

Swimming into prominence: the zebrafish as a valuable tool for studying human myopathies and muscular dystrophies

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A new and exciting phase of muscle disease research has recently been entered. The application of next generation sequencing technology has spurred an unprecedented era of gene discovery for both myopathies and muscular dystrophies. Gene-based therapies for Duchenne muscular dystrophy have entered clinical trial, and several pathway-based therapies are doing so as well for a handful of muscle diseases. While many factors have aided the extraordinary developments in gene discovery and therapy development, the zebrafish model system has emerged as a vital tool in these advancements. In this review, we will highlight how the zebrafish has greatly aided in the identification of new muscle disease genes and in the recognition of novel therapeutic strategies. We will start with a general introduction to the zebrafish as a model, discuss the ways in which muscle disease can be modeled and analyzed in the fish, and conclude with observations from recent studies that highlight the power of the fish as a research tool for muscle disease.

Why zebrafish?

There are numerous factors that make zebrafish a particularly appealing model organism. Such advantages have been extensively enumerated in several outstanding recent reviews and include especially (a) the ability to quickly generate a large number of offspring (100–300 embryos per cross), (b) the rapid *ex utero* development of the zebrafish, (c) the optical transparency of zebrafish embryos and larvae, (d) the ease of genetic manipulations and (e) the ready ability of the zebrafish embryo to absorb drugs [1,2]. In addition, zebrafish offer several specific qualities as a model for human muscle disorders. They have reproducible, quantitative and easily measured motor behaviors that are present from 1 day of life onward. Their skeletal muscle shares many molecular and histological features with mammalian muscle (Fig. 1A–C) and appears nearly identical to human

muscle at the ultrastructural level. This includes preservation of the components of the dystrophin-associated glycoprotein complex, the excitation–contraction coupling machinery and the contractile apparatus, three fundamental muscle structures that are key to studying and understanding human muscle disease pathogenesis [3–7]. In addition, skeletal muscle is the largest and most prominent organ system of the developing zebrafish and, due to the optical transparency of the zebrafish, can be easily visualized in live animals. Finally, the phenotypes of several zebrafish models more closely approximate the severity of the human clinical presentation than corresponding mouse models [8–10]. For example, dystrophin-deficient zebrafish exhibit a severe motor phenotype by 4 days of life and die by 2 weeks of life, while dystrophin-deficient *mdx* mice have a very mild

Abbreviations

TALEN, transcription activator-like effector nuclease; ZFN, zinc finger nuclease.

phenotype that only moderately impacts muscle function and survival [11,12].

How are zebrafish models of muscle disease made?

There are three primary techniques for generating zebrafish models of muscle disease, with the basic overall strategy centered on manipulating a gene in a manner analogous to what is observed in human patients. Morpholino-based gene knockdown is a rapid but transient strategy, forward genetic screens identify germline mutations primarily based on muscle-related phenotypes, and genome editing techniques create targeted mutations in specific genes of interest. Despite some limitations, particularly related to recapitulating dominant mutations and specific point mutations, these techniques have been used with great success to model numerous muscle diseases (Table 1).

Morpholino-mediated knockdown has emerged as an exceptionally useful technology, particularly for examining gene loss of function in zebrafish embryos (although some dominant muscle diseases have been modeled as well with morpholinos). This is largely because of the rapidity by which ‘morphant’ zebrafish can be created and analyzed. Morpholinos are synthetic oligonucleotides similar to RNA with an uncharged morpholino ring in place of a ribose. When targeted against the start codon or splice sites of a gene of interest, morpholinos can transiently reduce or eliminate

protein expression by inhibiting translation or pre-mRNA processing. Morpholinos provide a means to knock down gene expression quickly and with relatively low cost. Most notably, as discussed below, morpholino-mediated gene manipulation has emerged as a vital approach for validating new gene mutations discovered by next generation sequencing [4,5,13].

Because of their widespread use, it is important to point out some of the caveats associated with morpholinos in zebrafish. Many genes are duplicated in the teleost genome, making it critical to look carefully for multiple putative orthologs to a human gene of interest when designing a knockdown experiment. Morpholino knockdown is transient, and the efficacy of morpholinos typically fades by 3 days post fertilization. Gene knockdown may only be partial in many cases, making it important to confirm loss of expression using western blot or antibody staining. Additionally, morpholinos may have off-target effects. To ensure that a phenotype is gene-specific, careful validation of morpholino knockdown is necessary. Controls for morpholino experiments include injecting a mismatched control, using multiple morpholinos targeted to the same gene, and rescuing a morpholino phenotype with co-injection of capped mRNA.

Forward genetic screens have proved to be an excellent source of zebrafish mutants that model human disease. The first muscle mutants emerged from the original ENU mutagenesis-based screen by Nüsslein-Vollhard and colleagues, and include the first identified

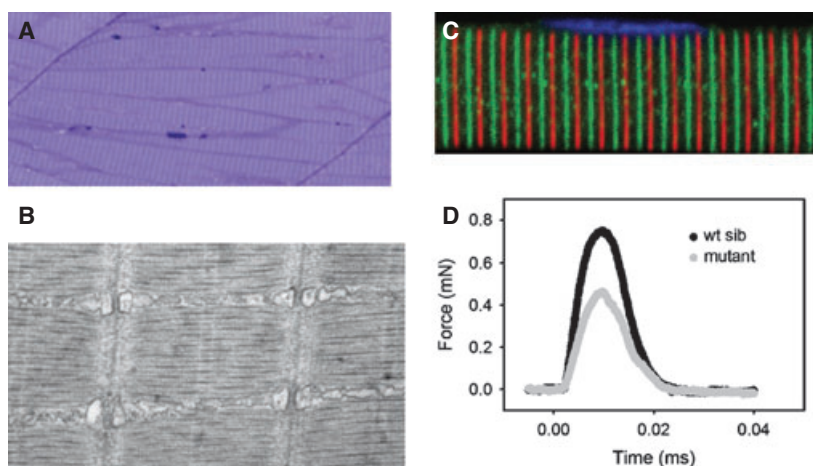


Fig. 1. Properties of zebrafish skeletal muscle. (A) Toluidine blue stained semi-thin longitudinal section from a 3-day-old zebrafish (20 \times). Note the obvious striations corresponding to the Z lines as well as the rare, eccentric nuclei. The thick vertical lines connecting the myofibers are the myotendinous junctions. (B) Electron microscopy of 7-day-old zebrafish skeletal muscle. All the salient features of mammalian muscle (Z bands, sarcomeres, thin and thick filaments, triad) are observed in fish muscle at this age (10 500 \times). (C) Immunostaining of an isolated myofiber from a 3-day-old zebrafish (120 \times). Green is tropomodulin and red is actinin. This co-stain highlights the ability to detect and visualize common markers of skeletal muscle structure. (D) Maximal force generation curve for wild-type (black) and *candyfloss* (gray) embryos. These curves recapitulate force curves measured in mouse muscle and human myofibers.

Table 1. Zebrafish models of human muscle diseases.

Human gene	Zebrafish gene	Zebrafish model	Reference	Human condition
DMD	<i>dmd</i>	Morpholino	[4]	Duchenne muscular dystrophy
		Mutant (<i>sapje</i>)	[11,59]	
		Mutant (<i>sapje-like</i>)	[34]	
LAMA2	<i>lama2</i>	Mutant (<i>caf</i>)	[13]	Congenital muscular dystrophy (MDC1A)
		Mutant (cl501)	[17]	
ITGA7	<i>itga7</i>	Morpholino	[60]	Congenital muscular dystrophy
LMNA	<i>lmna</i>	Morpholino	[61,62]	Multiple forms of dystrophy
COL6A1	<i>col6a1</i>	Morpholino	[9]	Ullrich congenital muscular dystrophy; Bethlem myopathy
COL6A3	<i>col6a3</i>	Morpholino	[9]	Ullrich congenital muscular dystrophy; Bethlem myopathy
DUX4		Overexpression	[63, 64]	Facioscapulohumeral muscular dystrophy
DNAJB6	<i>dnajb6b</i>	Morpholino	[65]	Limb-girdle muscular dystrophy
DAG1	<i>dag1</i>	Morpholino	[3]	Limb-girdle muscular dystrophy (MDDGC9)
		Mutant	[16]	
SGCD	<i>sgcd</i>	Morpholino	[61,66,67]	Limb-girdle muscular dystrophy
TCAP	<i>tcap</i>	Morpholino	[61,68]	Limb-girdle muscular dystrophy
DYSF	<i>dysf</i>	Morpholino	[32,56]	Myoshi myopathy; limb-girdle muscular dystrophy
POMT1	<i>pomt1</i>	Morpholino	[43]	Multiple forms of dystroglycanopathy
POMT2	<i>pomt2</i>	Morpholino	[43]	Multiple forms of dystroglycanopathy
FKRP	<i>fkpr</i>	Morpholino	[21,40,41,69]	Multiple forms of dystroglycanopathy
FKTN	<i>fktn</i>	Morpholino	[21,69]	Multiple forms of dystroglycanopathy
ISPD	<i>ispd</i>	Morpholino	[46]	Multiple forms of dystroglycanopathy
B3GNT1	<i>b3gnt1</i>	Morpholino	[45]	Walker–Warburg syndrome
GTDC2	<i>gfdc2</i>	Morpholino	[44]	Walker–Warburg syndrome
MTM1	<i>mtm1</i>	Morpholino	[7]	Myotubular myopathy
DNM2		Overexpression	[70]	Centronuclear myopathy
CCDC78	<i>ccdc78</i>	Morpholino	[36]	Congenital myopathy with internal nuclei and cores
NEB	<i>neb</i>	Morpholino	[20]	Nemaline myopathy
RYR1	<i>ryr1b</i>	Mutant (<i>ryr</i>)	[5]	Multiple forms of congenital myopathy
SEPN1	<i>sepn1</i>	Morpholino	[71,72]	Multiple forms of congenital myopathy
DES	<i>desma</i>	Morpholino	[61,73]	Myofibrillar myopathy
	<i>desmb</i>	Morpholino	[73]	
CAV3	<i>cav3</i>	Morpholino	[74]	Caveolinopathies

zebrafish mutant strains for dystrophin (*sapje*), titin (*runzel*) and laminin $\alpha 2$ (*candyfloss*) [14,15]. More recently, ENU and transposon-based screens have been performed by Kunkel and Beggs (using birefringence as a screen) and by Kuwada and colleagues (using impaired motility as the output) and have resulted in the identification of several strains with mutations in muscular dystrophy and congenital myopathy genes [16–19]. Finally, the Wellcome Trust Sanger Institute has created an emerging source of genetic mutants based on a saturating ENU mutagenesis screen (http://www.sanger.ac.uk/Projects/D_rerio/zmp/). Their mutation resource contains a large bank of strains with mutations that are identified based on a gene sequencing strategy called TILLING. By using TILLING, they have identified mutations in several genes associated with human muscle disease, although only a few resulting mutant strains have been characterized (including *neb*^{hu2849} and *dag1*^{hu3072}) and the majority of the strains have yet to be analyzed [20,21].

Until recently, there has not been a method for the targeted generation of stable zebrafish knockouts. However, new advances in genome editing technologies have made it possible to generate gene-specific lesions in the zebrafish genome. Two of these technologies, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), utilize the non-specific cleavage domain of the FokI endonuclease to introduce a double-stranded break in genomic DNA. In the case of ZFNs, these cleavage domains are targeted to a precise genomic location by a pair of DNA binding zinc finger domain assemblies [22]. TALENs are a similar form of genomic editing that utilize DNA binding domains from transcription activator-like (TAL) effectors, and Ekker and colleagues have developed a publicly available TALEN toolkit for targeting the zebrafish genome [23,24]. Both ZFNs and TALENs have been successfully used to generate somatic and germline mutations in zebrafish embryos; however, efficiently engineering a target-specific

domain remains a bottleneck with both technologies. A third technology utilizing bacterial CRISPR (clustered regularly interspaced short palindromic repeats) offers a promising alternative approach for inducing targeted lesions in genomes [25,26]. CRISPR-based targeting is RNA guided, bypassing some of the technical challenges involved in assembling appropriately targeted ZFNs or TALENs. In zebrafish embryos, CRISPR-based targeting has been shown to induce targeted gene lesions in somatic cells with high efficiency [27,28]. These new genome editing technologies open up exciting avenues for zebrafish research, and while muscle disease mutants have yet to be published, they promise ultimately to be a transformative technology in the generation of zebrafish models.

How are zebrafish models of muscle disease analyzed?

As the number of zebrafish models for muscle disease expands, a useful repertoire of techniques has been developed in parallel for the analysis of muscle structure and function in zebrafish. First, developing zebrafish embryos exhibit highly stereotyped motor behaviors that can be easily measured without the need for special equipment [29]. Zebrafish embryos begin coiling in a steady rhythmic pattern independent of stimulation within the first day of life, providing a simple measure of early slow-muscle-dependent muscle function (Video S1) [7,9,20]. By 2 days post fertilization, zebrafish embryos have developed a robust escape response, rapidly swimming away from mechanical stimuli (Video S2). Beginning at approximately 3 days post fertilization, larvae begin swimming spontaneously and swim behaviors such as velocity and turn frequency can be precisely quantified, especially through the use of proprietary imaging systems like the Noldus DanioVision or the Viewpoint Zebra-box. Together, these sequential motor behaviors provide simple and non-invasive measures of muscle function and can thus serve as a platform for assessing pharmacological and genetic interventions.

A number of zebrafish muscle studies have utilized electrophysiological and biomechanical measurements adapted from other model organisms. Several studies have used *in vivo* patch clamp recording of larval skeletal muscle to establish whether a motor defect is caused by defects in the central nervous system, the neuromuscular junction or the muscle fiber itself. To characterize the *relatively relaxed* and *accordion* behavioral mutants, Kuwada and colleagues analyzed the fictive swimming response from both mutants and demonstrated normal rhythmic input to the muscle in response to tactile stim-

ulation, showing that the primary functional defect must be downstream of myofiber excitation from the nervous system and neuromuscular junction [5,30]. Similarly, injecting current into zebrafish muscle can evoke contractions, and this technique has been used to measure muscle fatigue and relaxation in models of muscle disease [7,30,31]. To directly measure muscle force generation, Brooks and colleagues developed a novel system adapted from studies of murine muscle (Fig. 1D). Using this system, they showed that a zebrafish model of nemaline myopathy exhibits defects in contractile properties nearly identical to those observed in nebulin knockout mice and patient myofibers [20].

In addition to these functional measures of muscle output, the striking similarities between fish and mammalian muscle has allowed important insights to be gained from the analysis of zebrafish muscle using both standard light microscopy analysis and transmission electron microscopy. Skeletal muscle can be readily visualized in whole zebrafish using immunohistochemistry or by using fluorescent markers driven by muscle-specific promoters. Furthermore, the transparency of zebrafish embryos permits elegant optical assays of muscle structure and function that would not be possible in mammalian systems. Roostalu and Strahle recently used high-resolution imaging to visualize real-time membrane resealing in myofibers from whole embryos, elucidating a sequential repair program that occurs following sarcolemmal damage [32]. *In vivo* calcium imaging is another optical assay that has provided insight into the nature of muscle defects. Calcium flux in zebrafish myofibers is monitored *in vivo* by injecting embryos with chemical (such as dextran green) or genetically encoded calcium indicators (such as Gcamp3), which can reveal defects in calcium release or uptake [5,30].

One notable observation in developing zebrafish muscle is that myofiber integrity can be easily viewed using an optical phenomenon called birefringence. When viewed under polarized light, detached myofibers appear as dark patches in the skeletal muscle of zebrafish embryos (Fig. 2). This phenomenon can be directly quantified to measure loss of muscle integrity in dystrophic larval muscle, and it has been particularly useful in identifying novel muscle mutants in forward screens and for testing therapies in known dystrophy models [14,33,34]. Figure 2 depicts birefringence patterns for several well-characterized mutants. This simple visual assay can be combined with other measures of muscle integrity to provide substantial insight into the dynamics of myofiber damage, retraction and death in dystrophic mutants. Evans blue dye, a small molecule that only enters damaged myofibers,

can be injected into zebrafish larvae to assay for sarcolemmal damage. Currie and colleagues have visualized myofiber detachment in real time in *candyfloss* and *sapje* dystrophic mutants by anesthetizing embryos for the first 3 days of life, which preserved muscle integrity by eliminating any muscle contraction during development [8,13]. When anesthetic was washed out, fish embryos embedded in agarose or methylcellulose contract against surrounding media, inducing myofiber detachment.

For experiments that cannot easily be performed in an intact embryo, myofibers from the developing zebrafish can be isolated and maintained *ex vivo* [34a]. Such preparations contain a mixture of myofibers and other cell types, with myofibers representing the predominant cellular subtype. A range of experimentation is feasible on isolated myofibers – including immunocytochemistry, calcium imaging and electrophysiological recording – and these preparations can allow more precise measurements of myofiber size and protein subcellular localization.

Models of muscle disorders

As summarized in Table 1, zebrafish have provided useful models for many different muscle disorders. Recent reviews, in particular by Peter Currie, have extensively summarized several of these models [10,35].

While we present a brief listing of the major available models in Table 1, this review will instead focus on a few notable studies that reflect the power of the zebrafish to provide insight into muscle disease.

The zebrafish and new gene discovery: the perfect partner for next generation sequencing

It is estimated that the genetic basis of disease is not known for 25–50% of all genetic muscle disorders. This knowledge gap presents a significant barrier to clinical practice and to research into disease pathogenesis and therapy development. Next generation sequencing, including especially whole exome capture followed by massively parallel sequencing, is rapidly filling in the missing genetic pieces. A particular challenge with next generation sequencing, however, is that it identifies numerous variants of unclear significance that are not present in the existing variant databases. Given that sequencing efforts are often focused on isolated probands with a similar disease or small families with very rare myopathies, identifying the true pathogenic variants is often difficult in the absence of supporting experimentation. The zebrafish has turned out to be the ideal system for validating novel variants as it provides a rapid way of genetically modeling and testing a newly identified putative mutation.

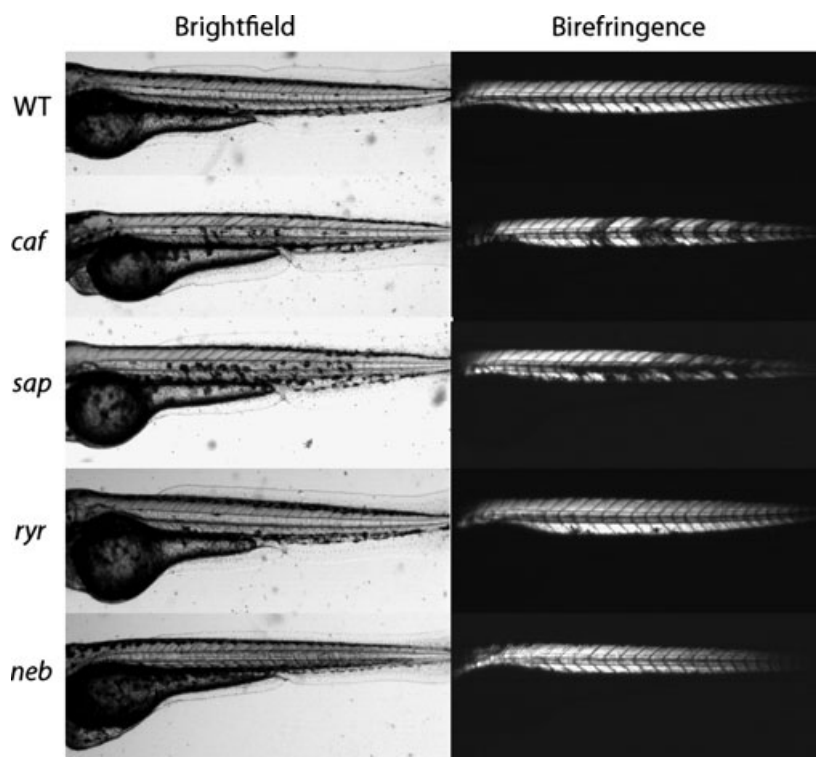


Fig. 2. Birefringence in representative zebrafish models of human muscle disease. Left, Bright-field images of the trunk and tail regions of the following zebrafish models: wild-type (WT), *candyfloss* (*lama2* mutant, *caf*), *sapje* (dystrophin mutant, *sap*), *relatively relaxed* (*RYR1* mutant, *ryr*) and *neb* (*nebulin* mutant). Right, Birefringence images as obtained by viewing zebrafish with a plane polarizing filter. Note the patchy birefringence of *caf* and *sap*, indicative of muscle detachment in these models, and the reduced birefringence in *neb*, indicative of the generalized and global sarcomeric disorganization.

There are now multiple examples, both in the muscle literature and for other monogenetic disorders, where zebrafish modeling has proved critical for the validation of a new gene mutation. We present a recent example from our laboratory for a novel congenital myopathy, and an example from Hans van Bokhoven and colleagues describing a new gene associated with a severe form of congenital muscular dystrophy.

CCDC78 mutation as the cause of a unique congenital myopathy

We identified five individuals in a three-generation dominant pedigree with moderate distal weakness, excessive fatigue, and a muscle biopsy with the unusual combination of excessive central nuclei, core-like lesions, and desmin and actin accumulations [36]. We used the combination of single nucleotide polymorphism linkage on the pedigree and whole exome sequencing of two affected individuals to identify a splice site sequence variant in the previously uncharacterized gene *CCDC78*. Based on RT-PCR data from patient lymphoblastoid cells, we predicted that this variant resulted in disease by allowing inclusion of an in-frame intron of *CCDC78*. To validate the pathogenicity of this variant, we developed a morpholino to zebrafish *ccdc78* targeting the exon–intron boundary affected by the human sequence variant. Injection of this morpholino into the developing zebrafish results in aberrant processing of *ccdc78* RNA, with inclusion of the in-frame intron and production of a higher molecular protein. The consequences associated with this morpholino included impaired motor function and abnormal larval morphology. Most notably, the morpholino-induced histopathological changes in the muscle, including myofibrillar disruption, triad abnormalities, and desmin and RYR1 accumulations, mirror exactly the findings observed in the patient biopsies. The similarities in both RNA processing and histopathology in the zebrafish morphants enabled us to conclude that the sequence variant in *CCDC78* identified in the patients was the cause of disease.

Mutations in *ISPD* cause Walker–Warburg syndrome

Mutant and morphant zebrafish have provided exceptionally useful models for a clinically diverse group of muscle disorders known as dystroglycanopathies, recessive disorders characterized by reduced *O*-mannose glycosylation of α -dystroglycan (*DAG1*). The phenotypes of dystroglycanopathies range from relatively mild limb-girdle muscular dystrophy to Walker–

Warburg syndrome, a severe form of congenital muscular dystrophy associated with eye and brain defects. *DAG1* plays an essential role in mammalian development, limiting the usefulness of mouse models in studying dystroglycanopathies, and complete knockout of dystroglycan or certain dystroglycan processing genes leads to embryonic lethality in mice [37–39]. While the *dag1* glycosylation pathway appears to be largely conserved in zebrafish, it is not essential for early development, and knockdown of most known dystroglycanopathy genes have been shown to recapitulate many aspects of human disorders [21,40–45].

Recently, van Bokhoven and colleagues identified mutations in the *ISPD* gene in individuals from nine families with Walker–Warburg-like features [46]. To elucidate the role of *ISPD* in this disorder, they injected zebrafish with morpholinos to *ispd*. Consistent with histopathology in patient muscle and other models of dystroglycanopathy, *ispd* morphants exhibit hypoglycosylation of *dag1*. The *ispd* morphants also have a dystrophic muscle phenotype, and sarcolemma integrity is disrupted. In addition to muscle abnormalities, zebrafish recapitulate a severe hydrocephalus phenotype seen in patients. These findings demonstrate the value of zebrafish in validating and characterizing rare variants associated with muscle disease. Of note, morpholino knockdown has also been used to functionally validate mutations in *GTDC2* and *B3GNT1*, two additional genes in which mutations were identified in patients with Walker–Warburg and related dystroglycanopathies. Both *gtdc2* and *b3gnt1* morphants exhibited severe muscle, eye and brain defects, as well as reduced glycosylation of α -dystroglycan [44,45].

The zebrafish as a tool for identifying novel pathogenic mechanisms

Much of what is known concerning disease pathogenesis in most skeletal muscle disorders has been derived from the combination of cell culture studies and experimentation in murine models. The zebrafish has recently entered the arena of pathogenesis studies and has helped reveal novel aspects of disease that were not previously detected in mice or cultured cells. Here we describe findings in two such studies and highlight the contributions they have made to the understanding of pathomechanisms in human muscle disease.

Vitamin-based rescue in a zebrafish model of muscular dystrophy

Using a *dag1* morphant zebrafish model, Henry and colleagues demonstrated a remarkable rescue of

dystrophic muscle by supplementation with coenzyme NAD⁺ [47]. In untreated *dag1* morphants, muscle degeneration began at 3 days post fertilization. Morphants supplemented with NAD⁺ exhibited reduced fiber detachment, improved organization of the surrounding basement membrane and increased swim speed in touch-evoked escape responses. Intriguingly, the group achieved a similar rescue by the simple addition of a vitamin supplement containing niacin, a precursor to NAD⁺.

To identify the mechanism underlying the protective effect of NAD⁺ supplementation, they used morpholinos against components of the dystrophin–glycoprotein complex or the $\alpha7\beta1$ integrin complex, two of the major adhesion complexes in muscle. Although they demonstrated that full NAD⁺-mediated rescue is dependent on expression of either *dag1* or integrin $\alpha7$, they find that an additional receptor (integrin $\alpha6$) is also required for rescue. These findings suggest that integrin $\alpha6$ may be part of a previously unrecognized integrin adhesion complex in skeletal muscle. Therefore, along with identifying a novel therapeutic strategy, this study highlights the utility of the zebrafish model in teasing out the molecular underpinnings of muscle disorders.

Oxidative stress in a zebrafish model of RYR1-related myopathies

Ryanodine receptors are calcium release channels that play a critical role in muscle excitation and contraction. Mutations in ryanodine receptor 1 (*RYR1*) cause a diverse range of dominant and recessive muscle disorders, including especially central core disease and minicore myopathy [48–53]. The spontaneous *relatively relaxed* (*ryr*) zebrafish mutant, identified by a slow swimming phenotype, carries an insertional mutation in *ryr1b*, the fast-twitch fiber zebrafish ortholog to *RYR1* [5]. Using a differential RNA microarray based approach to identify expression, our laboratory found that *ryr* zebrafish exhibit upregulation of numerous genes associated with cellular stress, and follow-up biochemical studies uncovered increased intracellular oxidant activity [54]. Based on this result, *ryr* zebrafish were treated with the anti-oxidant *N*-acetylcysteine (NAC), and treated larvae exhibited significant increases in swimming behavior and substantial improvements in muscle histopathology. These results were corroborated by Ana Ferreiro and colleagues [54] by the observation of increased oxidative stress in myotubes derived from patients with *RYR1*-related myopathies, suggesting that this is a clinically relevant pathomechanism, and NAC is now considered an

excellent therapeutic candidate for patients with *RYR1* mutations.

Zebrafish as an ideal system for novel drug discovery

Large-scale drug screens are not feasible in mammals due to cost and time required but are well suited to invertebrate models or to cultured cells. However, such assays in invertebrates or cells are often far removed from the human disease and often lack functional outcome measures that provide relevance for the identification of drugs that will ultimately translate to the clinical setting. In principle, the zebrafish offers the ideal combination of invertebrate advantages (small size, rapid development, large numbers) and vertebrate biological relevance. In practice, only one large-scale drug screen (described below) has been published to date using a zebrafish model of muscular dystrophy. However, the success of zebrafish screens in other disease spheres – in particular melanoma, where a novel drug identified in a large-scale screen has successfully translated to the clinic – has engendered optimism that screens in models of muscle disease will yield similar results [55].

Kunkel and colleagues performed a large-scale drug screen in dystrophin-null zebrafish (*sapje* and *sapje-like*), using birefringence (which is a marker of muscle integrity and is markedly abnormal in *sapje*) as the outcome measure [56]. This straightforward readout permitted the rapid screening of 1120 chemicals for compounds that prevented the loss of muscle organization that typically occurs in *sapje* during the first few days of development. In the initial screen, they tested pools of eight chemicals on embryos from heterozygous *sapje* parents, with the expectation that approximately 25% of embryos would exhibit abnormal birefringence when treated with a pool of non-effective compounds. Chemical pools that had reduced numbers of embryos with abnormal birefringence ($\leq 7.5\%$) were then separated and individually screened to determine which compounds were associated with the decrease. Using this two-step screening strategy, they identified seven compounds that preserved muscle integrity in *sapje* mutants. They further demonstrated that long-term treatment with one compound, a phosphodiesterase (PDE) inhibitor, was able to both extend survival and restore muscle integrity in older affected *sapje* zebrafish. Given that PDE inhibitors have been shown to modulate disease in mouse models of Duchenne muscular dystrophy and in Duchenne muscular dystrophy patient myotubes, this result provides important proof-of-principle of the suitability of zebrafish as a

tool for drug development [57,58]. In addition, the study highlights the tremendous potential of the zebrafish in large-scale drug screens, and provides a path forward for drug discovery in zebrafish models of muscle disease.

Conclusions and prospective

In this review, we have outlined the utility of the zebrafish as a model for human muscle diseases and have highlighted several recent publications that illuminate the power of the model for gene discovery and therapy development. At present, the most important area of impact for the fish model has been as a tool for validating new mutations discovered by next generation sequencing, and we predict that such experimentation will become increasingly important as whole exome and genome sequencing are applied more extensively to isolated probands and individual families. An ongoing challenge related to the application of zebrafish to mutation validation is the proper use of the system. We believe the strongest and most support outcomes to measure in the fish are those that reflect on specific features of the human disease, such as dystrophic changes (like patchy birefringence) or specific histopathological features (like rods or actinin accumulations in models of nemaline myopathy), and not on non-specific outcomes like motor function and overall fish morphology. In addition, while speed is often desired for new mutation validation, the need for well considered and controlled zebrafish experiments is paramount to reduce the chance that non-pathogenic variants are falsely considered to be disease causing.

Moving forward, perhaps the most exciting area for fish research relates to therapy development, as the ability to perform large-scale chemical and/or genetic screens in the zebrafish has amazing potential. To date, research has only scratched the surface of this potential, and it is exciting to speculate that such experimentation, because it is based on whole organism screening, will yield unexpected disease insights and novel therapeutic targets. The true translatability of the zebrafish, however, is still an unknown, and will not truly be understood until targets first identified in the fish are brought to the clinical trial arena.

In all, we anticipate widespread and increased use of the zebrafish to study muscle disease and to develop therapies, as more and more researchers discover the ease of use of the system, the facility for genetic manipulation and the power of the muscle-related outcomes it provides.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Video S1. Spontaneous coiling in a 1-day-old embryo.

Video S2. Touch-evoked escape response in a 3-day-old embryo.