tvst

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Absence of Desmin in Myofibers of the Zebrafish Extraocular Muscles

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Received: June 4, 2020
Accepted: July 14, 2020
Published: September 1, 2020

Keywords: extraocular muscles; desmin; neuromuscular junction; myosin heavy chain; zebrafish; multiterminal en plaque endplates

Citation: Dennhag N, Liu J-X, Nord H, von Hofsten J, Pedrosa Domellöf F. Absence of desmin in myofibers of the zebrafish extraocular muscles. Trans Vis Sci Tech. 2020;9(10):1, https://doi.org/10.1167/tvst.9.10.1

Purpose: To study the medial rectus (MR) muscle of zebrafish (*Danio rerio*) with respect to the pattern of distribution of desmin and its correlation to distinct types of myofibers and motor endplates.

Methods: The MRs of zebrafish were examined using confocal microscopy in whole-mount longitudinal specimens and in cross sections processed for immunohistochemistry with antibodies against desmin, myosin heavy chain isoforms, and innervation markers. Desmin patterns were correlated to major myofiber type and type of innervation. A total of 1382 myofibers in nine MR muscles were analyzed.

Results: Four distinct desmin immunolabeling patterns were found in the zebrafish MRs. Approximately a third of all slow myofibers lacked desmin, representing 8.5% of the total myofiber population. The adult zebrafish MR muscle displayed en grappe, en plaque, and multiterminal en plaque neuromuscular junctions (NMJs) with intricate patterns of desmin immunolabeling.

Conclusions: The MRs of zebrafish showed important similarities with the human extraocular muscles with regard to the pattern of desmin distribution and presence of the major types of NMJs and can be regarded as an adequate model to further study the role of desmin and the implications of heterogeneity in cytoskeletal protein composition.

Translational Relevance: The establishment of a zebrafish model to study the cytoskeleton in muscles that are particularly resistant to muscle disease opens new avenues to understand human myopathies and muscle dystrophies and may provide clues to new therapies.

Introduction

Desmin is the major intermediate filament protein present in adult skeletal myofibers. It plays a crucial role in the organization of the cytoskeleton of myofibers by linking adjacent myofibrils at the Z-discs and linking peripheral myofibrils to the sarcolemma and the nuclear membranes.[1,2] Desmin is the first muscle-specific structural protein detected during muscle differentiation,[3] and it is such a fundamental component of myofibers that it is regularly used as a marker of muscle tissue.[3] In desmin knockout mice, the myoblasts differentiate and build myofibers, but the highly used muscles develop a typical pathologic phenotype, with a myopathy characterized by myofiber damage, subsequent myofiber regeneration, and eventually fibrosis and reduced muscle strength. Desmin, although not necessary for muscle development, has therefore previously been assigned a fundamental role in the maintenance of myofiber integrity.[4,5] However, this paradigm was challenged when we reported that desmin is lacking or is weakly present in a subgroup of normal and intact myofibers in both adult and fetal human extraocular muscles (EOMs).[6,7] Desmin is therefore not necessary for maintenance of myofiber integrity in the EOMs and can no longer be regarded as a ubiquitous muscle protein.[6]

EOMs have a very complex architecture and myofiber type composition that is thought to reflect their highly specialized function in the execution of the different types of eye movements.[8] The myofibers first reported to lack or only have trace amounts of desmin in the human EOMs are those containing myosin heavy chain slow tonic (MyHCsto), and most of them also contain MyHCslow (MyHCI), the so-called MyHCsto/I myofibers.[6,9]

Myofibers containing MyHCsto show slow tonic contractile properties, responding to stimulation with long-lasting contractures, and are thought to play roles in the tonic activity required in fixation and in slow eye movements.[8] These MyHCsto myofibers are multiply innervated, displaying small motor endplates along their length, the so-called en grappe motor endings.[8,10] There are, in addition, two other major myofiber types in the human EOMs: those containing MyHCII and those lacking both MyHCII and MyHCsto/I but containing MyHCeom.[9] The myofibers containing MyHCII are typically innervated by a single large en plaque motor ending.[8] Finally, most of the myofibers containing MyHCeom display multiterminal en plaque motor endings.[10] We have recently reported that there is a complex relation between desmin content and the type of neuromuscular junction in different myofiber types in the human EOMs.[7]

Onset of *desmin* expression in zebrafish (*Danio rerio*) embryos has been reported at 9 hours postfertilization (hpf), just subsequent to that of *myoD*, in muscle precursor cells in the somites.[11] *Desmin* then continues to be expressed throughout somitogenesis in a manner similar to that of other vertebrates, reaching its peak of expression at 24 hpf before reaching lower expression levels in trunk muscle as well as the heart and fin buds.[11] At the protein level, zebrafish desmin shares conserved intermediate filament domains in the head and rod regions with human desmin.[11] The teleost genome has undergone a duplication event, causing a significant portion of genes to segregate in terms of function, retain a similar redundant functionality, or alternatively lose functionality in one of the two genes.[12] As a result, zebrafish express both *desma* and *desmb* with 73% and 76% homology to the human *desmin* gene, respectively.[13] However, functional redundancy for these genes has yet to be determined. At 48 hpf, *desmb* expression levels are 2500-fold lower than *desma* and no upregulation has been detected upon *desma* deletion.[13] Desmin aggregation has also been reported in the *desma*^{ct122aGT} knockin model[13] in the trunk muscle of 3 to 5 days postfertilization (dpf) zebrafish embryos, similar to mouse and human desmin missense mutation aggregation.[14]

The development of the zebrafish EOMs starts as early as 60 hpf but is not considered complete until 72 hpf.[15] At this point, all six eye muscles are present and spontaneous eye movements measured by the optokinetic response reflex can be observed. Functionally, the EOMs continue to develop until approximately 96 hpf, when optokinetic response reflex results are similar to those of adult zebrafish.[15]

The microanatomy of the adult zebrafish EOMs shows similarities to that of the human EOMs. The zebrafish lateral rectus muscle has previously been reported to display both en grappe and en plaque motor endings.[16,17] Neuromuscular junction (NMJ) density has been reported to be higher at the muscle origin and to be concentrated around smaller diameter myofibers, but only in a restricted portion of the lateral rectus.[16] The relationship between different myofiber types in the zebrafish EOMs and their innervation patterns is unknown.

In the present study, we investigated whether myofibers in the EOMs of the zebrafish also show absent or reduced content of desmin, whether the different types of motor endings described in the human EOMs (single en plaque, en grappe, and multiterminal en plaque) are present in zebrafish, and whether there is a correlation between desmin content and myofiber type and/or type of motor ending, with the aim of determining whether zebrafish may be an adequate model to study the role of desmin and the implications of heterogeneity in cytoskeletal protein composition.

Materials and Methods

Zebrafish Husbandry

Zebrafish were maintained under standard conditions in the Umeå University Zebrafish facility. Transgenic lines *tg (mylz2:GFP)*^{i135} and *(smyhc1:LY-Tomato)*^{i261} were used and are described below.[18–20] All animal experiments were conducted in accordance with the European Community's Council Directive (86/609/EEC), complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and had approval from Umeå Animal Experimental Ethics Board.

Muscle Samples

Adult zebrafish were euthanized using ethyl 3-aminobenzoate methane sulfonate (Tricaine) before the heads were mounted separately on cardboard,

rapidly frozen in propane chilled with liquid nitrogen, and stored at –80°C until sectioned. Adult zebrafish tissue was serially sectioned (12–16 µm) in a cryostat (Reichert-Jung, Leica, Heidelberg, Germany), mounted on glass slides, and stored at –20°C until processed for immunofluorescence.

We surveyed all the EOMs in the zebrafish and ensured that they were rather similar. We then chose to focus on the medial rectus (MR) muscle because of its position that ensures easier and complete dissection with good anatomical muscle integrity in the adult fish. To dissect adult MR muscle, euthanized double *tg (mylz2:GFP)*^{i135}*/tg (smyhc1:LY-Tomato)*^{i261} or single *tg (mylz2:GFP)*^{i135} zebrafish were fixed to a cardboard plate using pins. Part of the skull bone along with the brain was swiftly removed to increase EOM exposure to paraformaldehyde (PFA) while keeping muscles in their original condition, and the whole head was fixed in 2% PFA for 1.5 to 2 hours at room temperature (RT). Thereafter, the eyes were carefully removed along with the base of the skull to keep EOM anatomy as intact as possible. The MR muscle was carefully removed from the bone, and a piece of the eyeball was allowed to remain attached to the muscle to not disturb tendon insertions.

Antibodies and Immunofluorescence

Rabbit polyclonal antibody ab15200[21] (1:100; Abcam, Novakemi AB, Sollentuna, Sweden) was used to detect desmin. Mouse monoclonal antibody (mAb) F310 against MyHCII and mAb S58 against MyHCslow (1:10; The Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, IA) were used to label fast and slow myofibers, respectively. Motor endplates and axons were detected by Alexa Flour 647–conjugated α-bungarotoxin labeling (1:300; Molecular Probes, Eugene, OR) and mouse mAb SV2 (1:100, The Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa).

Zebrafish sections were briefly washed in phosphate-buffered saline (PBS) and blocked in 1% blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany) with 4% Triton-X for 1 hour. Primary antibodies were applied and the slides were incubated for 48 hours at 4°C. Slides were then washed extensively in PBS before secondary antibodies were applied and incubated overnight, followed by extensive washing and coverslip mounted using 80% glycerol.

Zebrafish embryos were immunolabeled using a standard whole-mount approach, as previously described.[22] Adult whole MR muscles were treated using the same standard whole-mount immunofluorescence strategy as for embryos, with the following exceptions: fixation was limited to 1.5 to 2 hours in RT, washing buffer was PBX (1× PBS, 1% Triton-X), and blocking was obtained with 1% blocking reagent in 4% PBX, 5% dimethyl sulfoxide (DMSO). The incubation was extended to 4 days for primary antibodies and to 1 day for secondary antibodies. Last, the MR muscles were flat mounted under a coverslip in 80% glycerol before imaging.

Muscle cross sections and whole-mount adult EOMs and whole-mount embryonic zebrafish were imaged using a Nikon A1 confocal microscope (Nikon, Tokyo, Japan). The images were processed using the ImageJ (National Institutes of Health, Bethesda, MD) and Adobe Photoshop software (Adobe System, Mountain View, CA).

Myofiber Typing

To show all fast myofibers in our zebrafish, transgenic expression of *tg (mylz2:GFP)*^{i135} was used. This transgenic line has previously been extensively characterized[18,19] and expresses green fluorescent protein (GFP) specifically in fast myofibers containing *myosin light chain 2 polypeptide*. Slow myofibers in zebrafish predominantly express *smyhc1* and, to a lesser extent, *smyhc2* and *smyhc3*.[23] Where longitudinal visualization of the EOMs was needed, we used *tg (smyhc1:LY-Tomato)*^{i261} to identify the slow muscle fibers.[24] For statistical analysis in cross sections, we used the S58 antibody, previously shown to immunolabel a broader spectrum of *smyhc* -positive myofibers.[20] For simplicity, myofibers identified as described above are henceforth termed fast and slow myofibers, respectively. The myofibers were classified into four categories based on their transgenic expression and/or immunoreactivity with antibodies against myosin isoforms: (1) myofibers expressing tg (mylz2:GFP)^{i135} were classified as fast myofibers containing MyLCfast, (2) myofibers labeled by antibody S58 against MyHCslow were classified as slow myofibers containing MyHCslow, (3) myofibers expressing both tg (mylz2:GFP)^{i135} and labeled by antibody S58 against MyHCslow were classified as hybrid myofibers containing MyLCfast and MyHCslow, and (4) myofibers lacking expression of tg (mylz2:GFP)^{i135} and unlabeled by the S58 antibody against MyHCslow were classified as EOM-like myofibers.

Statistics

The number of myofibers, type of myosin content, and desmin immunolabeling patterns were counted on nine cross-sectioned MR muscles from five 13-month-

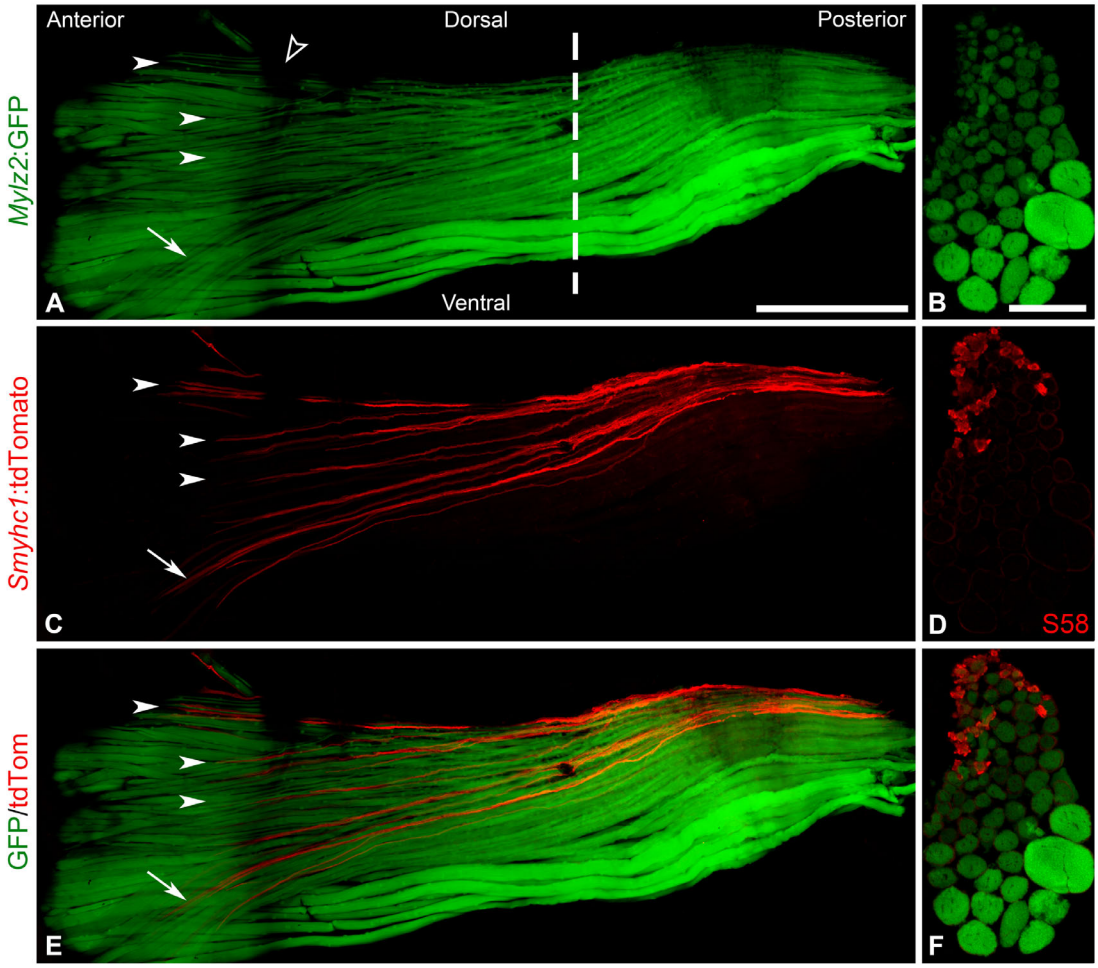

Figure 1. The myofiber organization of the zebrafish MR muscle. Longitudinal (A, C, E) and cross-sectional (B, D, F) view of adult whole-mount MR expressing *mylz2*:GFP (fast, A, B), *smyhc1*:tdTomato (slow, C, D), and merged (E, F). *Open arrowhead* indicates a shade created by the sclera, *white arrowheads* indicate slow myofibers (*smyhc1*:tdTom positive) spreading across the muscle, and the *white arrow* indicates a subgroup of myofibers reaching diagonally across the muscle. The dashed line indicates approximate level where cross sections of the MR shown were cut in B, D, F. *Scale bar* in A applies also to C and E: 500 µm; *scale bar* in B applies also to D and F: 50 µm.

old sibling *mylz2*:GFP transgenic adult zebrafish immunolabeled with S58. A total of 1382 myofibers were counted and evaluated. All data are presented as mean ± standard deviation (SD). Data were collected, processed, and plotted in Microsoft Excel (Microsoft, Redmond, WA).

Results

Myofiber Organization of the Adult Zebrafish MR

On whole-mount longitudinally orientated adult MR, the anterior part of the muscle, which attaches to the eye, was very flat and consisted almost of a single layer of myofibers (Fig. 1). On the posterior end, the muscle was cylindrical in the proximity of the tendon. Large-diameter fast myofibers (*mylz2*:GFP positive) dominated the ventral, global side of the muscle (Figs. 1A, 1B). Small-diameter slow and fast myofibers (*smyhc1*:tdTom and *mylz2*:GFP positive, respectively) were restricted to the dorsal, orbital side of the muscle, with occasional slow myofibers mixed in among fast myofibers (Figs. 1C–F). As the muscle became thinner and wider toward the anterior end, the thin fast and slow myofibers spread out across the surface of the muscle (Figs. 1A, 1C, 1E) with a subgroup reaching diagonally from the most posterior part of the muscle all the way to the most anterior end of the orbital side of the muscle (Figs. 1A, 1C, 1E). On average, the MR muscle contained 153.6 ± 25.2 myofibers and displayed a majority of fast myofibers (*mylz2*:GFP positive), followed by slow myofibers (S58 positive), fast/slow positive hybrid myofibers (*mylz2*:GFP/S58 positive), and fast/slow

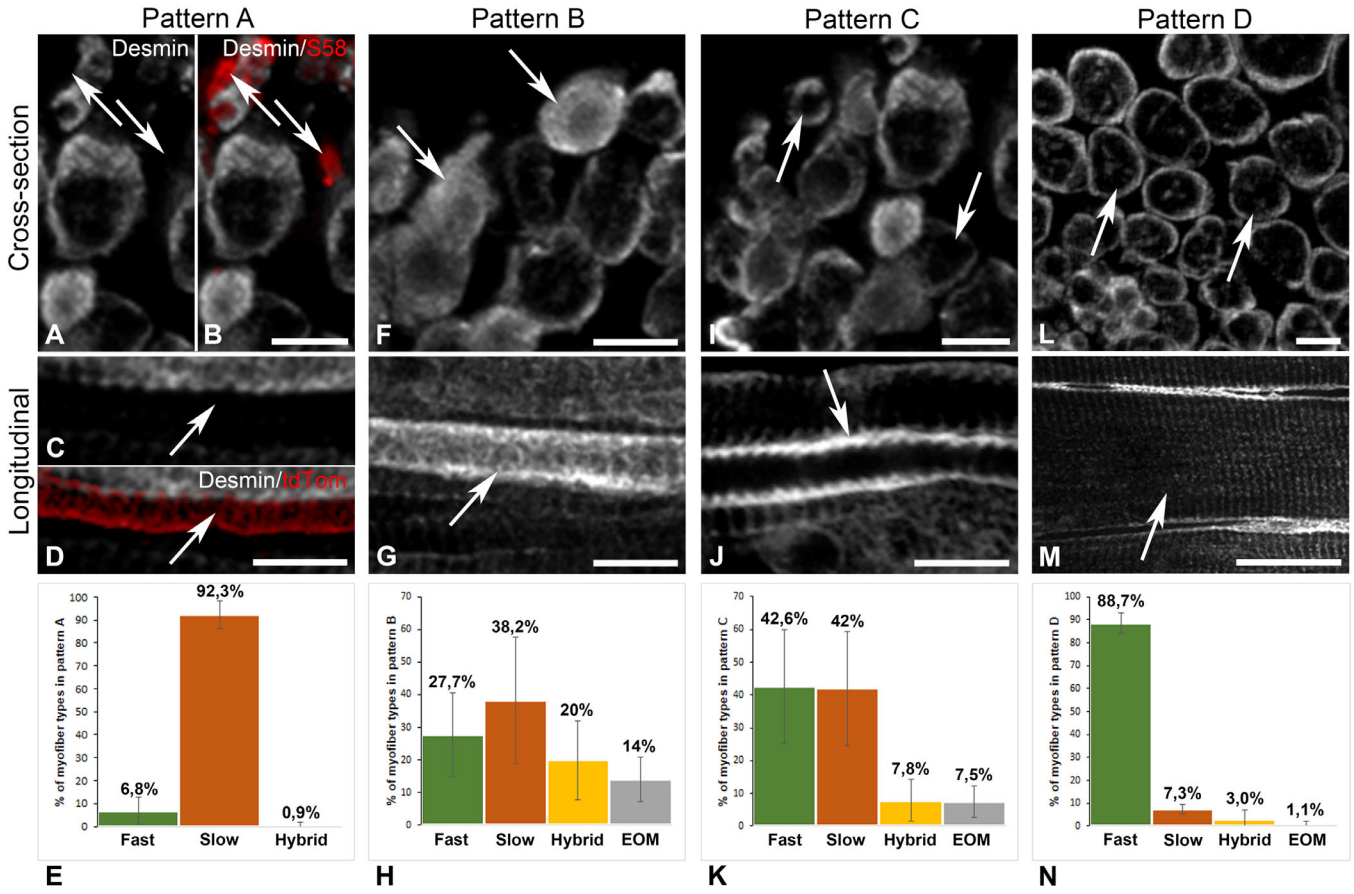

Figure 2. Desmin immunolabeling patterns of the adult zebrafish MR muscle. Examples of the four patterns of immunoreactivity with antibodies against desmin in cross (A, B, F, I, L) and longitudinal (C, D, G, J, M) views of MR muscle. Pattern A myofibers were completely unlabeled by the antibodies against desmin (A–D, *arrows*) but were identified with antibodies against MyHC slow (B) or transgenic expression of *smyhc1*:tdTom (D). Pattern B myofibers had desmin both subsarcolemmaly and inside the remaining myofiber area (F, G, *arrows*). Pattern C myofibers had only subsarcolemmal immunolabeling (I–J, *arrows*), and pattern D myofibers displayed desmin immunolabeling subsarcolemmaly as well as sparsely inside the myofiber (L–M, *arrows*). The frequency of each desmin immunolabeling pattern among the different myofiber types is shown in E, H, K, and N, respectively. *Scale bars*: 10 µm.

Table 1. Distribution of Myofiber Types in the Zebrafish Medial Rectus

Myofiber Type	Mean ± SD, %
Fast	58.5 ± 7.1
Slow	28.4 ± 5.4
Hybrid	7.8 ± 4.9
EOM-like	5.2 ± 1.9

negative myofibers (*mylz2*:GFP/S58 negative), henceforth termed EOM-like myofibers (Table 1).

Most Myofibers Lacking Desmin Contained Slow Myosin in the Adult Zebrafish MR

Most of the myofibers in the adult zebrafish MRs were labeled with the antibodies against desmin and revealed four immunolabeling patterns in cross sections and longitudinal view (Fig. 2). For pattern A, desmin immunolabeling was completely absent in the whole myofiber cross section (Figs. 2A, 2B) and longitudinal view (Figs. 2C, 2D). For pattern B, desmin immunolabeling was present both subsarcolemmaly and in the remaining area of the myofiber cross section and longitudinal view (Figs. 2F, 2G). For pattern C, desmin was present only subsarcolemmaly (Figs. 2I–J), and for pattern D, desmin was detected strongly to moderately subsarcolemmaly and only sparsely in small aggregates inside the myofiber (Figs. 2L–M). Pattern D was found to be the most abundant, followed by patterns B and C. Pattern A was found to be the least numerous (Table 2).

Next, the different desmin immunolabeling patterns were correlated with myofiber types. Among the myofibers completely lacking desmin immunolabeling (pattern A), 92.3% were labeled as slow

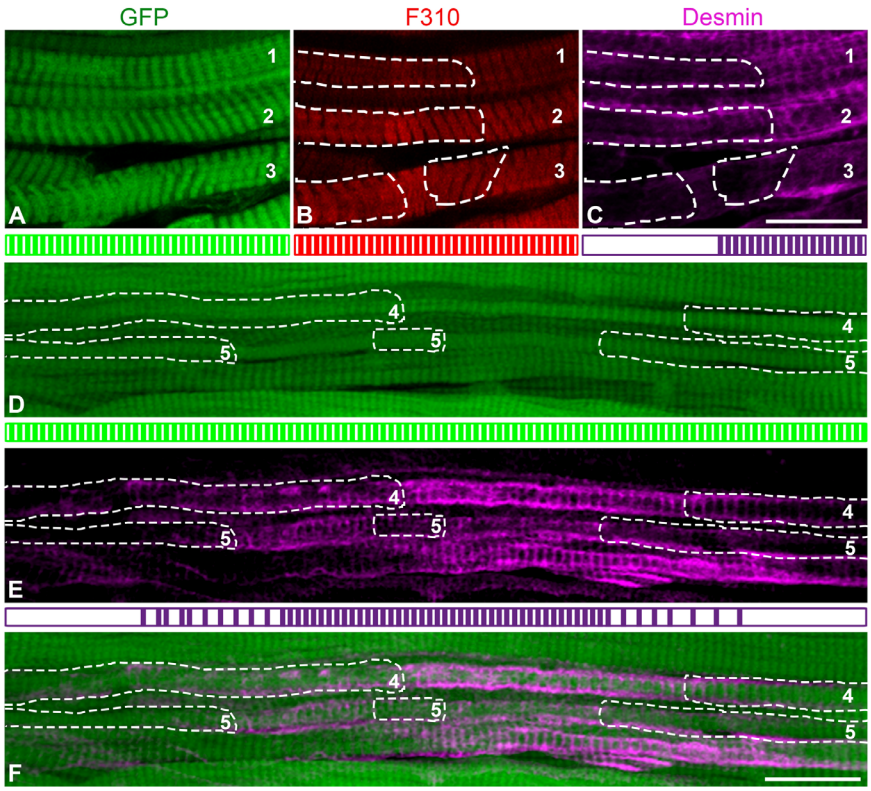

Figure 3. Desmin immunolabeling intensity varied along the length of individual myofibers. Longitudinal view of adult zebrafish MR showing fast *mylz2*:GFP-positive (A, D), F310 fast-positive (B), desmin-positive (C, E), and merged image (F) myofibers. Examples of different levels of desmin immunolabeling intensity along the length of individual myofibers are outlined by *dashed lines*. A schematic illustration of the labeling pattern along the myofiber is provided underneath each figure. Numbers indicate the same myofibers in A–C and D–F, respectively. *Scale bars*: 10 µm.

Table 2. Distribution of Desmin Immunolabeling in Zebrafish Medial Rectus

Pattern	Mean ± SD, %
A	8.5 ± 6.5
B	21.2 ± 5.1
C	22.9 ± 8.2
D	47.8 ± 5.1

myofibers with the S58 Ab, whereas it was seldom seen in hybrid myofibers (0.9%; Fig. 2E). Pattern B was found in a more evenly distributed manner among the different myofiber types, with the majority of myofibers identified as slow myofibers (38.2%; Fig. 2H). Pattern C was found most often in fast myofibers expressing *Mylz2*:GFP (42.6%) and slow myofibers (42%; Fig. 2K). Pattern D was predominantly found in fast myofibers expressing *mylz2*:GFP (88.7%; Fig. 2N). Additionally, when studying longitudinal slow myofibers (*smyhc1*:tdTom positive), we noted that weakly positive myofibers showed a more intense desmin immunolabeling, and in contrast, slow myofibers intensely expressing *smyhc1*:tdTom were weakly labeled with desmin antibodies (Supplementary Figs. S1A–C).

In general, the desmin patterns described above appeared homogeneous along the myofiber length, in longitudinal sections. Interestingly, occasional myofibers showed a varying level of desmin immunolabeling along their myofiber length (Fig. 3), comprising intense labeling pattern B and weak or no labeling inside the myofiber, as described for pattern C (Figs. 3C, 3E). Both *mylz2*:GFP and myosin immunolabeling with the mAb F310 against MyHCII showed homogeneous intensity levels along the length of the same myofibers (Figs. 3A, 3B, 3D).

Slow Myofibers of the Zebrafish Embryo EOMs Lacked Desmin at 5 dpf

Because adult zebrafish MR muscles contained 8.4% myofibers lacking desmin, we asked whether desmin was initially present in all myofibers or not. To investigate the presence of desmin in fast and

slow myofibers in the EOMs of zebrafish embryos, *mylz2*:GFP and *smyhc1*:tdTomato transgenic zebrafish were crossed and the presence of desmin in specific myofiber types was analyzed throughout embryogenesis. Because the MR muscle was challenging to visualize in the embryo due to its location deep within the skull, we chose to focus on the EOMs that were more accessible.

At 48 hpf, no fast (*mylz2*:GFP positive) or slow (*smyhc1*:tdTom positive) EOMs had formed and no desmin could be detected (Supplementary Figs. S2A–D). At 56 hpf, desmin and fast myofibers (*mylz2*:GFP positive) could be detected in the lateral rectus (LR), and in the superior rectus (SR), some slow myofibers (*smyhc1*:tdTom positive) were present (Supplementary Figs. S2E–H). Desmin was present in the EOMs, but at this early stage, we were unable to determine the identity of many of the desmin-expressing myofibers in the SR (Supplementary Figs. S2E–H). However, desmin was not detected in any zebrafish myofibers in EOMs clearly identified as slow (*smyhc1*:tdTom positive) at this stage (Supplementary Fig. S2). At 3 dpf, slow and fast myofibers (*smyhc1*:tdTom and *mylz2*:GFP positive) could be detected in separate regions in the ventrally located inferior oblique (IO) and inferior rectus (IR) muscles, but no desmin was detected in these muscles at this stage (Figs. 4A–C). On the dorsal side, slow myofibers (*smyhc1*:tdTom positive) were detected in the superior oblique (SO), SR, and LR muscles, and fast myofibers (*mylz2*:GFP positive) could be detected in the lateral part of LR (Figs. 4D–F). At 5 dpf, desmin was coexpressed with *mylz2*:GFP (fast) in IO, IR, SO, and SR muscles (Figs. 4H, 4K). However, no desmin was detected in the slow myofibers (*smyhc1*:tdTom positive) of these EOMs (Figs. 4G–L), indicating that desmin was not present in slow myofibers in the EOMs of zebrafish embryos at 5 dpf.

Adult Zebrafish MR Contained Both Single En Plaque, Multiple En Grappe, and Multiterminal En Plaque Neuromuscular Junctions

To investigate the NMJs of adult zebrafish EOMs, whole MR muscles were fixed in situ and subsequently dissected from the heads of double transgene *mylz2*:GFP/*smyhc1*:tdTom zebrafish. Next, we analyzed the NMJs using α-bungarotoxin as well as a mAb against synaptic vesicle 2 (SV2). SV2 and α-bungarotoxin immunolabeling was shown to practically overlap (Supplementary Fig. S3), and thereafter, only α-bungarotoxin was used to label NMJs. The whole MR muscle was scanned using a confocal microscope to render a three-dimensional (3D) image of all myofibers and their NMJs. A general summary of the results obtained is provided in Table 3. The MR muscle was richly innervated (Fig. 5, Fig. 6), and when the myofibers of the global layer were followed throughout the length of the entire muscle, both single en plaque and multiterminal en plaque, defined as three or more en plaque NMJs within 200 µm on the same myofiber, were frequently identified (Figs. 5A–C, F–H). Desmin immunolabeling was found to be slightly more intense around the edges of the NMJs and was weaker directly underneath NMJs (Figs. 5D, 5E, 5I, 5J). In 3D, desmin was found adjacent to these NMJs, delineating a small groove in the subsarcolemma, where the NMJs were positioned (Supplementary Figs. S4A, S4B). No slow myofibers marked by *smyhc1*:tdTom were identified in the global layer; therefore, en grappe motor endplates are described only in the orbital layer.

The orbital layer, consisting of smaller diameter fast myofibers (*mylz2*:GFP positive) and slow myofibers (*smyhc1*:tdTom positive) displayed en plaque, multiterminal en plaque, and en grappe NMJs (Fig. 6). Similar to the above description of the global layer, fast myofibers in the orbital layer displayed single en plaque (Figs. 6A1–A3) and multiterminal en plaque NMJs along their length (Figs. 6B1–B3). However, in contrast to the global layer, multiterminal en plaque NMJs were only found in the vicinity of the nerve entry into the MR muscle. Slow myofibers displayed en plaque (Figs. 6C1–C3, D1–D3) as well as en grappe NMJs (Figs. 6E1–E3). Most NMJs on fast myofibers displayed a similar desmin immunolabeling pattern as described for the global layer. Sporadically, desmin immunolabeling was lacking adjacent to the subsarcolemma directly beneath the NMJ (Figs. 6A4, A5, B4, B5). Additionally, slow myofibers displayed either unchanged desmin immunolabeling or areas lacking desmin immunolabeling near NMJs (Figs. 6E4, E5). Lastly, en grappe NMJs were also found on myofibers completely lacking desmin (Figs. 6F1–F5).

Discussion

Desmin is a fundamental cytoskeletal protein with a pivotal localization and role, linking adjacent myofibrils and keeping them aligned inside the myofiber. In addition, desmin links the myofibrillar apparatus to the subsarcolemma, the nuclei, and the mitochondria[25–27] and plays a role in cell signaling and gene regulation.[28,29] It is therefore of importance to identify an

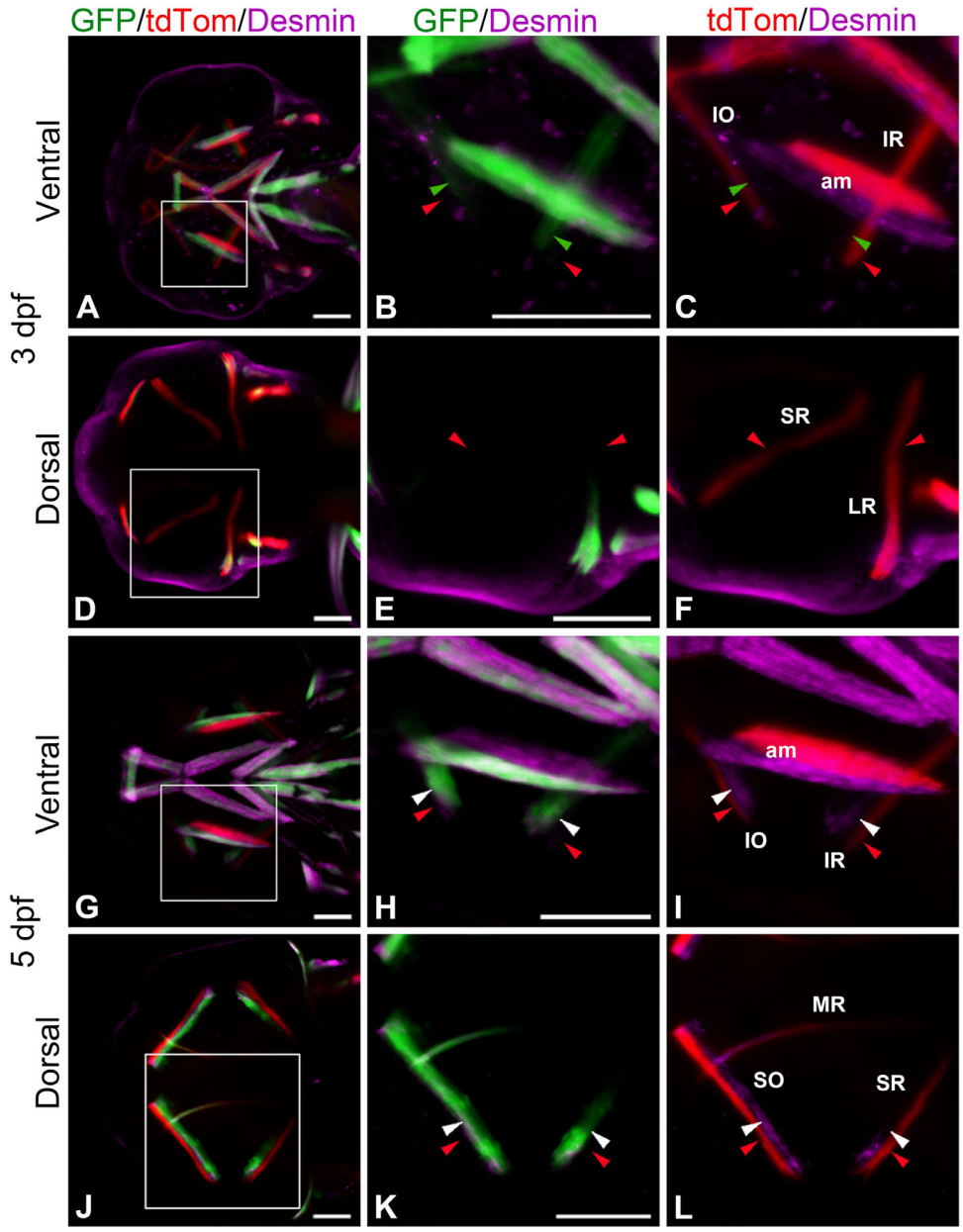

Figure 4. Desmin was not present in the myofibers containing *smyhc1*:tdTomato in the zebrafish embryo EOMs. Ventral and dorsal view showing desmin (*magenta*) in fast/slow double transgenic (*smyhc1*:tdTomato (*red*)/*mylz2*:GFP (*green*) positive) embryos at 3 dpf (A–F) and 5 dpf (G–L). *Squares* indicate approximate area of magnification in panels to the right. *Red arrowheads* indicate slow *smyhc1*:tdTom-positive myofibers, *green arrowheads* indicate fast *mylz2*:GFP-positive myofibers, and *white arrowheads* indicate *mylz2*:GFP/desmin double-positive myofibers. Desmin was only present in *mylz2*:GFP-positive myofibers and was lacking in *smyhc1*:tdTomato-positive myofibers. am, anterior mandibulae muscle. *Scale bars*: 100 µm.

adequate model to study the biological implications of the lack of desmin in subgroups of myofibers and in relation to NMJs. This knowledge is in addition expected to shed light on the special properties of the EOMs and, in particular, their ability to resist disease. The present study was therefore undertaken to evaluate whether the zebrafish EOMs showed similarities to the human EOMs regarding desmin expression and could therefore be used as such a model in the future.

The zebrafish MR muscle consisted of myofibers that could be divided into four major myofiber categories on the basis of their myosin composition: slow myofibers containing *smyhc1*, fast myofibers containing *mylz2*, hybrid slow/fast myofibers (*smyhc1*/*mylz2* positive), and slow/fast double-negative myofibers (*smyhc1*/*mylz2* negative). We refer to the double-negative myofibers as EOM-like myofibers as a parallel to those described in human EOMs,[9] as these

Table 3. Summary of Desmin Immunolabeling in Correlation to Myofiber Type

Type of NMJ	Myofiber Type	Desmin at NMJ
Single en plaque	Fast	+ (–)
	Slow	+ (–)
	EOM like	+ (–)
Multiterminal en plaque	Fast	+ (–)
En grappe	Slow	+ (–)
	EOM like	+ (–)

Desmin was most often present in the immediate vicinity of all types of NMJs examined (denoted +), irrespective of myofiber type. Occasionally, however, desmin was absent (denoted (–)) directly underneath or in the close vicinity of NMJs, without apparent correlation to myofiber type.

myofibers are unlabeled with antibodies against both MyHCslow and MyHCII. The zebrafish EOM-like myofibers were not immunolabeled by the S58 ab or expressed *mylz2*:GFP, normally labeling all slow and fast muscle, respectively, within the zebrafish trunk and cranial musculature.[18] It is currently unclear what type of MyHC is present in the EOM-like myofibers in the zebrafish, although there is likely no zebrafish MyHC that is directly homologous to human MyHCeom. Human MyHCeom displays 75.9% homology to the zebrafish *fmyhc2.2* gene, previously shown to be expressed in the cranial and trunk musculature of 3-dpf zebrafish embryos.[30] However, MyHCeom also shows similar homology to several other MyHC genes expressed in similar locations in the zebrafish.

We found four distinct patterns of immunolabeling in the zebrafish MR with antibodies against desmin. Interestingly, 92.3% of myofibers lacking desmin contained slow myosin and represented almost a third of all slow myosin-positive myofibers. Slow myosin-positive myofibers also displayed all three remaining desmin staining patterns. These results are in line with previously reported results from human EOMs[6,7] and suggest a possible relationship between lack of desmin and MyHCslow. However, it should be stated that 6.8% of myofibers lacking desmin were also identified as fast in zebrafish (Fig. 2), and this is in line with our previous report on lack of desmin in myofibers containing MyHCeom in the human EOMs.[7] Thus, lack of desmin is not exclusively linked to the presence of MyHCslow. We have also found lack of desmin in myofibers containing MyHCslow in the EOMs of rabbit, rat, and mouse (unpublished results). Altogether, these results indicate that lack of desmin in a subgroup of slow myofibers in the EOMs is an evolutionary conserved feature shared between vertebrate species.

Interestingly, we also found that desmin immunolabeling intensity varied along the length of some myofibers in the zebrafish MR muscle without apparent relation to NMJs. The same myofiber could shift from pattern B to pattern C (Fig. 3). Local disorganization and lack of desmin have previously been reported in myofibers of the upper airway in snorers and sleep apnea patients,[31] as well as in patients with cardiomyopathy,[32] but lack of desmin has previously only been described in healthy tissue in relationship to NMJs in the human EOMs.[7,10] It is possible that these are further specialized myofibers showing a variable desmin content along their length.

Additionally, in the zebrafish embryo, no slow EOM myofibers (*smyhc1*:tdTom positive) overlapped with desmin immunolabeling at 5 dpf. Previous results from knockout mice show that desmin is not needed for normal EOM development,[33] and in fetal human EOMs, the myotubes containing MyHCslow lack desmin.[17] In this study, we showed that desmin was

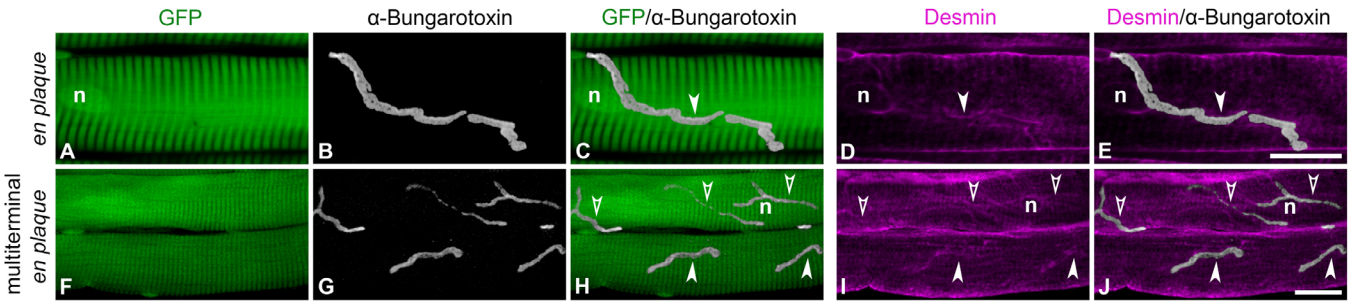

Figure 5. NMJs in the global layer of the zebrafish MR muscle. Longitudinal view of large-diameter fast myofibers in the global layer identified with *mylz2*:GFP (A, F), treated with α-bungarotoxin (B, G), and merged image of *mylz2*:GFP/α-bungarotoxin (C, H), desmin immunolabeling (D, I), and merged image of desmin/α-bungarotoxin (E, J). These myofibers displayed single en plaque (A–E) and multiterminal en plaque NMJs (F–J, *arrowheads*). Arrowheads indicate slightly increased desmin immunolabeling intensity in the vicinity of single (D, E) and multiterminal NMJs (I, J). NMJs on the same myofiber are marked with the same *arrowhead* type in H–J. n, nucleus. *Scale bar*: 25 µm.

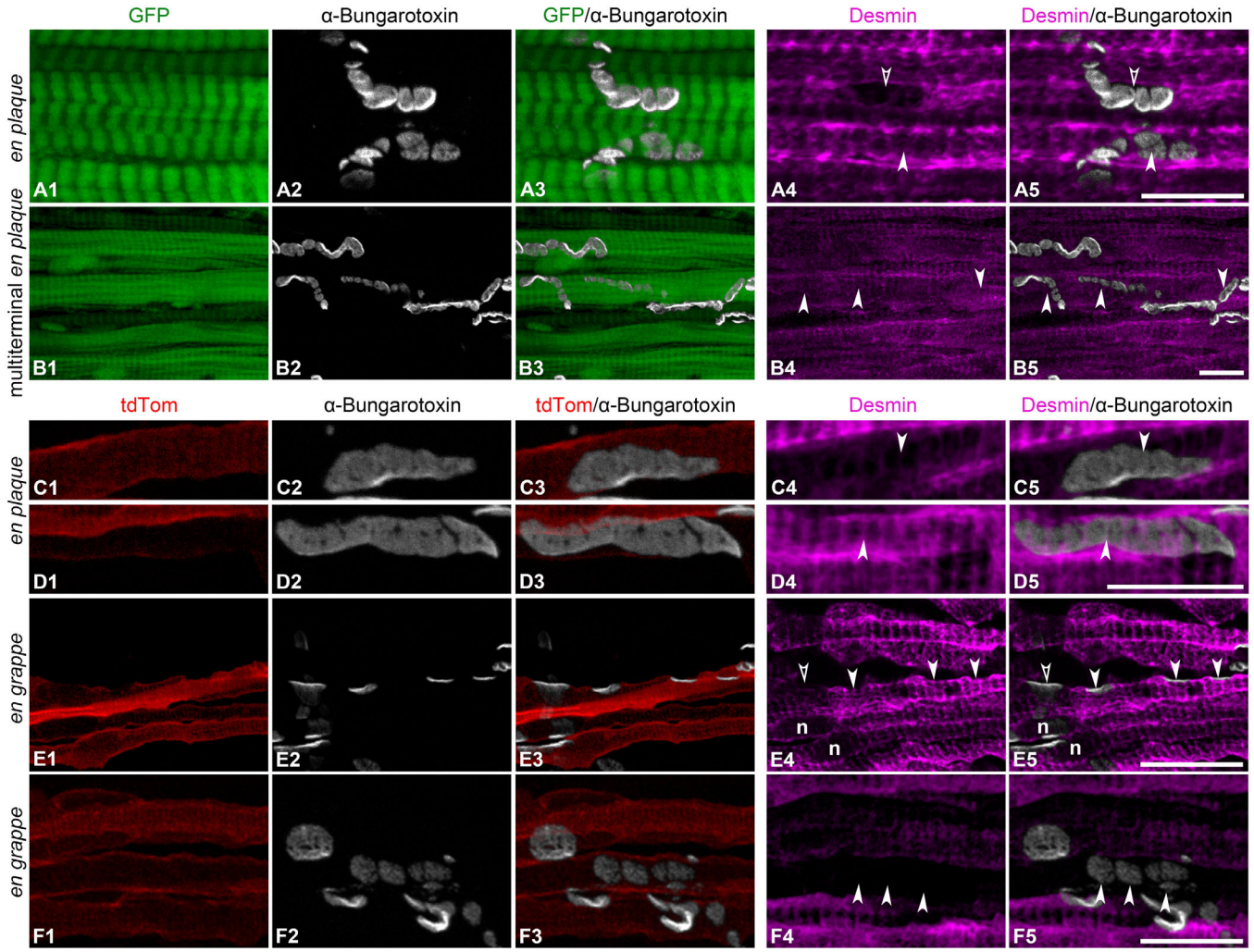

Figure 6. NMJs in the orbital layer of the zebrafish MR muscle. Longitudinal view of the thinner fast myofibers of the orbital layer labeled with *mylz2*:GFP (A1, B1), *smyhc1*:tdTom (C1, D1, E1, F1), α-bungarotoxin (A2, B2, C2, D2, E2, F2), and desmin (A4, B4, C4, D4, E4, F4). Merged image of *mylz2*:GFP/α-bungarotoxin (A3, B3), *smyhc1*:tdTom/α-bungarotoxin (C3, D3, E3, F3), and desmin/α-bungarotoxin (A5, B5, C5, D5, E5, F5). Fast myofibers displayed single en plaque motor endings, either lacking (A4, *open arrowhead*) or containing (A4, *arrowhead*) desmin, as well as multiterminal en plaque motor endings (B1–B5, *arrowheads*) with unchanged or slightly more intense desmin immunolabeling (B4, B5, *arrowheads*). Slow myofibers displayed single en plaque (C1–D5, *arrowheads*) as well as en grappe NMJs (E1–F5), with desmin unchanged near single en plaque motor endings (C4, C5, D4, D5) and occasionally lacking desmin in the vicinity of en grappe motor endings (E4, E5, *open arrowhead*). Slow myofibers lacking desmin displaying en grappe NMJs were also identified (F1–F5, arrowheads). *Scale bars*: 10 µm.

initially not present in the developing slow myofibers in the EOMs of zebrafish embryos, but it switched on during maturation in a subgroup of these myofibers and was present in EOMs of the adult animal in a similar manner to that of adult human EOMs. These results could indicate that some myofibers are predetermined not to express desmin, raising questions about the time point and regulation of desmin expression. Further studies are needed to address these questions.

Adult zebrafish MR muscle was found to contain the NMJ types typical of EOMs, including the multiterminal en plaque NMJ recently discovered in human EOMs.[10] Desmin immunolabeling in the vicinity of NMJs was found to be unchanged, slightly enhanced, or lacking in some myofibers, generally mimicking results recently published in human EOMs.

In summary, the present study showed that the pattern of desmin distribution in the MR of zebrafish was rather similar to that of human EOMs. We propose that the powerful tools of transgenic lines and whole-mount immunolabeling available combined with 3D reconstruction techniques make zebrafish an excellent model for continued research on the roles of desmin and lack thereof in the EOMs.

Acknowledgments

Supported by grants from the Swedish Research Council (2018-02401; Stockholm, Sweden), County Council of Västerbotten (Umeå, Sweden), Ögonfonden (Stockholm, Sweden), and Kronprinsessan Margaretas Arbetsnämnd för Synskadade (Valdemarsvik, Sweden).

Disclosure: **N. Dennhag,** None; **J.-X. Liu,** None; **H. Nord,** None; **J. von Hofsten,** None; **F. Pedrosa Domellöf,** None

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