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Sequence and expression of the zebrafish alpha-actinin gene family reveals conservation and diversification among vertebrates

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Abstract

alpha-actinins are actin microfilament crosslinking proteins. Vertebrate actinins fall into two classes: the broadly-expressed *actinins* 1 and 4 (*actn1* and *actn4*) and muscle-specific actinins, *actn2* and *actn3*. Members of this family have numerous roles, including regulation of cell adhesion, cell differentiation, directed cell motility, intracellular signaling and stabilization of f-actin at the sarcomeric Z-line in muscle. Here we identify five zebrafish actinin genes including two paralogs of *ACTN3*. We describe the temporal and spatial expression patterns of these genes through embryonic development. All zebrafish actinin genes have unique expression profiles, indicating specialization of each gene. In particular the muscle actinins display preferential expression in different domains of axial, pharyngeal and cranial musculature. There is no identified avian *actn3* and approximately 16% of humans are null for *ACTN3*. Duplication of *actn3* in the zebrafish indicates that variation in *actn3* expression may promote physiological diversity in muscle function among vertebrates.

INTRODUCTION

Actinins are a family of actin-binding proteins that have diverse functions (Otey and Carpen, 2004). Despite the radical spectrum of actinin function, most members of the actinin family are highly conserved, suggesting very strict evolutionary constraints act upon these genes (Beggs et al., 1992). All model tetrapods have four actinin family members except avian genomes, which have three. All members possess an N-terminal actin-binding domain (ABD) followed by four α -helical spectrin-like repeats and a C-terminal calmodulin (CaM) domain with two EF-hand motifs. Actinins function as anti-parallel dimers, resulting in a flexible, rod-like structure able to bind f-actin at each of its two N-terminal heads (Chan et al., 1998). Actinins 1 and 4 are referred to as cytoplasmic or non-muscle actinins. They bind to actin filaments throughout the cytoplasm and have been shown to decrease their affinity to actin stress fibers and focal adhesion scaffolds upon calcium treatment (Burridge and Feramisco, 1981). In addition to binding actin via their ABD, actinins 1 and 4 directly bind a multitude of other proteins along their spectrin repeats, presumably regulating their localization or function (Otey and Carpen, 2004).

In mice, *Actn4* is required for maintaining podocyte function in the kidney glomerulus (Weins et al., 2007). Supporting a role regulating the actin cytoskeleton architecture, *Actn4* knockouts display disorganization of podocyte microfilaments in response to changes in hydrostatic pressure (Michaud et al., 2009). In humans, dominant negative alleles of *ACTN4* are associated with Focal and Segmental Glomerulosclerosis (FSGS; OMIM:603278) due to uncoupling of Ca^{+2} -binding from microfilament release by ACTN4, resulting in unresponsive and actin-bound populations of ACTN4 (Kaplan et al., 2000; Weins et al., 2007).

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Actinins 2 and 3 are highly expressed in muscle fibers, where they function to maintain microfilament spacing at the Z-disc (Geeves and Holmes, 1999). The muscle-specific actinins have different activities than the non-muscle actinins. While they bind actin microfilaments, they are unable to bind calcium with their CaM domain EF-hands and are largely restricted to the Z-discs of striated muscle and analogous actin-rich dense bodies (Beggs et al., 1992; Geeves and Holmes, 1999). Muscle-specific actinins are expressed during the earliest stages of myofibril formation in the zebrafish (Costa et al., 2002). The zebrafish actinins localize to the Z-disc early in myofibrillogenesis (Costa et al., 2003; Sanger et al., 2009). Expression of particular muscle-specific actinins correlates with differences in muscle physiology. Muscle actinins have been shown to bind to amorphin and members of the calsarcin family and localize them to the Z-disc (Chowrashi et al., 2002; Frey and Olson, 2002). These proteins may in turn associate with calcineurin to regulate differentiation of myofiber types (Serrano et al., 2001). ACTN2 and ACTN3 may interact with different complements of proteins to regulate muscle physiology. In adult human muscle, ACTN2 is present in all skeletal muscle, while ACTN3 is active in only a subset of type II (fast) muscle fibers (Mills et al., 2001; North and Beggs, 1996). In mouse skeletal muscle, there is an incomplete overlap in *Actn2* and *Actn3* expression, leaving some muscle fibers expressing only *Actn3* (Mills et al., 2001). There is also evidence that human ACTN2 and ACTN3 molecules can form heterodimers *in vitro* and *in vivo*, indicating potential additional physiological complexity resulting from overlapping expression (Chan et al., 1998). A null allele of ACTN3 containing a premature stop codon at Arginine 577 is estimated to be homozygous in 16% of the human population (North et al., 1999). Further analysis revealed a significant positive selection for ACTN3^{R577X} homozygotes among female endurance athletes, indicating that loss of ACTN3 promotes an efficient muscle phenotype (Yang et al., 2003). There is also a negative correlation for ACTN3 mutation in elite sprinter athletes who have predominately fast twitch muscle (Yang et al., 2003). This concordance with performance in humans suggests that the presence or absence of the full-length ACTN3 protein significantly impacts muscle physiology and performance. Supporting this hypothesis, mice lacking ACTN3 activity show a shift to aerobic metabolism, resulting in efficient muscle function and increased endurance (MacArthur et al., 2007).

Taken together, the above observations indicate diverse functional roles for this highly related gene family. We chose to examine the temporal and spatial regulation of zebrafish actinins as a first step in understanding the functional requirements of actinins in teleost development. We identified clear zebrafish homologs of all tetrapod actinins including two of the three splice isoforms of human ACTN1 as well as two orthologs of human ACTN3. We found that the non-muscle *actn1* is widely expressed. *actn4* displays higher expression in the notocord. We observed expression of *actn2*, *3a* and *3b* in overlapping sets of muscle, implying a contribution of these genes to physiological specialization of muscles. In particular *actn2* is highly expressed in cardiac muscle and a subset of slow twitch skeletal muscle while *actn3a* and *actn3b* are highly expressed in the axial muscles and have differential expression in cranial and pharyngeal muscles. The diverse expression patterns of the actinins indicate substantial specialization of individual genes within this highly related gene family. The duplication of *actn3* and divergent expression of both paralogs in the zebrafish contrasts with its positive selection for a non-functioning allele in human populations and absence in chicken. These observations indicate that diversification of *actn3* expression or activity may be an effective mechanism for regulating muscle physiology in response to selective pressure.

RESULTS AND DISCUSSION

Identification of zebrafish alpha-actinin homologs

Zebrafish alpha-actinin family members were initially identified by reciprocal BLAST searches using annotated human actinins to identify homologs in the zebrafish genome (see Experimental Procedures). We were able to identify five unique zebrafish actinin genes, located

on linkage groups 7, 13, 17 and 21 as well as another actinin that has not yet currently been placed on any of the 24 zebrafish chromosomes as of the release of genome build Zv8 (Sanger Center). The zebrafish actinins sorted into existing phylogenetic clades when aligned with other vertebrate family members (Fig. 1A). The *actn3* clade revealed the presence of two distinct zebrafish *actn3* members (Fig. 1A). In light of the apparent dispensability of ACTN3 in humans and mice (Beggs et al., 1992; MacArthur et al., 2007) the presence of two active and conserved *actn3* genes in zebrafish may represent differences in muscle physiology and adaptation among vertebrates. These two zebrafish Actn3 proteins show a striking sequence divergence with only 92.9% identity. This falls within the range of percent identity conserved between the zebrafish genes and their human homologs, with these pairs showing at least 82.5% identity (*actn3a::ACTN3*) and at most sharing 93.4% identity (*actn1 iso b::ACTN1 iso b*) at the amino acid level (Fig. 1B). The assignment of gene identity was confirmed by analysis of syntenic conservation between human and zebrafish actinin gene regions. Each homolog had clear local conservation of homologous genes in the corresponding genome including the duplicated *actn3* genes (Fig. S1). No additional actinin genes were identified by searches of EST or genomic databases, therefore we expect that these five actinins represent the full complement of zebrafish actinin genes.

Calcium binding is a feature of CaM domain EF-hands (Burridge and Feramisco, 1981; Haiech et al., 1991), and previous work has shown that microfilament binding of non-muscle actinins can be regulated by intracellular Ca^{+2} levels while muscle-specific actinin activity is not (Arimura et al., 1988; Burridge and Feramisco, 1981; Janssen et al., 1996; Witke et al., 1993; Beggs et al., 1992; Tang et al., 2001). We aligned the first 2 EF-hands of human and zebrafish actinins to determine their conservation. We observed that both EF-hands found in Actn3a and 3b were 100% identical and differ from the human ACTN3 at 4 of 63 amino acids (Fig. 1C). The Actn2 EF hands were more divergent with changes in 12 of 63 amino acids. Actn2, Actn3a and Actn3b all have substitutions at the critical Y position in EF hand 1 that is essential for Ca^{2+} binding, indicating that like mammalian muscle actinins they are not regulated by calcium (Beggs et al., 1992; Tang et al., 2001). We identified two splice isoforms of *actn1* both of which are homologous to isoforms identified in humans. These isoforms utilize alternate exons within the EF hand domains (Fig. 1C). The c isoforms of both human and zebrafish have substitutions at positions essential for Ca^{2+} binding and lack 5 amino acids between the EF hands, indicating these isoforms are likely similar to the muscle actinins in their calcium binding activity. Actn1 isoform b and the Actn4 protein contain all of the essential amino acids for calcium binding.

Temporal expression profile of zebrafish alpha-actinin genes

If the actinins have specific functions during development of the zebrafish embryo we would expect the expression to vary during development of the embryo. We performed RT-PCR reactions to determine the temporal profile of each actinin (Fig. 2). We tested a broad range of time points beginning with maternal ovary and developing oocytes through 8 days post fertilization (dpf) larval stage cDNA samples. To determine the level of expression, we performed RT-PCR reactions with 25, 30 or 35 rounds of amplification for each actinin to obtain at least one sample within a linear amplification range. All primer pairs spanned at least one intron to distinguish between cDNA and genomic DNA amplification. Transcripts for the cytoplasmic actinins *actn1* and *actn4* are present maternally and persist throughout embryogenesis, with *actn4* appearing to be more abundant in whole-embryo cDNA samples. The muscle actinins were expressed at low levels maternally. *actn3b* expression persisted at low levels through gastrulation. All muscle actinins were robustly expressed by 24 hours post fertilization (hpf) and continued to be expressed through the time course.

Tissue specific expression of zebrafish actinins

We performed *in situ* hybridization reactions in order to visualize which tissues expressed each actinin during development. We included an early time point (64-cell stage) prior to initialization of zygotic transcription to observe maternal message as well as stages associated with the beginning of gastrulation (shield stage), blastopore closure (bud stage), onset of segmentation and somitogenesis (5- and 10-somite stages) as well as later larval stages during myogenesis in the maturing somite mesoderm, pharyngeal arches and cranium (24, 36, 48 and 96 hpf).

***actn1* and *actn4*: the non-muscle actinins**—*actn1* and *actn4* mRNAs were ubiquitously expressed at maternal stages through segmentation (data not shown). However at 36 hpf, enrichment of *actn1* was observed in the notochord and skin (Fig. 3F) in transverse sections. *actn1* was also expressed throughout the 36 hpf somite but was markedly absent from the central nervous system (Fig. 3F').

At 36 hpf, *actn4* was not widely expressed. *actn4* was highly expressed in the notochord and the developing gut, but was excluded from neural tissues at these larval stages (Fig. 3G&G').

***actn2*, *actn3a*, *actn3b*: the muscle actinins during axial muscle development**—

In our RT-PCR analysis we detected maternal expression of all three muscle actinins. In whole mount *in situ* hybridization *actn2* was distributed ubiquitously at maternal stages and at shield stage (data not shown).

In contrast to mammalian limb muscle, the zebrafish axial muscle of the trunk and tail displays strict separation of slow and fast muscle into distinct spatial domains (Devoto et al., 1996). Lineage tracing experiments have shown that slow (Type I) muscle fibers arise primarily from adaxial cells adjacent to the notochord (Blagden et al., 1997; Devoto et al., 1996). Subsequently, these cells migrate laterally and by 24 hpf, these cells have expanded dorsoventrally to create a sheet of slow muscle fibers extending along the lateral edge of the myotome (Hollway and Currie, 2003; Devoto et al., 1996). Fast (Type II) muscle fibers arise from the somite and compose the majority of the *myoD*-positive zebrafish myotome by 24 hpf (reviewed in Ochi and Westerfield, 2007). This spatial separation allowed us to examine fiber type specific restriction of expression of the muscle-specific actinins if it was present.

The first specific expression of *actn2* was at 15 somites with expression in the early differentiating adaxial cells immediately adjacent to the notochord (Fig. S3). In transverse sections, expression of *actn2* was highest in these adaxial muscle progenitors. At 24 hpf, *actn2* was expressed in a mediolateral set of muscle fibers extending dorsoventrally along the lateral sides of the somite (Fig. 3C&C'). This expression pattern is similar to the location of slow muscle cells. In order to determine if the muscle-specific actinins had expression patterns synonymous with slow or fast fiber types by 24 hpf, we performed RNA *in situ* hybridization with genes known to be restricted to slow or fast fibers in the zebrafish myotome. *slow-specific troponin C* (*stnnc*) (Elworthy et al., 2008) is expressed in slow fibers (Fig. 3A&A'), while *myosin heavy polypeptide 2* (*myhz2*) (Peng et al., 2002) is exclusively expressed in fast muscle fiber during this time (Fig. 3B&B'). *actn2* was expressed in the same pattern as *stnnc* and in an opposite pattern to *myhz2*. *actn2* is also strongly expressed in the heart at 24 hpf (Fig. 3C). Strikingly, by 36 hpf and continuing to 48 hpf, *actn2* is absent from the slow muscle of the myotome and is only expressed in the heart while *stnnc* continues to be expressed in the slow muscle (Fig. 3H,H'&H'' and data not shown).

actn3a and *actn3b* expression was first detected by *in situ* hybridization at 10 somites (14 hpf) by whole mount *in situ* hybridization (data not shown). This expression was restricted to adaxial cells, but began to expand laterally by 15 somites (Fig. S3). By 24 hpf, both *actn3a* and

actn3b were strongly expressed throughout the somites (Fig 3D,I,E&J). This expression appears broader than the expression of the fast muscle marker *myhz2* (Peng et al., 2002) and may encompass the domain of the slow muscle marker *stnnc* and *actn2*. At 36 hpf and 48 hpf, strong gene expression of *actn3a* and *actn3b* is still observed and appeared to encompass the entirety of the somites (Fig 3I&J and data not shown). Because we do not observe expression of *actn2* in axial musculature at 48 hpf, it is likely that *actn3a* or *actn3b* or both are expressed in all slow and fast fibers at this stage.

By 96 hpf, *actn2* was once again expressed in axial muscle (Fig 4A&A'). Unlike earlier expression, where the *actn2* expression pattern was similar to the antibody staining profile of Slow-Type MyHC and the expression pattern of *stnnc* in all of the slow fiber domains of the 24 hour myotome (Blagden et al., 1997; Fig. 3A), at 96 hpf, expression of *actn2* is restricted to a narrow row of slow muscle cells that make up the presumptive lateralis superficialis muscle, with no expression detected in the sheath of Type I (slow) muscle cells that reside in the lateral periphery of the myotome at this stage (Elworthy et al., 2008; Devoto et al., 1996). *actn2* is also expressed in the inferior obliquus muscle along the ventrolateral edge of the myotome (Fig. 4A inset). There are similarities between *actn2* expression at this stage of development and expression of a MyHC gene *mMYH_{C1}* that is restricted to the lateralis superficialis from three to seven dpf in the Medaka axial muscles (Ono et al., 2006). This restriction of *actn2* to only a small set of muscle fibers in the 96 hpf myotome contrasted with the expression patterns of *actn3a* and *actn3b*, which were both expressed throughout the 96 hpf larval myotome (Figure 4A',B'&C'). Both *actn3a* (65%, n=17) and *actn3b* (65%, n=14) showed variable lateral, superficial patches of expression (Fig. 4B). These patches were located in the trunk and anterior tail from multiple clutches and may indicate transient high level expression of *actn3* genes.

Muscle actinins display different patterns of expression in the pharyngeal and cranial muscles

We examined the expression of the muscle actinins in the cranial and pharyngeal musculature of 96 hpf zebrafish (Table 1, Fig. 4). These embryonic muscles have been shown to be composed of slow and fast muscle and are thought to maintain similar morphology and function as their adult forms (Schilling and Kimmel, 1997; Hernandez et al., 2005). These muscles are likely to have diverse physiological specializations for their specific functions.

Differential expression of *actn2* in mandibular and hyoid arches—We observed low levels of *actn2* in most muscles (Table 1). However, one exception to this was the levator arcus palatine (lap) muscle, a predominant muscle associated with compression of the buccal cavity and opercula (Winterbottom, 1973). This muscle is responsible for constant perfusion of oxygenated water over respiratory gills, and expressed *actn2* strongly (Fig 4J). Interestingly, the largest differences in expression between *actn2* and both *actn3* paralogs were observed in the lateral muscles of Arch I and Arch II, the mandibular and hyoid arches, respectively. More specifically, all three cases of decreased *actn2* expression relative to the other muscle actinins were in muscles associated with either mandibular closing or opercula movement. The dilator opercula functions to withdraw, or open, the operculum while adductor mandibulae muscles are responsible for the forced, medial closure of the mandibular cartilages. In the mandibular arch, adductor mandibulae and dilator operculi muscles showed markedly less expression of *actn2* than either *actn3* gene (Table 1, Fig 4F-H&J-L).

Differential expression of *actn3* paralogs in branchial arches and somite-derived anterior muscles—With one exception, we observed large differences between *actn3a* and *actn3b* expression within the five branchial arches (pharyngeal arches three through seven). Specifically, *actn3b* was the dominant *actn3* paralog expressed in transversus ventralis

and dorsal pharyngeal wall muscle pairs. While *actn3a* staining was observed in these muscles, it was reduced (Fig 4K&L). The transversus ventralis muscles insert on ceratobranchial cartilages and control gill bar movements while the dorsal pharyngeal wall muscles insert on the lateral and dorsal edges of the branchial arches and expand the buccal chamber (Hernández, 2000; Winterbottom, 1973).

Conversely, *actn3a* was observed to be the preferred *actn3* paralog expressed in sternohyoideus muscles at 96 hpf (Fig 4G&H). The sternohyoideus muscles are derived from somitic mesoderm, which migrates anteriorly and inserts into hyoid cartilages (Huang et al., 1999; Schilling and Kimmel, 1997; Winterbottom, 1973). The sternohyoideus muscles have been shown to express predominantly fast muscle fibers throughout larval development and are the dominant muscles that rapidly open the hyoid jaw during feeding (Hernandez et al., 2005).

Expression of *actn2*, *3a* and *3b* in the sternohyoideus muscles—The cranial and pharyngeal muscles have segregated populations of slow and fast muscle fibers with slow muscle fibers surrounding the central fast muscle fibers (Hernandez et al., 2005). In order to determine if the zebrafish actinins are restricted to slow- or fast-specific domains of these muscles, the expression of the muscle-specific actinin genes was analyzed by sectioning the large, easily identified 96 hpf sternohyoideus muscles. We used *stnnc* as a marker for slow fibers and *myhz2* as a fast fiber marker. *actn2* was expressed in a small number of cells restricted to the outside of the bundle of fibers composing the sternohyoideus muscle (Fig. 4O). This pattern appears to be identical to the slow muscle fibers expressing *stnnc* (Fig. 4M). Thus, at least in the case of this muscle, *actn2* expression is restricted to the slow muscle cell population. In contrast to the peripheral punctate expression of *actn2*, both *actn3a* and *actn3b* are expressed in many of the fibers of the sternohyoideus (Fig 4P&Q). As described above, *actn3a* expression was much greater than *actn3b* in these muscles.

These differences in expression patterns within muscles of the head and pharynx indicate diversification of gene expression associated with specific muscles. The complement of actinins expressed in different muscle fibers may direct their contractile or physiological properties. Muscles associated with feeding require fast, strong contractions, while those associated with respiration require slower and more regular contractions. Also, differential regulation of the actinins may reflect a difference in embryological origin and subfunctionalization of the duplicated *actn3a* and *actn3b* in lineages that have different origins. However, one of the differences we have described between expression of the *actn3* paralogs is in the transversus ventralis, while other muscles derived from the same arch maintain similar expression of both paralogs. The changes could also be associated with neofunctionalization and expression in teleost specific muscles. However, we do not see differences in the teleost specific intermandibularis posterior or interhyodeus (Diogo et al., 2008).

CONCLUSION

This work is the first comprehensive examination of alpha-actinin expression in vertebrates. We have used a bioinformatics approach to identify members of the *alpha-actinin* gene family in the zebrafish genome. We identified clear orthologs of all identified mammalian actinin genes, including two splice isoforms of *actn1* homologous to those described for human *ACTN1*. We also identified two paralogs of human *ACTN3*. We characterized the temporal profile of expression of each family member through 8 days of zebrafish development. By whole mount *in situ* hybridization we observed that the widespread expression of *actn1* was conserved. We observed that the onset of unique and restricted expression domains of the muscle-specific actinins corresponded to the onset of muscle differentiation, and that each of the three genes had distinct expression patterns.

Muscle-specific actinins display different expression patterns in different vertebrates. In humans, *ACTN2* is expressed in all skeletal muscle fibers, with *ACTN3* restricted to a subset of Type II (fast) fibers (Mills et al., 2001; North and Beggs, 1996). Chickens do not have an *actn3* gene, nor could we identify any other avian *actn3* sequences (data not shown). In zebrafish *actn2* is transiently expressed in axial slow muscle, but is then down regulated in most slow muscle cells of the trunk and tail. In cranial and pharyngeal muscles the relative levels of *actn2* vary, with strong expression in the levator operculi but not in other muscles. Sections of the sternohyoidus muscle indicated that *actn2* is restricted to the slow muscle fiber compartment of this muscle. We tentatively conclude that *actn2* expression is restricted to a subset of slow muscle fibers in many muscles. However a more comprehensive analysis is required to support this conclusion. Zebrafish *actn3a* and *actn3b* are the dominantly-expressed muscle actinins, and one or the other are expressed throughout the developing musculature. The overlapping but distinct expression patterns of the *actn3* genes indicate that there may be functional differences between the two genes that arose in duplicated *actn3* genes following the teleost genome duplication (Taylor et al., 2003; Volff, 2005; Woods et al., 2005). However, we searched the current genome assemblies of medaka (HdrR) and stickleback (BROAD S1) and found only a single *actn3* gene in either genome (data not shown). This indicates that the retention of *actn3* duplicates may not be widespread in teleosts, and that the differences may represent subfunctionalization of the *actn3* genes in zebrafish and a unique requirement for non-overlapping domains resulting in the maintenance of both *actn3* paralogs. The presence of duplicated *actn3* genes in zebrafish is in direct contrast to the absence of functional *ACTN3* in many humans and chickens. Neither humans nor mice nor chickens require *ACTN3* for viability, and loss of *ACTN3* is associated with changes in muscle performance (MacArthur et al., 2007; Yang et al., 2003; North et al., 1999).

In contrast to the differences in *Actn3*, we observed striking conservation in the *Actn1* isoforms in zebrafish and humans, including conservation of the calcium insensitive isoform c. The conservation of some members of this family and divergence of others indicates that the functions of actinins are diverse. The zebrafish will be an exciting platform for investigation of the roles that the actinins play in development, disease and evolution.

Experimental Procedures

Fish care—All fish maintenance was performed according to Rice University and NIH animal care procedures. Embryos were collected and reared at 28°C in E3 embryo media. To eliminate pigmentation after 24 hpf, 1-phenyl-2-thiourea (PTU) was added to the E3 fish media at 0.003% (w/v) to inhibit melanin production. All staging was performed as described (Kimmel et al., 1995).

Accession numbers—A list of actinin sequences used to create the phylogenetic tree in Figure 1 is available (Supplemental Table S1). Zebrafish actinin sequences were identified using a reciprocal BLAST approach. Annotated *Homo sapiens* and *Mus musculus* gene entries for each of the four actinins were used in a BLAST search against the zebrafish genome build (Wellcome Trust Sanger Institute) to identify putative zebrafish homologs. These gene sequences were then queried against the human and mouse genomes to ensure all zebrafish actinin genes were identified.

Full length gene sequencing—Full length *actinin* genes were PCR-amplified. Overlapping internal primer sets were used for sequencing and can be found in Supplemental Table S2.

Sequence analysis—Alignment of tetrapod alpha-actinins was performed by nearest neighbor joining of the actinin protein sequences or the translations of our sequenced actinins

in table S1. The zebrafish actinin protein sequences obtained in our analysis were used for this alignment. The alignment and identity of the coordinating amino acids in the EF hands is after Beggs et al., 1992; Tang et al., 2001. Initial synteny conservation was established by searching the Synteny Database (Catchen et al., 2009). Database output was updated from the latest builds of zebrafish and human pseudogenes were removed. Accession numbers for zebrafish loci were updated with gene names from Vega (http://vega.sanger.ac.uk/Danio_rerio/index.html) or Zv8 (http://www.ensembl.org/Danio_rerio/Info/Index). Where no gene identity has been annotated the accession number was used even in cases where the Synteny Database established identity.

RT-PCR—RNA was prepared from the indicated stages by trizol lysis and purification (Invitrogen). cDNA was prepared with the ImPromII cDNA synthesis kit (Promega) according to the manufacturer's protocol with the substitution of random pentadecamers for random hexamers. PCR was performed using the primers in Table S3 at appropriate annealing temperatures. The PCR was conducted for the indicated cycle number, and then the product was analyzed by agarose gel electrophoresis and Ethidium bromide staining. *odc* primers were used to standardize the output for analysis (Draper et al., 2001).

in situ probe cloning and in situ hybridization—For construction of anti-sense riboprobe suitable for *in situ* hybridization, zebrafish α -actinin and *stnnc* cDNAs were amplified with the high fidelity Phusion polymerase (Finnzymes/New England Biolabs, F-530L) and cloned into pGEM-T EZ vector (Promega, #A1360). *myhz2* plasmid was obtained from ZIRC (CB-38) Zebrafish International Resource Center (ZIRC).

In order to compensate for the high degree of relatedness among the zebrafish alpha-actinins, where possible, EST data obtained from the zebrafish Ensemble Genome Browser (Wellcome Trust Sanger Institute) that yielded 3' UTR sequence (verified through NCBI BLAST searches) was targeted in conjunction with coding sequence for cloning to ensure probe specificity for each zebrafish α -actinin gene. Primer sequences are listed in Supplemental Table S4. Probe homology between the *actn* genes is presented in Table S5. Relative probe positions are in Figure S2.

Whole mount *in situ* hybridization staining was done essentially as described (Schulte-Merker et al., 1994; Thisse et al., 1993). Embryos and larvae were manually dechorionated and treated with 0.04% Tricaine (Sigma) prior to fixation in 4% (w/v) paraformaldehyde. Fixed embryos aged 24, 36 or 48 hpf were treated with ProteinaseK (Fisher) at a concentration of 10 μ g/ml in PBST for 5, 10 or 15 minutes, respectively. Stained embryos and larvae were semipermanently whole mounted in GMM (10% (v/v) methyl salicylate (Sigma, M-6752) in Canada balsam (Sigma, C-1795) for imaging. Photomicrographs were obtained using an AxioCam MRc5 camera (Zeiss) mounted on an Axiovert 200M inverted stereomicroscope (Zeiss). The relative expression of *actn2*, *actn3a* and *actn3b* at 96 hpf was determined using either expression in the levator arcus palatine as a reference for high level of expression for all muscles, or comparatively scoring the relative intensities between the three genes for each individual muscle independently. Both scoring methods yielded similar results.

Histology—For cryosectioning, whole mount stained embryos were equilibrated overnight in a solution of 7.5% sucrose in PBST at 4°C to reduce dehydration artifacts when samples were later frozen. Embryos were equilibrated for 1 to 6 hours in OCT embedding medium (Sakura Finetek USA, 4583), then oriented in OCT at 25°C, frozen on dry ice, and stored or sectioned at -20 to -25°C. Sections were obtained using a Harris-Cryostat (Int'l Equip. Co., model CTD) at a thickness of 10 μ m or more and captured on FrostPLUS positively charged glass slides (VWR, 48311). EosinY (Sigma) was used to counter stain *in situ* specimens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The zebrafish genome contains 5 actinin genes representing the 4 major classes of mammalian actinins. **A:** Jukes-Cantor nearest neighbor tree of zebrafish, tetrapod and Drosophila actinins. **B:** Percent identity of Human and zebrafish actinins. **C:** Alignment of EF hands 1 and 2 of Human and zebrafish. Coordinating residues X, Y, Z, -Y, -X and -Z are indicated. Positions with conservation of Actn1 iso c with the muscle specific actinins are highlighted in grey.

Figure 2.

Temporal expression profiles of zebrafish actinin genes as shown by semi-quantitative PCR. Each actinin gene shows a unique and dynamic regulation of expression through early development. cDNA samples used for each PCR reaction are indicated by column and range from adult ovary and oocytes to 8 dpf larval cDNA. The upper band of the *actn1* doublet was sequenced to show it is a product from an unrelated locus.

Figure 3.

Expression of the actinin gene family at 24-48 hpf. Expression of the actinins by whole mount in situ hybridization reveals distinct patterns for most genes. A-J: lateral whole mount views with insets showing higher magnification dorsal or lateral views of somite gene expression, insets are magnified lateral (C, Gb, I, J), dorsal (A, B, D, E, Ga) or anteroventral (H) views; A'-J': transverse sections of axial gene expression taken at level indicated by blue lines in A-J. **A, A'**: Expression of slow-specific marker *stnnc* at 24 hpf. Expression was observed in slow muscle fibers including muscle pioneer cells (arrow) and in peripheral slow muscle sheath. **B, B'**: Expression of fast-specific marker *myhz2* at 24 hpf. Expression is seen in most of the somite but no expression is seen in the muscle pioneer cells (arrow). **C, C'**: Expression of *actn2* at 24 hpf. Expression is seen in the heart (arrowhead) and in the slow muscle pioneers (arrows). **D, D', E, E'**: Expression of *actn3a* (D, D') and *actn3b* (E, E') at 24 hpf. Both *actn3a* and *actn3b* are widely expressed in the somite. Expression is also seen in the slow muscle pioneer cells (arrows). **F, F'**: Expression of *actn1* at 36 hpf. Expression was observed in the skin (arrow), notochord (arrowhead) and generally expressed in the somite. Expression was not observed in the neural tube (nt) or other components of the CNS. **G, G'**: Expression of *actn4* at 36 hpf. Expression is seen in the notochord (arrows) as well as the developing gut (arrowhead). **H, H'**: Expression of *actn2* at 48 hpf. Expression is seen in the heart (arrowheads) but is no longer observed in the slow muscle fibers of the myotome (arrow). **H''**: Expression of *stnnc* at 48 hpf. Expression can be seen in the peripheral sheath of slow muscle fibers (arrow). **I, I', J, J'**: Expression of *actn3a* (I, I') and *actn3b* (J, J') at 48 hpf. Both *actn3a* and *actn3b* are broadly expressed in most or all of the myotome muscle fibers.

Figure 4.

Muscle-specific actinins are differentially expressed in axial and craniofacial musculature at 96 hpf. **A-C**: lateral whole mount views. Inset **Aa**: transverse section at the posterior gut level; insets **Ab**, **B** and **C** are higher magnification lateral views of somite expression. **A'-C'**: higher magnification lateral views of craniofacial expression from **A-C**. **D**: camera lucida representation identifying muscles of zebrafish at 96hpf (abbreviations listed in Table 1). **E-I**, **F'-H'**: Ventral views of craniofacial expression, with **E-I** having a ventral focal plane and **F'-H'** being more a dorsal focal plane. Jaw structures and sternohyoideus muscles have been removed in **G'** for visibility of more dorsal muscles. **J-N**: Transverse sections at the level of the heart. **A,A'**: Expression of *actn2* at 96 hpf. Expression is seen in the heart (arrow) as well as the lateralis superficialis (white arrowhead) and inferior obliquus (black arrowhead). In the cranial musculature, strong *actn2* expression was seen in lap muscles (green arrowhead). Inset **Ab** shows broad expression of *stnnc* at 96 hpf in the slow fibers of the myotome. **B&B'**: Expression of *actn3a* at 96 hpf. Expression was seen in the axial musculature (white arrowhead, superficial expression). Expression was also seen in cranial and pharyngeal muscles and strong expression is seen in the sh (arrowhead). **C&C'**: Expression of *actn3b* at 96 hpf. Expression was seen in the axial musculature. Weak expression was seen in the sh (arrowhead). **D**: Craniofacial muscle groups are colored to demarcate pharyngeal arches of origin: red, mandibular arch (pharyngeal arch 1, P1); green, hyoid arch (P2); yellow, branchial arches 1-5 (P3-P7); purple, extraocular/cranial muscle; pink, somite-derived muscle; orange, cardiac muscle. **E**: *stnnc* was expressed uniformly in most cranial and pharyngeal muscle. Expression was light in sh muscles (white arrowhead). **F-H**: Expression of the muscle-specific actinins in

sh muscles was variable between homologs. *actn2* was weakly expressed in the sh (arrowhead, F), *actn3a* was very strongly expressed (arrowhead, G), and *actn3b* was moderately expressed (arrowhead, H). **I:** *myhz2* was expressed strongly in sh muscles (arrowhead). **J:** *actn2* expression was weak in tv and dpw muscles (white and green arrowheads, respectively) compared to its strong expression in lap muscles (black arrowhead). **K&L:** *actn3a* (K) was weakly expressed in tv (white arrowheads), dpw (green arrowheads) and lap (black arrowheads) muscle pairs. *actn3b* (L) expression was high in all pharyngeal muscles. **M-Q:** *stnnc* was expressed in the periphery of the sh (arrowhead, M). *myhz2* was expressed in the center of the sh (arrowhead, N). *actn2* was expressed in the periphery of the sh (arrowhead, O). *actn3a* was expressed in the center of the sh (arrowhead, P). *actn3b* was expressed in the center of the sh (arrowhead, Q).

Table 1

Relative expression of muscle actinins in 96 hpf cranial and pharyngeal muscles. Locations of muscles are indicated by their abbreviations in Figure 4 G. Expression levels are identified as: n/a not analyzed, – no expression, +/- weak variable expression, + weak consistent expression, ++ moderate expression, +++ strong expression

Region	Muscle	actn2	actn3a	actn3b
mandibular arch	adductor mandibulae	+	++	+++
	dilator operculi	+	++	+++
	intermandibularis anterior	+/-	+	++
	intermandibularis posterior	+/-	+/-	-
	levator arcus palatini	+++	+++	+++
hyoid arch	adductor hyoideus	++	++	+++
	adductor operculi	+	+++	+++
	hyohyoideus	+/-	+	++
	interhyoideus	+/-	+/-	+
	levator operculi	n/a	n/a	n/a
branchial arches	dorsal pharyngeal wall 1-5	+	+	+++
	rectus communis	n/a	n/a	n/a
	rectus ventralis	n/a	n/a	n/a
	transversus ventralis 1-5	++	+	+++
extraocular	inferior oblique	+	+/-	+
	inferior rectus	-	+/-	-
	lateral rectus	++	++	+++
	medial rectus	+	+	+/-
	superior oblique	+	+/-	+
	superior rectus	+	++	+
somite	sternohyoideus	+/-	+++	+