Impaired embryonic motility in \textit{dusp27} mutants reveals a developmental defect in myofibril structure

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Running title: Myofibril defects in \textit{dusp27} mutants
Keywords: zebrafish, dusp27, motility, myofibrillogenesis, muscle

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Summary

An essential step in muscle fiber maturation is the assembly of highly ordered myofibrils that are required for contraction. Much remains unknown about the molecular mechanisms governing the formation of the contractile apparatus. We identified an early embryonic motility mutant in zebrafish caused by integration of a transgene into the pseudophosphatase dual specificity phosphatase 27 (dusp27). Dusp27 mutants exhibit near complete paralysis at embryonic and larval stages, producing extremely low levels of spontaneous coiling movements and a greatly diminished touch response. Loss of dusp27 does not prevent somitogenesis but results in severe disorganization of the contractile apparatus in muscle fibers. Sarcomeric structures in mutants are almost entirely absent and only rare triads are observed. These findings are the first to implicate a functional role of dusp27 as a gene required for myofiber maturation and provide an animal model for analyzing the mechanisms governing myofibril assembly.

Introduction

Animal models of neuromuscular disorders are an invaluable resource for identifying new genes that are required for normal development of the neuromuscular system (Pappalardo et al., 2013) and elucidating the pathogenic mechanisms of human disease genes (reviewed in (Berger and Currie, 2012)). In zebrafish, precise genetic processes governing muscle and nerve development result in highly stereotyped early behaviors that are easily observed. The earliest motor behavior occurs within 18 hours of fertilization (hpf), with internally generated spontaneous coiling movements (Grunwald et al., 1988). By 21 hours, embryos show a vigorous tail contraction in response to touch (Saint-Amant and Drapeau, 1998) and a few hours later also respond to light (Kokel et al., 2010). Rapid neural and muscular maturation enable this fast development of behavior. The first muscle fiber contractions occur just minutes after primary motor neurons emerge in a given somite, and large trunk flexions occur while somitogenesis is still ongoing (Myers et al., 1986). The first mutagenesis screens in zebrafish identified
genetic mutants effecting nerve and muscle development by the disruptions caused to embryonic motility (Felsenfeld, 1990; Westerfield et al., 1990). Subsequently, systematic genetic screens and characterization of spontaneous mutations in wildtype fish stocks lead to the isolation of genes required for many aspects of early motility, including neuronal specification, axon pathfinding, neuromuscular junction formation, muscle differentiation and myofiber contraction (Granato et al., 1996; Hirata et al., 2007).

The high efficiency of transposon mediated transgenesis has facilitated high throughput transgenesis efforts, including enhancer and gene trapping screens (Kawakami et al., 2004). The random insertion of a transgene into or nearby a gene may disrupt expression sufficiently to cause a mutant phenotype (Sivasubbu et al., 2006; Nagayoshi et al., 2008). A key advantage of gene disruption by transgenes is that mutations can be easily mapped, avoiding the time-consuming linkage analysis required to identify mutations induced by chemical exposure or radiation. We recently performed an enhancer trap screen aimed at generating neuronal specific transgenic lines useful for manipulating discrete cohorts of neurons (Bergeron et al., 2012). Of the integrations that we have mapped so far, 17% (7/41) are in an exon or the first intron of a gene making them potentially mutagenic. One instance is the enhancer trap line Et(REx2-SCP1:Gal4ff)y241 (y241), in which we noted a strong embryonic motility phenotype. Here we show that this line harbors a transgene insertion that disrupts expression of the dual specificity phosphatase 27 (dusp27) gene. Mutants show severe disruption of muscle architecture, including almost complete disorganization of the contractile apparatus. The y241 mutant is a new myopathy model that may help to elucidate the molecular mechanisms governing the formation of the contractile machinery within myofibers.

**Results**

During maintenance of the y241 line, we observed embryos from multiple heterozygous incrosses that showed poor touch responsiveness. Consistent with the presence of a recessively segregating mutant allele, 24.4% of 2 dpf embryos (154/630) responded to a
tail touch with a weak 'shiver' instead of vigorous forward swimming (Fig. 1A). This phenotype was never observed in an outcross of y241 transgenics to a wildtype strain of the same genetic background confirming a recessive mode of inheritance (0/168 embryos). In addition, pectoral fin movement was almost completely absent.

Morphologically, mutant embryos show edema surrounding the heart, curvature of the tail and a reduction in eye size (Fig. 1B). Heart beat frequency was reduced in 2 dpf embryos (siblings: 167 ± 3 beats per minute (bpm), mutants: 147 ± 5 bpm, n = 10 larvae; t_{13.6} = 3.2, p = 0.006). The tail curvature defect in mutants normalizes by 4 dpf. Edema also resolves in around half of the mutants such that larvae are morphologically normal, apart from a failure to inflate swim bladders. These larvae continue to show a reduced touch response and also fail to generate a normal acoustic startle response. The acoustic startle response is a rapid swimming movement in zebrafish that begins with a stereotyped flexion of the body to one side (C-bend). To quantify the movement defect, we analyzed kinematic parameters of C-bends of 5 dpf larvae to acoustic startle stimuli (Burgess and Granato, 2007). Larvae responded with a reduced body curvature (Fig. 1C, D) but normal C-bend duration (Fig. 1E). This suggests that the swimming defect is not due to uncoordinated timing of left-right muscle contractions, but reflects reduced muscle contraction. As short-latency acoustic startle responses are all-or-nothing events independent of the intensity of the acoustic stimulus (Burgess and Granato, 2007), the reduction in motor response can not be ascribed to attenuated sensory reception.

To confirm that the defect in y241 mutants was primarily a motor defect and not a sensory defect, we examined 'spontaneous coiling', a transient behavior starting at around 18 hpf in which embryos show vigorous tail movements that are generated by circuitry intrinsic to the spinal cord (Saint-Amant and Drapeau, 1998; Downes and Granato, 2006). We used automated video recording and image analysis to quantify spontaneous coiling (supplementary figure 1) and found that this behavior in mutants was strongly reduced in magnitude from the earliest timepoints when compared to wildtype sibling embryos (Fig. 1F). We also observed that despite a strong reduction in the magnitude of the tail coiling in mutants (supplementary videos 1-2) the frequency of coiling events was
normal (24 hpf, siblings: 0.087 ± 0.006 Hz, mutants: 0.100 ± 0.016 Hz, n = 29 and 10 embryos respectively, t\textsubscript{11.5} = 0.75, p = 0.46). This again suggests that neuronal control of the timing of contractions was not impaired. As the timing of tail movements is normal but the magnitude of contractions is markedly reduced in both spontaneous coiling and acoustic startle responses, reduced motility in y241 embryos is likely due to a primary defect in muscle or neuromuscular junctions leading to reduced contractile strength. Moreover, because there is a reduction in the earliest muscle contractions at the outset of spontaneous coiling, the defect is likely to represent a structural or functional abnormality rather than a progressive breakdown of muscle integrity.

Linker-mediated PCR mapping determined that the transgene integration site in y241 is on chromosome 1 at nucleotide 619473 (zebrafish genome Zv9; Fig. 2A). Genotyping showed that all mutant larvae (n = 128) were homozygous for the transgenic allele, while unaffected siblings (n = 41) were either wildtype or heterozygous for the transgenic allele making it unlikely that an independently segregating mutation is responsible for the mutant phenotype. Measurement of swim distances in response to touch revealed no difference between wildtype embryos and embryos heterozygous for the transgene (t\textsubscript{29} = 0.50, p=0.62).

Transcripts of the zebrafish homolog of dual-specificity phosphatase 27 (dusp27) map near this chromosomal location, but it was unclear whether the integration site fell within the gene or was in the upstream promoter region. We used 5' RACE to determine the transcriptional start site of dusp27 and found that the transgene insertion site is immediately after the last base of exon 1 thereby disrupting a near consensus 5' splice donor site (Fig. 2A,B). Exon 1 contains a single ATG that is not in frame with the large open reading frame that begins near the start of exon 2. Dusp27 is a member of the dual-specificity phosphatase family which share sequence similarity in the catalytic phosphatase domain. Zebrafish Dusp27 is most strongly similar (80% identity) to human DUSP27 in this domain, with weaker similarity in the N-terminal region and relatively poor identity (40%) in the extended C-terminal region which in both species is markedly
enriched in serine and glutamate residues (Fig. 2C). An unusual feature of Dusp27 is the loss of the highly conserved cysteine residue at the base of the catalytic loop in dual specificity phosphatase (DUSP) proteins suggesting that Dusp27 is not an active phosphatase (Fig. 2D). As the integration in y241 likely disrupts splicing of dusp27, we measured transcript levels using quantitative RT-PCR (qPCR) for a region of dusp27 encoded by exons 4-5 downstream of the integration site. dusp27 expression is reduced to 3.6% of wildtype levels in mutant larvae (Fig. 2E), a finding confirmed by in situ hybridization for dusp27 expression in mutants (supplementary fig. 2A), indicating that the transgene does indeed disrupt mRNA expression.

The transgene in y241 includes two neuronal restrictive silencing elements (NRSE) designed to suppress expression of the reporter outside the nervous system (Bergeron et al., 2012). NRSE elements frequently occur at a distance from the genes they regulate, and act by binding the Rest protein which recruits a protein complex to regulate chromatin structure (Huang et al., 1999; Ding et al., 2009). We therefore speculated that the transgene might also silence genes adjacent to dusp27 and used qPCR to measure the expression level of the two adjacent genes (rpl24 and pou2f1a) and fourth gene cul4a with a large intron encompassing dusp27, rpl24 and pou2f1a. Expression of each of these genes was reduced to about 40% of the level in siblings, a reduction that was significant for rpl24 and cul4a (Fig. 2E). To test whether the reduction in the expression of genes closest to the transgene integration site contributes to the mutant phenotype, we injected a morpholino against rest into y241 homozygous embryos (Gates et al., 2010) that we previously demonstrated can reverse transgene silencing in non-neuronal tissues (Bergeron et al., 2012). Mutants injected with rest morpholino remained almost completely unresponsive to a touch stimulus, indistinguishable from mutants injected with control morpholino (2 dpf embryos: n = 7 y241 homozygotes injected with control morpholino, n = 10 homozygotes injected with rest morpholino). This result affirms that the mutant phenotype is not caused by Rest mediated silencing of genes proximal to the insertion site, but rather by disruption of dusp27.
To verify that loss of *dusp27* produces the mutant phenotype, we injected wildtype embryos with a translation blocking morpholino against *dusp27* that was able to strongly suppress protein translation from an mRNA encoding a fusion of the morpholino target sequence to GFP (Fig. 3A). At 2 dpf morphants were morphologically normal but showed pericardial edema and tail curvature (n = 17/22 embryos; Fig. 3B) similar to phenotypes seen in the *y241* mutants. These phenotypes were not observed in embryos injected with control morpholino (n = 32). Like *y241* mutants, *dusp27* morphants responded to a touch stimulus with only a weak shiver while embryos injected with control morpholino executed a vigorous swim bout in response to touch. To quantify the movement defect in morphants, we measured spontaneous coiling. Morphants showed a dose dependent reduction in movement throughout the entire period of spontaneous coiling, excluding the possibility that reduced movement reflected developmental delay (Fig. 3C, supplementary movies 3-4). The close similarity of morphological and behavioral phenotypes caused by transgenic insertion into *dusp27* and injection of morpholino against *dusp27* demonstrates that loss of *dusp27* expression produces a profound locomotor impairment. This conclusion is supported by the rescue of the mutant histological phenotype by restoring expression of *dusp27* (see below).

At 24 hpf, *dusp27* mRNA is expressed in the somites (Fig. 4A-C) and several regions of the brain including the midbrain roof (tectum). Kaede expression in *y241* embryos recapitulates this pattern (Fig. 4D-F). Kaede is first detected in somites at 15 somites, is strongly expressed by 24 hpf and continues to be expressed in fast muscle fibers through 6 dpf. The earliest cells within the somites to express Kaede are found in medial positions near the notochord, and at the lateral edge of the somites (Fig. 4F). *dusp27* mRNA was expressed at 15 somites (supplementary fig 2B) but too weakly to assess whether spatial organization was similar to the transgene. Within the brain, transgene expression is first seen in the optic tectum at 24 hpf, is robust by 48 hpf and continues through 6 dpf (supplementary fig. 2C). Expression of a GFP-dusp27 fusion protein in muscle fibers revealed a regular striated pattern, indicating that dusp27 may localize to sarcomeres (Fig. 4G-H). To determine whether abnormal neuronal activity contributes to the
phenotype, we raised embryos from 6 hpf to 24 hpf in the sodium channel blocker
tricaine (0.01%) to suppress electrical activity (Frazier and Narahashi, 1975). After
washing out the tricaine, mutant embryos showed a very low level of spontaneous
movement indistinguishable from mutants raised without tricaine (coiling activity in
mutants raised in embryo medium only: 367 ± 60 a.u. ; with tricaine 384 ± 33 a.u. ; t35.1 =
-0.23, p = 0.82) indicating that the motor impairment is not a developmental consequence
of aberrant neuronal activity.

The near normal timing but greatly reduced amplitude of muscle contractions suggests
that y241 mutants have a primary defect in the neuromuscular junction or muscle. As
alpha-bungarotoxin staining of neuromuscular junctions in mutant larvae failed to reveal
an impairment (Fig. 5A) and dusp27 is strongly expressed in muscle, we examined
muscle structure. The characteristic chevron-shaped morphology of the somite
boundaries was not disrupted and somite width was close to normal in mutants (at 4 dpf
wildtypes: 82.4 ± 0.9 µm ; mutants 78.8 ± 0.4 µm) indicating that somitogenesis is not
disrupted in mutants. Supporting this, whole mount in situ hybridization with a myoD
probe on 10 somite stage offspring (n = 40) from y241 heterozygote parents did not
reveal embryos with altered expression indicative of abnormal somite patterning
(supplementary fig. 3A). In addition, localization of dystrophin demonstrated that somite
boundaries are intact (supplementary fig. 3B). Muscle integrity in zebrafish is readily
assessed by examining birefringence, an optical effect caused by the diffraction of
polarized light through myofilaments (Granato et al., 1996). Birefringence was abnormal
in y241 mutants, suggesting a disorganization of muscle fibers (Fig. 5B). Indeed muscle
striation is lost in mutants (Fig. 5C) and muscle fibers appear disorganized in toluidine
blue stained semi-thin sections through the tail (Fig. 5D). In zebrafish transgenic
reporters controlled by UAS element containing promoters are susceptible to stochastic
silencing, leading to mosaic reporter expression (Akitake et al., 2011). To determine if
the observed muscle defects were structural or an indirect result of the loss of attached
muscle fibers, we took advantage of mosaic expression of the UAS:Kaede transgene to
visualize individual fast muscle fibers. Fast muscle fibers were multinucleated (Fig. 5E
and supplementary fig. 4) and extended between somite boundaries in mutants, suggesting that muscle cells fuse and extend normally (Fig. 5E). Slow-twitch muscles were correctly located at the superficial aspect of the somite as revealed by F59 staining (Devoto, S. H. et al., 1996) (Fig. 6A) and immunofluorescence against engrailed protein revealed normal localization of muscle pioneers at the horizontal myoseptum (supplementary fig. 3C)(Devoto, S.H. et al., 1996). These findings suggested that muscle cells are present and correctly localized but internally disorganized in y241 mutants which lead us to examine the subcellular architecture of myofibers.

Slow-twitch muscle myosin heavy chain showed a regular striated pattern in sibling larvae which was not present in y241 mutants indicating abnormal thick filament assembly in slow-twitch fiber sarcomeres (Fig. 6A). In contrast α-actinin staining showed regular striation in mutant slow twitch fibers demonstrating that z-lines were present (Fig. 6B). The selective maintenance of Z-lines despite disorganization of other sarcomere components due to genetic mutation or pharmacological treatment has been previously observed (Funatsu et al., 1990 ; Li et al., 2009). In fast muscle fibers, staining against myosin light chain using the F310 antibody (Zeller et al., 2002) and tropomyosin with CH1 (Lin et al., 1985) demonstrated severe disruption of thick and thin filaments respectively (Fig. 7A-B). Unlike in slow muscle fibers, z-lines were also disrupted in fast twitch fibers (Fig. 7C, also apparent in isolated fast twitch myofibers, supplementary fig. 4). Mosaic expression of the GFP-dusp27 transgene in y241 homozygotes selectively rescued the striated pattern of α-actinin staining further confirming that loss of dusp27 is the basis of the structural phenotype (supplementary fig. 3D). The giant sarcomeric protein titin and proteins involved in excitation coupling - ryanodine receptors and dihydropyridine receptors - also failed to show striated expression within fast muscle fibers (Fig. 7D-E, supplementary fig. 3E). Expression of these muscle markers confirms that differentiated muscle cells are present in mutant embryos. However these data suggest a near complete disorganization of the sarcomere and triad in fast muscle fibers. To verify this, we performed electron microscopy. Muscle tissue from y241 mutants revealed massive disruption of the internal organization of myofibers (Fig. 7F). Although
occasional triadic structures were visible, the ultrastructure of the t-tubules, sarcoplasmic reticulum, and sarcomere were all highly disorganized in mutant embryos. These findings show that myofibrils are strongly disrupted in larvae lacking dusp27 function. As somitogenesis appears normal, but motility is lost from the earliest stages, our findings suggest that dusp27 mutants have a primary defect in myofibrillogenesis.

Discussion

Here we show the first functional characterization associated with the loss of dusp27. Genetic or morpholino mediated reduction of dusp27 leads to a strong reduction in embryonic motility in zebrafish embryos due to massive disruption of myofibrils. Grossly normal somite morphology and patterning in mutants suggests that the defect is not in the specification of muscle precursors. As the very first motor responses of the embryo already exhibit deficiencies and almost every aspect of myofiber internal organization is affected, it is likely that the mutation disrupts myofibril assembly rather than resulting in a progressive breakdown in muscle architecture (as might be observed in a dystrophy). Microarray analysis of genes activated during IGF-I induced differentiation of cultured mouse myoblasts supports this theory - DUSP27 (referred to as Hypothetical protein C130085G02) was found to be up-regulated 4.6 fold within 24h of IGF-I treatment (Kuninger et al., 2004).

Inconsistent nomenclature has been used to refer to dusp27. The closest human homolog to the zebrafish gene disrupted in y241 (61% identity over 310 amino acids) is currently designated by the HUGO Gene Nomenclature committee as dual specificity phosphatase 27 (Dusp27, HUGO #25034, genbank NM_001080426). Human Dusp27 is on chr 1q24, and like its zebrafish ortholog is adjacent to a gene encoding POU class 2 homeobox 1. Zebrafish dusp27 has a weaker match (43% identity over 169 amino acids) to human dual specificity phosphatase and pro isomerase domain containing 1 (DUPD1, HUGO #23481, genbank NM_001003892 on chr 10q22). DUPD1 has been referred to by several groups as Dusp27 (Friedberg et al., 2007; Devi et al., 2011; Lountos et al., 2011)
including in a recent review of DUSPs (Patterson et al., 2009). The only reports we are aware of which deal with Dusp27 are GWAS and microarray studies (Kuninger et al., 2004; Iio et al., 2010; Nielsen et al., 2010).

Dusp27 contains sequence with strong similarity to the catalytic domain of dual specificity phosphatases (DSPc domain). Dual-specificity phosphatases play a key role in regulating intracellular signaling pathways in a variety of physiological contexts by hydrolyzing phosphorylated tyrosine, serine and threonine residues (Patterson et al., 2009). Dusp27 belongs to the 'atypical' subclass of DUSPs, a miscellaneous group, members of which are generally small (less than 30 kDa) and lack an N-terminal CH2 domain but are not close phylogenetic relatives (Patterson et al., 2009). dusp27 is unusual among the atypical DUSPs in that it is large (130.2 kDa) and lacks the critical cysteine residue at the base of the catalytic cleft required for phosphatase activity. Despite its large size, outside of the DSPc domain, there are no recognizable protein domains in dusp27 making it difficult to predict its mode of action.

The best studied role for DUSPs is in negative feedback regulation of mitogen-activated kinase (MAPK) signaling cascades (reviewed in (Lawan et al., 2012)). MAPK phosphatases (MKPs) simultaneously dephosphorylate threonine and tyrosine residues within the MAPK activation loop to attenuate signaling in a wide variety of physiological contexts including immune function, embryonic development and metabolic homeostasis. Recent studies have demonstrated roles for MKPs during development in zebrafish: dusp4 is required for endoderm specification in zebrafish (Brown et al., 2008) and dusp6 is required for embryonic axis formation and specification of cardiac progenitor cells regulating heart size (Tsang et al., 2004; Molina et al., 2009). MAPK has a well established role in vertebrate somitogenesis. FGF signaling via MAP kinase regulates cell motility gradients in pre-somatic mesoderm that establish the rate of axis elongation and somite size (Sawada et al., 2001; Delfini et al., 2005; Benazeraf et al., 2010). During somitogenesis, MAP kinase activity is high in posterior pre-somatic mesoderm and downregulated during maturation of anterior somites (Sawada et al., 2001). We
speculated that defects in y241 mutants may be due to a failure to attenuate MAPK signaling. However raising embryos in U0126 which strongly inhibits ERK1/2 in zebrafish (Hawkins et al., 2008) did not restore motor function in mutants and no elevation in doubly phosphorylated ERK1/2 immunostaining was observed in mutants (data not shown), suggesting that the defect is not due to a hyper-activity in the MAPK pathway.

It is likely that Dusp27 does not function as a MKP. Two residues in the catalytic core of all active dual specificity phosphatases are not conserved in human and zebrafish Dusp27. In Dusp27 the nucleophilic cysteine is replaced by a serine residue. The same mutation introduced into Dusp6 produces a dominant negative protein (Tsang et al., 2004) and abolishes enzymatic activity in several other DUSPs (Zhou et al., 1994; Aoki et al., 2001). While biochemical characterization is essential to confirm the loss of enzymatic activity, it is likely that Dusp27 is a pseudophosphatase. Although catalytically inactive, pseudoenzymes frequently have essential roles, regulating cell function through protein interactions (Leslie, 2013). Like Dusp27, the DUSP-like pseudophosphatase STYXL1 has a serine in place of the catalytic cysteine but is nevertheless required for sperm production in mice (Wishart and Dixon, 2002). Catalytically inactive DUSPs may act by binding to and regulating the activity of related active DUSPs, competing with active DUSPs for phospho-protein targets or by regulating the subcellular localization of binding partners. The latter possibility is suggested by the finding that type II DUSPs, characterized by nuclear export sequences, may sequester MAPK in the cytoplasm and prevent phosphorylation of nuclear targets (Brunet et al., 1999).

Zebrafish models of neuromuscular disorders have helped to clarify the pathogenic mechanisms of human disease genes (for example (Telfer et al., 2012; Horstick et al., 2013; Mitsuhashi et al., 2013)). The genetic basis of numerous subtypes of congenital myopathies are in genes that provide structural integrity of the muscle contractile apparatus, or calcium homeostasis within myofibers (Nance et al., 2012). The availability of animal models with comparable genetic mutations has helped to identify molecular
pathways associated with disease pathogenesis and development of therapeutic strategies. This work is the first to reveal a role for the pseudophosphatase dusp27 in the maturation of the contractile apparatus in myofibers. The molecular pathways through which it acts are at present unknown but the near complete lack of movement from the earliest stages of embryonic motility together with the extensive disorganization of muscle fiber architecture argue for an early and essential role in myofibrillogenesis.

**Materials and Methods**

**Zebrafish husbandry.** All zebrafish in this study were maintained on a Tubingen long fin strain background. Transgenic Et(REx2-SCP1:Gal4ff)y241 fish were generated during an enhancer trap screen in which the reduced toxicity Gal4 variant Gal4FF (Asakawa et al., 2008) is downstream of a basal super core 1 promoter (Juven-Gershon et al., 2006) fused to tandem NRSE elements (Bergeron et al., 2012). The NRSE elements bind the transcriptional silencer Rest, which is expressed outside the nervous system, resulting in neural specific expression in 30% of lines obtained with the REx2-SCP1 promoter (Bergeron et al., 2012). y241 is a case in which robust non-neuronal expression is observed. Gal4 expression was visualized with Kaede reporter line Tg(UAS:Kaede)s1999t (Davison et al., 2007). y241 was maintained as a heterozygous stock as homozygotes are non-viable, dying by 11 days post fertilization (dpf) presumably due to inability to feed. All in vivo experimental protocols were approved by the local animal care and use committee.

**In situ hybridization & immunohistochemistry.** Larvae were fixed in 4% paraformaldehyde. Standard colorimetric whole-mount *in situ hybridization* (WISH) and immunohistochemistry was performed as described in (Bergeron et al., 2008). For WISH, probes were myoD (Weinberg et al., 1996) and a dusp27 fragment amplified by RT-PCR from 24 hpf embryonic RNA (primers 5-TCTCTCAGAGGAGGACAG and 5-CAGGTAAGCAGCTACCGAGCA) and cloned into pGEM-T Easy (Promega, Madison, WI). No staining was observed with the sense probe. Antibodies used for
immunofluorescence were anti-dystrophin (1:200; MANDRA1 (7A10) Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA), anti-RYR (1:200; 34C DSHB), anti-DHPR α-subunit (1:200; 1A, Affinity Bioreagents, Golden, CO), anti-α-actinin (1:200; A7811 Sigma, St Louis, MO), anti-engrailed (1:500; 4D9, DSHB), F59 (1:500; F59, DSHB), F310 (1:500, DSHB), CH1 (1:500; DSBH), anti-titin (1:500, T11, Sigma), anti-kaede (1:200; PM012, MBL International, Woburn, MA), anti-mouse IgG Alexa Fluor 488 and 555 (1:800 and 1:1000 respectively, Invitrogen, Carlsbad, CA).

Histology. For transmission electron microscopy samples were fixed in 2.5 % glutaraldehyde in 0.1 M Sorensen's buffer pH 7.4, overnight at 4 °C. After several buffer rinses, they were postfixed in one percent osmium tetroxide in the same buffer. Samples were rinsed in double distilled water to remove phosphate salt and then en bloc stained with aqueous 3 % uranyl acetate for one hour. The preparation was dehydrated in ascending concentrations of ethanol, rinsed two times in propylene oxide, and embedded in epoxy resin. The samples were ultra-thin sectioned 70 nm in thickness and stained with uranyl acetate and lead citrate. The sections were examined using a Philips CM100 electron microscope at 60 kV. Images were recorded digitally using a Hamamatsu ORCA-HR digital camera system operated using AMT software (Advanced Microscopy Techniques Corp., Danvers, MA). Semi-thin section preparations were made by cutting 0.5 µm sections through epon embedded embryos and subsequently staining with toluidine blue. Sections were imaged on an Olympus BX43 microscope. Birefringence was measured on 5 dpf embryos anaesthetized with tricaine. Images were taken using two polarizing filters on a Nikon AZ100 microscope, exposure was set at 100 µs for all images. α-bungarotoxin conjugated Alexa555 staining for acetylcholine receptors at neuromuscular junctions was performed as previously described (Ono et al., 2001).

Behavioral analysis. Larval startle responses in 5 dpf embryos were elicited using a minishaker (4810, Brüel and Kjaer, Norcross, GA) and tracked as previously described (Burgess and Granato, 2007) with the modification that kinematics for mutant larvae were manually extracted from curvature traces because responses were of too small...
magnitude to be automatically detected by Flote. Spontaneous coiling was measured in embryos arrayed in a 7 by 7 grid and illuminated with infra-red light (Advanced Illumination, Rochester, VT) while imaged at 1 Hz (µEye IDS-1545LE-M, 1stVision, Andover, MA). Analysis was performed by adding a new tracking module in the DAQtimer software (Yokogawa et al., 2012) implementing a similar algorithm to that described in (Myers et al., 1997). Briefly, pixels with an intensity change above a predetermined threshold between frames were counted and averaged each minute to provide a measure of overall movement, similar to counting ‘beam breaks’ to assess locomotor activity (supplementary figure 1). Note that this method provides an aggregate measure of activity, combining frequency of tail coiling with the magnitude of tail movements, and the temporal profile of activity therefore differs slightly from previous analyses (Myers et al., 1997; Saint-Amant and Drapeau, 1998). Supplementary videos 1 and 2 were taken using a Leica stereomicroscope and an Olympus DP72 camera.

Transgene mapping. y241 was mapped using linker-mediated PCR with DpnII linkers (Dupuy et al., 2005; Davison et al., 2007). Genotyping primers are x210g 5-GTCCTCAGAGCTGGAGATCG, x210r 5-CCCTGATCAGTGTGTGGTGT and x210t 5-TCAAGTAAAGTAAAAATCCCCAAAA. The wildtype band is 241 bp and transgenic band 121 bp. Sequencing of the bands confirmed that they are derived from the wildtype locus and the junction of the transgene and wildtype genomic DNA respectively.

RT-qPCR. Total RNA was extracted from 3 dpf larvae (5-10 pooled larvae per treatment group), using Trizol reagent (Invitrogen) following the manufacturer’s protocol. cDNA was synthesized from RNA samples using iScript Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA) using the oligo(dT) protocol. Dusp27 and neighboring genes cul4a (Ensembl ID ENSDART00000126069), rpl24 (ENSDART00000019227), and pouf1a (ENSDART00000064142) were targeted for RT-qPCR using the following primers: dusp27, 5-CATCGCTGAGCTGGAGATCG and 5-GGATGTTCATGCAGGTAG (spans exons 4-5); pouf1a, 5-GTTCGACCCACCACCATC and 5-
GAGCCTGAATAGTGGCCAGA (spans exons 3-5); cul4a, 5-
CTGCTGAGATGGGAAGCTG and 5-CTGTCTTCAGCACTGCGTGT (spans exons 5-6); rpl24, 5-TGAGGAGGTGTCGAAGAAGC and 5-GCACTTCAGGCTTTCTGTTTC (spans exons 3-4). The reference gene was efla, 5-CTGGAGGCCAGCTCAAACAT and 5-ATCAAGAAGAGTAGTACCAGCTAGCAT (Tang et al., 2007). RT-qPCR was performed using a CFX96 C1000 Touch Thermal Cycler (Bio-Rad) and SsoAdvanced SYBR Green Supermix (Bio-Rad). For all genes, technical triplicates were used for each of the three biological replicates (different clutches of embryos). From standard curves, all target primer efficiencies were between 90% and 110%; standard curve r² values were greater than 0.98 indicating low replicate variability. Expression data was then analyzed with CFX manager 3.0 software (Bio-Rad) using the ΔΔCq method to determine relative normalized expression of target genes (Livak and Schmittgen, 2001).

Morpholino injection. The translation blocking morpholino against dusp27 targets the region surrounding the start codon (GATCCTCCACAGACGACGCCATCA; Gene Tools, Philomath, OR). To confirm efficacy, we co-injected 2 ng of dusp27 or control morpholino with 50 pg in vitro transcribed mRNA containing the morpholino target sequence fused to the 5' of GFP.

Rescue. To make the UAS:GFP-dusp27 rescue construct, we synthesized zebrafish codon optimized emerald GFP (Genscript) and subcloned it into pT1UMP removing the stop codon (Yokogawa et al., 2012). Zebrafish dusp27 (ZGC dusp27 cDNA clone 7420637, Thermo Scientific) was cloned in frame after the c-terminus of GFP. Embryos were injected with 75 pg of the rescue construct.

Statistical analysis. SPSS (IBM, Armonk, NY) and Gnumeric (http://projects.gnome.org/gnumeric/) were used for statistical analysis. Statistical analysis was performed using Student's t-test with a significance threshold of 0.05. Variation is presented as mean ± s.e.m. throughout the text.
Acknowledgements

We thank Ben Feldman and the NICHD zebrafish core for assistance and for a critical reading of the manuscript.

Competing interests statement

No competing interests declared.

Author contributions

K.F., S.B., E.H., J.D., F.O. and H.B. conceived and designed the experiments. K.F., G.L., H.C., S.B., E.H., H.B. and F.O. performed the experiments. S.B., J.D., F.O. and H.B. analyzed the data. H.B. wrote the draft and all authors contributed to the final manuscript.

Funding

This work was supported by the Intramural Research Programs of the NICHD (HAB) and of the NIAAA (FO), and National Institutes of Health grants 1K08AR054835 (JJD) and T32 NS007222 (EH).

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**Figure legends**

Figure 1. Embryonic motility is impaired in y241 mutant embryos

(A) Swimming response to a tactile stimulus is severely impaired in 2 dpf y241 mutants. Image frames are at 10 ms intervals from a touch stimulus delivered with a fine needle. Siblings (top panels) swim rapidly forward while mutants (lower panels) respond with a barely perceptible shiver. (B) y241 mutants (bottom panel) show edema adjacent to the heart (asterisk), tail curvature (arrow) and smaller eyes. Scale bar 300 µm. (C) Curvature trace for 120 ms during an acoustic startle response in a sibling (black) and y241 mutant (red) embryo. The stimulus was delivered at 30 ms (arrow). (D) The magnitude of the initial C-bend in y241 mutant acoustic startle responses is reduced compared to wildtype embryos (n = 6 larvae each) while (E) the duration of the initial C-bend is not significantly different. * p < 0.001. (F) Spontaneous coiling is strongly reduced in y241 mutants (grey, n=7) compared to wildtype siblings (black, n=37) during the entire period of early locomotor development. y-axis is arbitrary units. Inset, average coiling during 24-28 hpf for the same larvae. * p < 0.001. Graphs show mean ± SEM

Figure 2. dusp27 is disrupted in y241 mutants

(A) Chomatogram showing sequence of genomic DNA from y241 wildtype siblings (top trace) and y241 mutants (bottom trace). Black upper case nucleotides are exon 1 of dusp27, lower case bases are intron 1 and orange nucleotides are derived from the transgene. (B) Structure of the dusp27 gene, shaded areas represent predicted coding sequence with the translation start site in exon 2 marked. The transgene integration immediately following exon 1 in y241 is represented, showing the orientation of the tol2 arms, Gal4FF and sv40 polyA signal. (C) The predicted proteins encoded by Dusp27 in zebrafish (NCBI XM_003197607) and human (NP_001073895) are 121.6 kDa and 130.2 kDa respectively with highest identity in the DSPc phosphatase domain (boxed). The C-terminal (red) has relatively poor homology, but both are highly enriched in serine and glutamate residues (total 24.6% and 29.6% of all amino acids in zebrafish and human respectively). (D) The catalytic cysteine (red) in the core of the phosphatase domain is
replaced by a serine in dusp27 in both human and zebrafish. For comparison, the four
paralogs with the highest identity to human Dusp27 are shown, each of which contains
the catalytic cysteine residue. (E) Expression of genes (cul4a: cullin4a; pou2f1a: POU
class2 homeobox 1a; rpl24: 60S ribosomal protein L24) in the immediate vicinity of the
y241 integration site as determined by RT-qPCR. Expression is calculated normalized to
efla, relative to the sibling group for each gene. Comparison between sibling and mutant
larvae from three independent clutches. # p < 0.05 ; * p < 0.001. Graphs show mean ±
s.e.m.

Figure 3. Morpholino knockdown of dusp27 phenocopies y241 mutants.
(A) Validation of morpholino efficacy for dusp27 knockdown using a Dusp27 N-terminal
sequence fused to GFP. (B) Morphology of embryos injected with control morpholino
(top panel) or 1 ng of morpholino against dusp27 (bottom panel). Embryos show
pronounced edema (asterisk). (C) Spontaneous coiling in embryos injected with control
morpholino (black, n=11), 1 ng dusp27 morpholino (blue, n=9) or 2 ng dusp27
morpholino (grey, n=9). Inset, average coiling during 24-28 hpf for the same larvae. * p
< 0.01. Graphs show mean ± s.e.m.

Figure 4. dusp27 is expressed during embryonic muscle development
(A-C) in situ hybridization for dusp27 expression at 24 hpf. Prominent expression is seen
in the somites (asterisk). (B) Lateral view above the yolk extension. (C) Cross section
through the trunk caudal to the yolk extension. (D-F) Kaede reporter expression in y241;
UAS:Kaede embryos. (D) Kaede expression is strongly expressed in trunk muscle at 24
hpf and (E) continues to label myofibers in 6 dpf larvae. Scale bar 50 µm. (F) Early
expression in somites is observed at 15 somites in a small number of cells adjacent to the
notochord (arrowheads), as well as at the lateral edge of the somite (asterisk). Scale bar
50 µm. (G-H) Expression of a GFP-dusp27 in muscle cells shows a striated pattern
(green, G) which alternates with α-actinin (red and merge, H). Scale bar 10 µm.

Figure 5. Muscle integrity is impaired in y241 mutants.
(A) α-bungarotoxin conjugated Alexa555 staining of 2 dpf wildtype sibling (left) and y241 mutant (right) embryos showing equatorially located neuromuscular junctions (brackets). Scale bar 50 µm. (B) Birefringence in live 5 dpf wildtype sibling (top) and y241 mutant embryos (bottom) detected using polarized light. Mutants show reduced and patchy birefringence. (C) Dodt gradient contrast illumination of muscle in 3 dpf larvae after removal of skin. Internal muscle striations are visible in myofibers stretching between somite boundaries in siblings (top) but not in y241 mutants (bottom). (D) 10 µm toluidine blue stained sections through the tail of 3 dpf wildtype sibling (top) and y241 mutants (bottom). (E) Kaede expression in highly variegated 24 hpf y241 ; UAS:Kaede sibling embryos (top panels) and mutants (bottom panels) with DAPI staining to reveal nuclear positions. Somite boundaries observed in brightfield images are indicated by dashed lines. Scale bar 30 µm.

Figure 6. Slow muscle myofibrils are partially disrupted in y241 mutants
(A) F59 staining against slow-twitch muscle myosin heavy chain in 48 hpf embryos shows the presence of slow muscle fibers at the lateral edge of the somite in both mutants siblings but disorganized thick filaments in y241 mutants. Scale bar 20 µm. Inset shows rotated transverse view through the somite. (B) α-actinin staining in slow twitch fibers shows preserved z-lines in y241 mutants. Scale bar 25 µm.

Figure 7. Fast muscle myofibrils are disrupted in y241 mutants.
(A-E) Immunofluorescence in fast twitch fibers in 48 hpf sibling and y241 mutant embryos. (A) F310 labeling of fast twitch fiber myosin shows disruption of thick filaments. Scale bar 25 µm. Inset shows rotated transverse view through the somite (merged view of DAPI in blue and F310 in red). (B) CH1 labeling of tropomyosin shows disruption of thin filaments. Scale bar 20 µm. (C) α-actinin staining shows loss of z-lines in fast twitch fibers Scale bar 25 µm. (D) anti-titin staining. Scale bar 30 µm. (E) DHPR in fast twitch fibers. Scale bar 25 µm. (F) Transmission electron microscopy in myofibers of 3 dpf y241 siblings and mutants, showing z-lines (arrows) and triads (arrowheads). Scale bar 500 nm.
Supplementary Material

Supplementary videos. Spontaneous coiling behavior in *dusp27* mutants and siblings (24 hpf).

1. Sibling embryos (23 hpf) show large magnitude tail flexions during spontaneous coiling events.
2. In *y241* mutants (23 hpf), spontaneous coiling events are observed, but with greatly diminished tail flexions.
3. Spontaneous coiling in embryos (28 hpf) injected with control morpholino.
4. Spontaneous coiling in embryos (28 hpf) injected with morpholino against *dusp27*.

Supplementary figure 1. Measurement of embryonic motor activity.

(A) Schematic of the apparatus used to record motor activity in embryos. Embryos are arrayed in a 49 well grid in a petri dish with temperature maintained at 28°C using a thermistor and heating element. A camera mounted above records images (B) at a 1 Hz framerate. (C) Motor activity is measured as the sum of the number of pixels which change in intensity greater or less than a predetermined threshold between frames. Top row shows a sequence of frames for the boxed well in (B), while the bottom row shows the same frames, with changed pixels highlighted and the total number of changed pixels indicated. To allow real-time processing, only every second pixel in the image is checked. (D) For validation, the coiling frequency was manually observed for 20 embryos (number of coiling events in a one minute interval) and the total number of pixel change events automatically measured for the same video sequence. Manual and automatically scored values are highly correlated Pearson r = 0.86, p < 0.001 confirming that the algorithm captures differences in motor activity. (E) For additional validation, embryos were exposed to varying concentrations of tricaine for 20 minutes (N = 14 each condition) before movement was measured over a 10 minute period. The y-axis shows the mean number of pixel changes per minute averaged over the recording period. 0.002% tricaine significantly reduced but did not eliminate movement while 0.02%
tricaine completely paralyzed embryos such that the number of pixel changes was not
significantly (n/s) different from baseline as measured in wells with no embryos. * p <
0.001.

Supplementary figure 2. Additional analysis of dusp27 expression.
(A) Muscle fibers labeled by anti-kaede staining (green) in y241 ; UAS:Kaede
heterozygotes (left) at 30 hpf co-express dusp27 (red, in situ hybridization), while dusp27
expression in mutants is strongly diminished. Note that the expression of dusp27 in fibers
not labeled by kaede is likely due to variegated expression of the UAS:Kaede transgene,
a commonly observed phenomenon in zebrafish UAS lines (Goll et al., 2009). Scale bar 20 µm. (B) in situ hybridization for dusp27 at 15 somites (right panel shows sense
control) (C) In 6 dpf brain, Kaede prominently labels deep tectal neurons that extend
neurites into the tectal neuropil (arrowheads) as well as neuronal groups in the retina,
cerebellum and hypothalamus (not shown). Scale bar 50 µm.

Supplementary figure 3. Additional analysis of muscle markers in y241 mutants
(A) In situ hybridization for myoD expression in 10 somite sibling (left) and y241 mutant
(right) embryos. After photographing, embryos were genotyped by PCR. (B)
Immunofluorescence staining for dystrophin (merged z-stack through muscle) shows
similar localization at somite boundaries in sibling and y241 mutants in 48 hpf embryos.
Scale bar 50 µm. (C) 4D9 staining of engrailed expression in muscle pioneer cells
indicates the presence of grossly normal densities of pioneer cells (arrows). Scale bar: 50
µm. (D) In y241 mutants injected with the GFP-dusp27 plasmid, muscle fibers expressing
GFP (left panel, arrowheads) recover a normal striated pattern of α-actinin expression
(middle panel) while regions of the somite without GFP expression do not show
patterned α-actinin expression (asterisk). Merged view on right. Scale bar 10 µm. (E) The
normal striated pattern of ryanodine receptor staining is disrupted in 48 hpf y241 mutant
embryos. Arrowheads point to somite boundaries. Scale bar 25 µm.
Supplementary figure 4. Immunofluorescence in isolated fast muscle fibers in y241 mutants
(A) α-actinin stain and (B) DAPI stain in isolated myofibers from 4 dpf sibling and y241 mutants. Merged image in (C). Scale bar 20 µm.

Resource Impact

Clinical issue: Congenital myopathies (CM) are muscle disorders in which infants present with generalized weakness, usually at birth. No treatments are available for any congenital myopathies and genetic diagnosis is difficult because many disease genes for CM remain unknown. A more complete understanding of the molecular mechanisms underlying different stages of myogenesis is therefore critical for the development of treatment and diagnostic options. Myofibrillogenesis is a key stage in myofiber maturation in which proteins are assembled into a contractile apparatus. Many sarcomeric proteins have been identified, but much less is known about the processes which enable them to assembly into regular arrays.

Results: The authors identified an embryonic motility mutant in zebrafish which displayed weak muscle contractions from the very earliest stages. They demonstrated that the motility defect was due to a transgenic insertion in dusp27 which disrupts gene expression. Dusp27 is a pseudophosphatase with no previously identified physiological function. Dusp27 is expressed in somites at early stages of development, however analysis of muscle tissue showed normal somitogenesis and patterning. In contrast, histological and electron microscopy revealed a near complete defect in assembly of the contractile apparatus in myofibers.

Implications and future directions: The finding that dusp27 is required for the formation of sarcomeres opens up a new line of investigation into the mechanisms governing myofibrillogenesis. The authors provide an animal model with well characterized genetic, histological and behavioral defects that will enable investigations into the molecular pathways that control sarcomere assembly.
Figure 1
Figure 2
**Figure 3**

(A) ctrl MO vs. dusp27 MO

(B) ctrl MO vs. dusp27 MO

(C) Graph showing movement (a.u.) over time (hpf) for Ctrl, 1, and 2 groups, with significant differences indicated by asterisks.
Figure 4