial pathology is also reported upon overexpression of mutant variants of the MFM genes αB-crystallin and Desmin in a cardiomyopathy mouse model [25]. Loss of ZASP/Cypher results in a skeletal muscle myopathy and heart failure in zebrafish [15] while ZASP-deficiency in mice causes embryonic lethality most likely due to the cardiac phenotype [7]. The MFM gene-specific functional, structural and ultrastructural alterations observed in the morphants that frequently mimic the situation in distinct MFM patients and MFM animal models strongly supports the use of the zebrafish

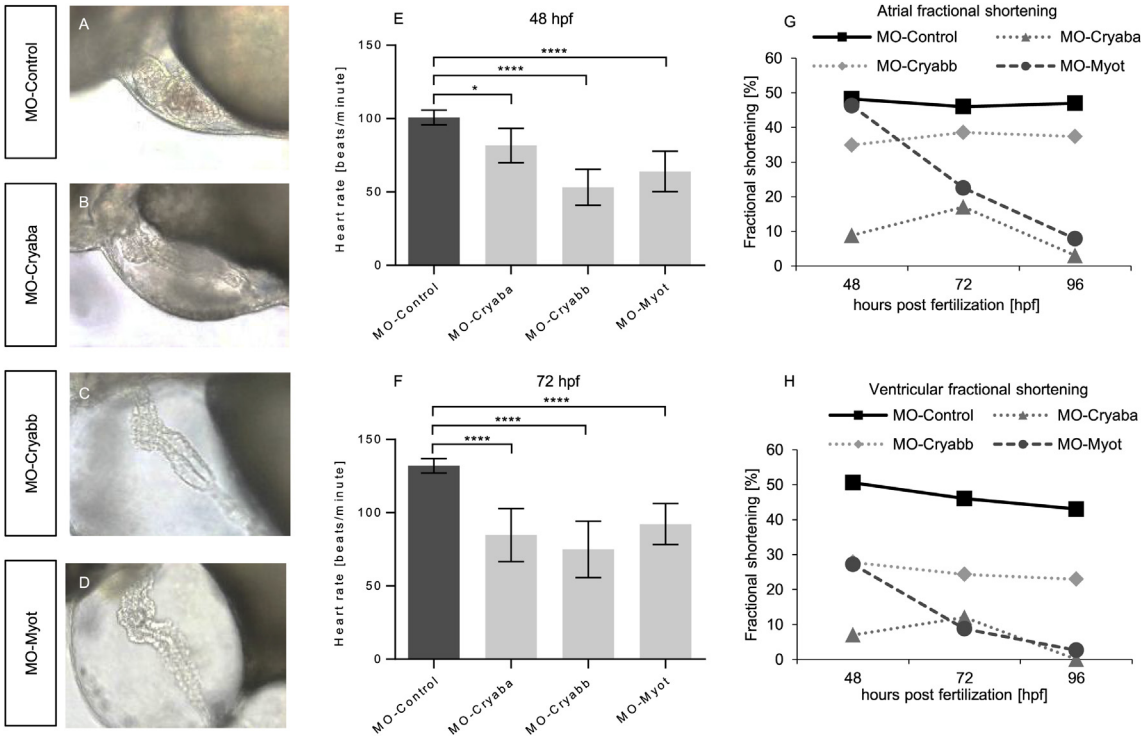

Fig. 4. Loss of MFM gene function results in a cardiomyopathy in zebrafish (A–D) Phenotype of MO-control (A), MO-Cryaba (B), MO-Cryabb (C) and MO-Myot (D) zebrafish hearts at 72 hpf. (E,F) Heart rates of MFM morphants at 48 (E) and 72 hpf (F) are significantly reduced. (G,H) Fractional shortening at defined developmental stages (48, 72, 96 hpf) of atrial (G) and ventricular (H) chambers.

as a model system to study the molecular functions of human MFM disease genes.

Interestingly, ultrastructural examinations of MFM gene deficient muscle cells in zebrafish did not reveal clear protein aggregation as described for human MFM [1,3,4], suggesting that the complete loss of MFM genes does not result in protein aggregation and that instead the presence of mutant MFM proteins might be responsible for aggregate formation. This finding is not surprising since the majority of identified MFM gene mutations seem not to result in a complete loss of gene function but rather act in a dominant-negative manner. A recent elegant study by Ruparelia and coworkers supports this hypothesis [12]. Here, overexpression of a MFM-associated BAG3 variant (BAG3-P209L) in the zebrafish muscle results in pronounced protein aggregation, whereas targeted BAG3 inactivation leads to severe myopathic phenotypes without protein aggregation [12]. Similarly, human filaminopathies due to haploinsufficiency cause distal myopathy without pathological protein aggregation, whereas mutations that lead to the expression of toxic proteins result in aggregation of mutant Filamin C protein together with many other proteins [26]. The analysis of these human MFM protein aggregates by laser microdissection-coupled mass spectrometry, led to the identification of known but also novel proteins that are enriched in the aggregates [27]. Whether these novel aggregate-enriched proteins play a role in the pathogenesis of MFM is not known. In an analogous manner to what we have shown in this study, these novel putative MFM genes can now be evaluated in functional genomic studies in zebrafish. Very recently, powerful genome editing strategies have emerged. TALEN and CRISPR/Cas9 technologies have been successfully used to generate stable knock-out but also knock-in zebrafish lines [28]. Therefore, the zebrafish is not only an excellent model system to study the *in vivo* roles of novel putative MFM disease genes by loss-of-function studies as shown here but might also be suitable to analyze the MFM pathology and pathomechanisms caused by individual gene mutations in patients using genetically engineered zebrafish that specifically express these genetic variants.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.149.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.149.

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