약학박사학위논문

Role of Akap12 in the Muscle Morphogenesis and Locomotion in Zebrafish

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약학과 의약생명과학 전공

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ABSTRACT

Role of Akap12 in the Muscle Morphogenesis and Locomotion in Zebrafish

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Swimming behavior in fish is driven by coordinated contractions of muscle fibers. As in other vertebrates, zebrafish skeletal muscle cells can be classified as slow or fast fibers on the basis of their physiological functions. In zebrafish, slow muscle cell migration is crucial for the formation of the muscle network; slow myoblasts, which arise from medial adaxial cells, migrate radially to the lateral surface of the trunk and tail during embryogenesis. At the level of the

horizontal myoseptum, adaxial cells at the medial surface do not migrate but rather remain associated with the notochord; these cells are termed muscle pioneers. Fast muscle cells are generated by the elongation and fusion of initially rounded lateral presomitic mesoderm cells after their slow muscle migration. These dramatic morphogenic changes during somitogenesis are critical for normal muscle contractility and function. Although the molecular mechanisms that specify myogenic fate have been extensively studied, relatively little is known about how the normal morphogenic movements of myoblasts lead to early locomotor behaviors. This study found that the zebrafish A-kinase anchoring protein (*akap*)*12* isoforms *akap12α* and *akap12β* are required for muscle morphogenesis and locomotor activity. Embryos deficient in *akap12* exhibited reduced spontaneous coiling, touch response, and free swimming. *Akap12*-depleted slow but not fast muscle cells were misaligned, suggesting that the behavioral abnormalities resulted from specific defects in slow muscle patterning; indeed, slow muscle cells and muscle pioneers in these embryos showed abnormal migration in a cell-autonomous manner. Taken together, these results suggest that *akap12* plays a critical role in the development of zebrafish locomotion by regulating

the normal morphogenesis of muscles.

Keywords : *akap12*; locomotion; muscle; morphogenesis; development; zebrafish

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LIST OF ABBREVIATIONS

AC; adenylyl cylase

AKAP; A-kinase anchoring protein

AS; anti-sense

bactin2; *beta actin 2*

CaM; calmodulin

cAMP; cyclic adenosine monophosphate

DSHB; Developmental Studies Hybridoma Bank

engrailed; *engrailed homeobox*

GFP; green fluorescent protein

GPCR; G-protein-coupled receptor

H&E; hematoxylin and eosin

hpf; hours post-fertilization

IP_3; inositol 1,4,5-triphosphate

MLC; myosin light chain

M-MLV; Moloney murine leukaemia virus

MO; morpholino oligomer

MP; muscle pioneers

myoD; *myogenic differentiation*

PAK; p21-activated kinase

PBDTT; 1X PBS, 0.1% Tween 20, 1% dimethyl sulfoxide, and 0.5% Triton X-100

PBS; phosphate-buffered saline

PKA; protein kinase A

PKC; protein kinase C

PLC; phospholipase C

Prim-5; primordium 5

prox1; *prospero homeobox 1*

ROCK; Rho-associated protein kinase

RTK; receptor tyrosine kinase

RT-PCR; reverse transcription polymerase chain reaction

S; sense

shha; *sonic hedgehog a*

smyhc1; *slow myosin heavy chain 1*

ss; somite stage

INTRODUCTION

1. Locomotion behavior of the zebrafish embryo and larva

Zebrafish embryos and early larvae exhibit well-characterized and stereotypic locomotor activity that includes spontaneous muscle contractions, touch-evoked coiling, and swimming. Spontaneous contractions of trunk muscles, the first manifestation of swimming behavior, begin abruptly at 18 h post-fertilization (hpf) when the rostral somites terminally differentiate into slow muscle cells (Felsenfeld et al., 1990; Grunwald et al., 1988; Saint-Amant and Drapeau, 1998). Spontaneous movement was found to occur in the absence of supraspinal input and independent of several neurotransmitter systems (Saint-Amant and Drapeau, 1998, 2000, 2001). Unlike other vertebrate preparations, spontaneous movement depends solely on electrical coupling through gap junctions (Brustein et al., 2003). Subsequently, zebrafish embryos are able to respond to mechanosensory stimulation with vigorous, propulsive coils as of 21 hpf (Granato et al., 1996; Muller and van Leeuwen, 2004; Naganawa and Hirata, 2011; Saint-Amant and Drapeau, 1998). By 27 hpf

dechorionated embryos will briefly and slowly swim away when touched. During the hatching period, the swim cycle frequency increases to a mature level but the larvae swim only infrequently and in bursts. After swim bladder is inflated, zebrafish can swim freely and execute spontaneous changes in direction. Thus embryonic and early larval locomotion of the zebrafish can be devided into different periods characterized by different patterns of locomotion (Fig. 1), similar to the early swimming behavior described in *Xenopus* (Kahn and Roberts, 1982; van Mier, 1988; van Mier et al., 1989). Aspects of the development of locomotion have been also described in vertebrates including chick (O'Donovan, 1989), rats (Kudo and Yamada, 1987), and zebrafish (Kimmel et al., 1974). This repertoire of behaviors is controlled by orchestrated contractions of skeletal muscle fibers.

Skeletal muscle is a major component of vertebrate anatomy, making up around 50% of the body mass of a human and around 80% of that of a fish (Jackson and Ingham, 2013) (Fig. 2). As in other vertebrates, zebrafish skeletal muscle cells can be classified as slow or fast fibers on the basis of their physiological functions (Cho et al., 1993; Close, 1972; Harris et al., 1989; Ochi and Westerfield, 2007).

For example, their shapes, orientations, and gene expression profiles are distinct and the two cell types have different speeds of contraction corresponding to their respective metabolic activities (Table 1). Slow-twitch (red) fibres are rich in mitochondria and therefore more efficient at using oxygen to generate ATP, resulting in a higher endurance level. Fast-twitch (white) fibres, by contrast, are better adapted to generating short bursts of strength or speed, but fatigue more rapidly due to their high contraction velocity (Jackson and Ingham, 2013; Schiaffino and Reggiani, 2011). The terms red and white are derived from the way these muscles appear during surgery or autopsies, but largely refer to the mitochondrial content of the muscle itself. Thus, a carefully orchestrated skeletal muscle developmental program is obviously necessary to generate normal embryonic locomotion.

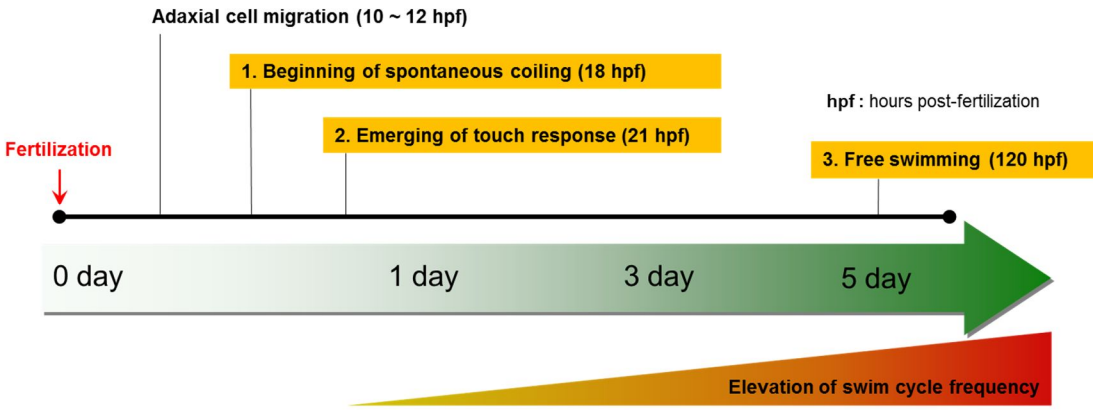


Figure 1. Stereotypic locomotor activity of zebrafish embryos

Spontaneous contractions begin at 18 hpf, subsequently, embryos are able to respond to touch stimulation at 21 hpf and zebrafish can swim freely and execute active changes in direction at 120 hpf.

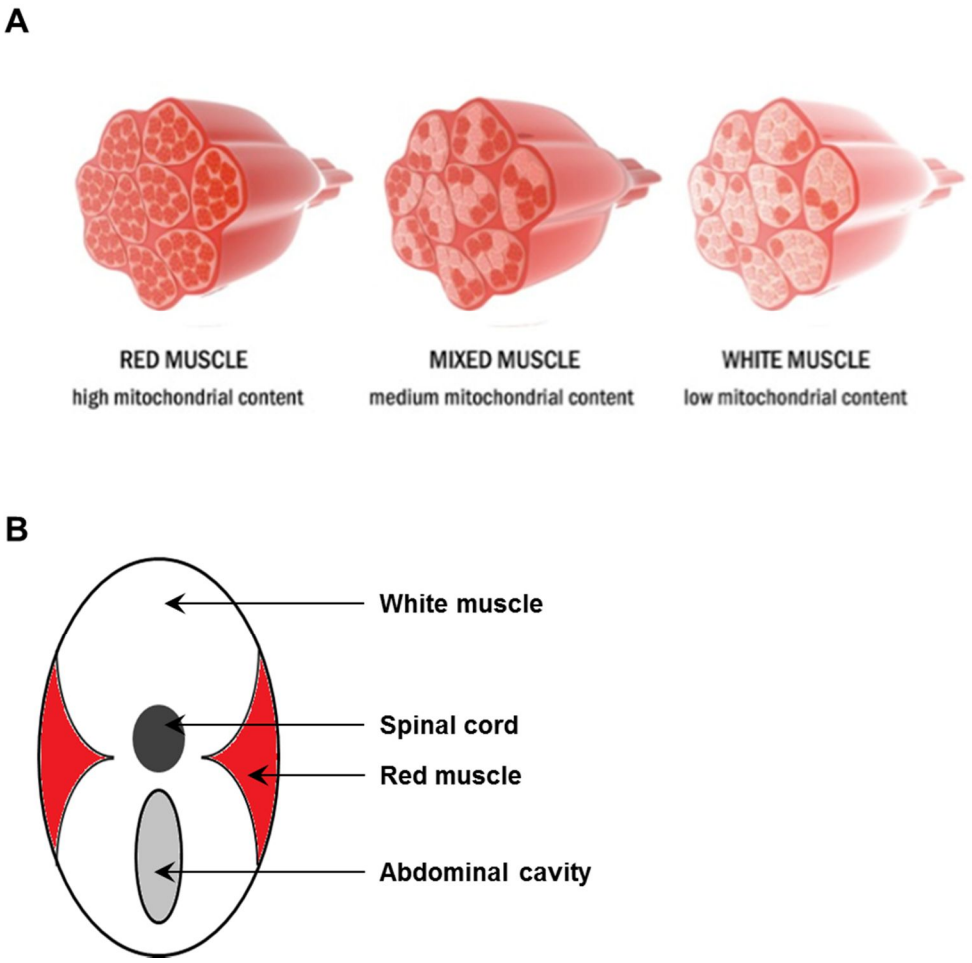


Figure 2. Slow (red) and fast (white) muscle fibers in mammals and fish

(A) Slow and fast muscles are generally fused with each other in mammals. Thus, muscle is divided into three types – white muscle, red muscle and mixed muscle (http://fitstar.com/high-intensity-

exercise/). (B) Drawing showing a transverse section of an adult zebrafish. Slow and fast muscle fibers can be unambiguously distinguished in zebrafish.

Table 1. Properties of slow versus fast muscle

	Properties
Slow muscle (red)	Small, less powerful muscle utilized long term (endurance muscle)
	Lower ATPase activity (slow muscle)
	Smaller number of muscle fibers in each motor unit
	High capacity for aerobic metabolism
	Red color due to high Myoglobin
Fast muscle (white)	Large, powerful muscle utilized for a short term
	High ATPase activity (fast muscle)
	Larger number of muscle fibers in each motor unit
	High capacity for anaerobic glycolysis
	Low Myoglobin

2. Muscle development

Muscle morphogenesis in zebrafish embryos proceeds via a series of events starting with early differentiation of the segmental plate, which comprises adaxial cells that form a single-cell layer adjacent to the notochord and give rise to slow muscle cells (Fig. 3). During segmentation, adaxial cells migrate radially to the lateral surface of the myotome, forming a subcutaneous layer of slow muscle fibers (Blagden et al., 1997; Devoto et al., 1996). At the level of the horizontal myoseptum, adaxial cells at the medial surface do not migrate but rather remain associated with the notochord; these cells are termed muscle pioneers (Ekker et al., 1992; Halpern et al., 1993; Hatta et al., 1991). Fast muscle cells are generated by the elongation and fusion of initially rounded lateral presomitic mesoderm cells after their slow muscle migration (Henry and Amacher, 2004; Moore et al., 2007). Three distinct types of embryonic muscle fibers and their development is summarized in Fig. 3 (Du et al., 1997). These dramatic morphogenic changes during somitogenesis are critical for normal muscle contractility and function. Specification of the slow muscle lineage is dependent upon Hedgehog signaling (Barresi et al., 2001; Blagden et al., 1997; Coutelle et al., 2001;

Currie and Ingham, 1996, 1998; Du et al., 1997; Lewis et al., 1999; Roy et al., 2001) (Fig. 4). In addition, it has been demonstrated that Hedgehog signaling impacts not only slow muscle differentiation but also medial fast fibers that is a small subpopulation of fast muscle and express Engrailed protein (Wolff et al., 2003). Although the molecular mechanisms that specify myogenic fate have been extensively studied (Arnold and Braun, 2000; Currie and Ingham, 1996), relatively little is known about how the normal morphogenic movements of myoblasts lead to early locomotor behaviors.

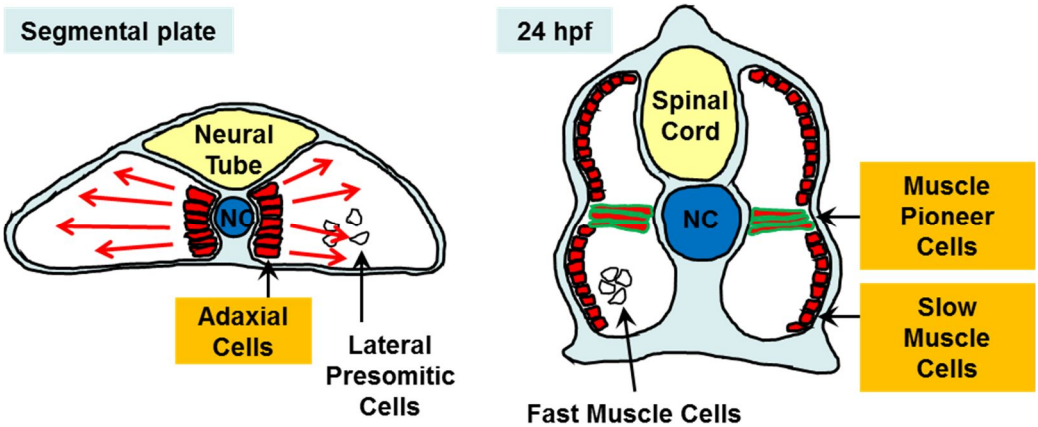


Figure 3. Development of zebrafish muscle fiber types

Schematic transverse section through the segmental plate (left) and the trunk of a 24 hpf embryo (right) (Du et al., 1997).

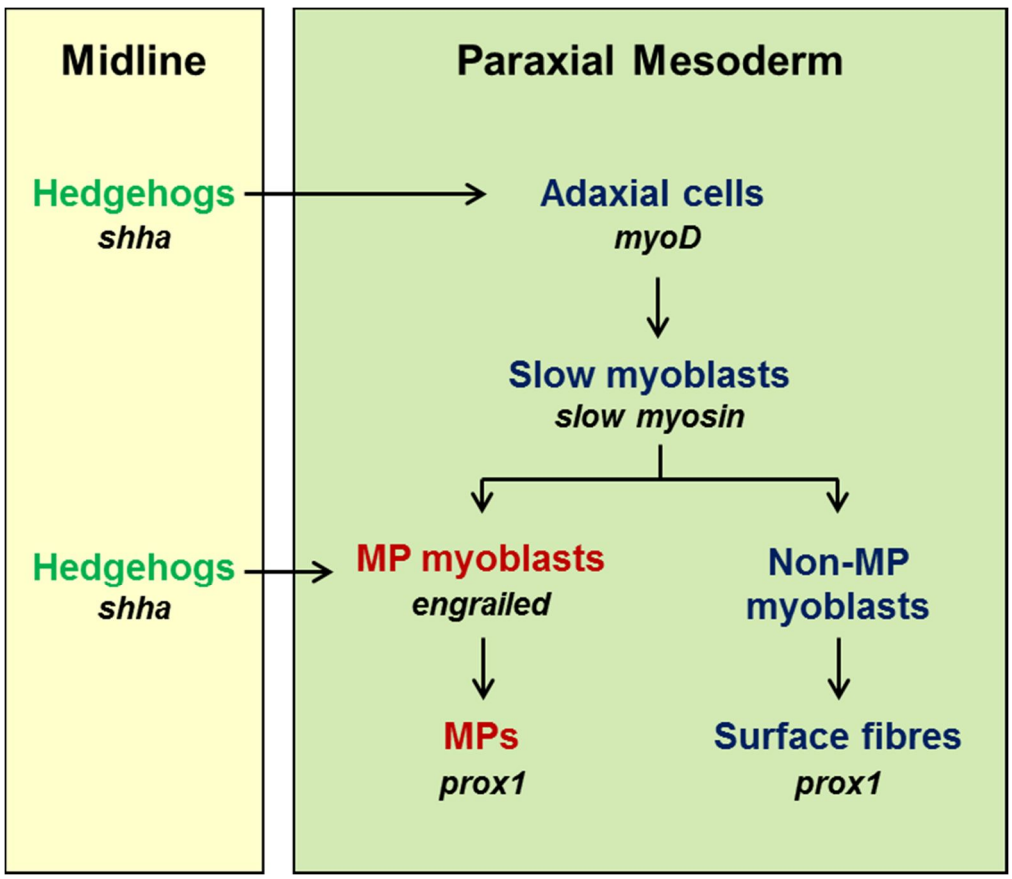


Figure 4. Schematic representation of the genetic pathway that regulates specification and differentiation of the slow muscle fibers

Hh proteins from the midline signal to the paraxial mesoderm. This results in the induction of myogenesis through the expression of myogenic regulatory genes in the adaxial cells. Hg signaling acts to

induce high levels of Engrailed (Eng) expression in the muscle pioneer precursors. The requirement of the homeobox gene *prox1* is restricted to the final stages of the slow fibers (Roy et al., 2001).

3. A-kinase anchoring protein (AKAP) 12

AKAPs (A-kinase anchoring proteins) are members of a diverse family of scaffold proteins that possess a binding domain for protein kinase A (PKA) and play critical roles in the coordinated interplay of multiple signaling pathways (Hausken and Scott, 1996). AKAPs are expressed in a wide range of species from worms, to mammals (Colledge and Scott, 1999) and contribute to the precision of intracellular signaling events by directing anchored enzyme pools to a subset of their physiological substrates at specific subcellular locations (Carnegie et al., 2009) (Fig. 5). Therefore, AKAPs provide a multivalent template for cell signaling that complies with organizing protein-protein interactions among low abundance proteins, providing a dynamic, reversible platform for signaling (Dodge and Scott, 2000). With regard to multivalency, AKAP12 displays docking sites for PKA, protein kinase C (PKC), protein phosphatases, the non-receptor tyrosine kinase Src, and for a prominent member of the superfamily of G-protein-coupled receptors (GPCRs), the β_2-adrenergic receptor (Fan et al., 2001; Lin et al., 2000) (Fig. 6). Additionally, AKAP encodes three polybasic domains that facilitate binding to phosphoinositols, four SV40 type nuclear localization signals (NLS),

and a domain that facilitates nuclear exclusion (Gelman, 2012). Four major PKC phosphorylation sites as well as one major tyrosine phosphorylation site of Akap12 were reported (Guo et al., 2011; Xia and Gelman, 2002). The AKAP12α isoform is post-translationally myristoylated (Myr) at its N terminus (Lin et al., 1996).

AKAP12 is a well-characterized tumor suppressor that controls cytoskeletal remodeling (Cheng et al., 2007; Gelman et al., 1998; Guo et al., 2011; Kwon et al., 2012; Lin et al., 1996; Weiser et al., 2008) and cell migration (Gelman et al., 2000; Lee et al., 2011; Su et al., 2010; Weiser et al., 2007; Yan et al., 2009a) by interacting with various signaling molecules such as PKA, PKC, F-actin, Src, and phospholipids. As a member of the AKAP family of scaffolding proteins (Diviani and Scott, 2001; Wong and Scott, 2004), *akap12* induces changes in cell shape and function during mesangial cell differentiation (Nelson et al., 1999).

In zebrafish, *akap12* is involved in the control of complex cell behavior in mesodermal cells undergoing gastrulation (Weiser et al., 2007). Interestingly, the change of the expression level of *akap12* has no effect on cell migration in scratch assays, but *akap12* is a potent inhibitor of cell invasion through matrigel (Weiser et al., 2007),

indicating that *akap12* is a key regulator of complex cell behaviors, not a simple regulator in cell migration.

Two isoforms of *akap12, α* and *β,* are encoded by the spliced transcripts in zebrafish. The 71 amino acids of *akap12α* and 8 amino acids of *akap12β* in the N-terminal region are encoded by small exons (1 and 2 for *α* and 3 for *β*). They then splice to a common exon 4, which encodes the nearly 1,525 remaining amino acids (representing 95.6% of the *α* isoform or 99.5% of the *β* isoform) (Kwon et al., 2012) (Fig. 7).

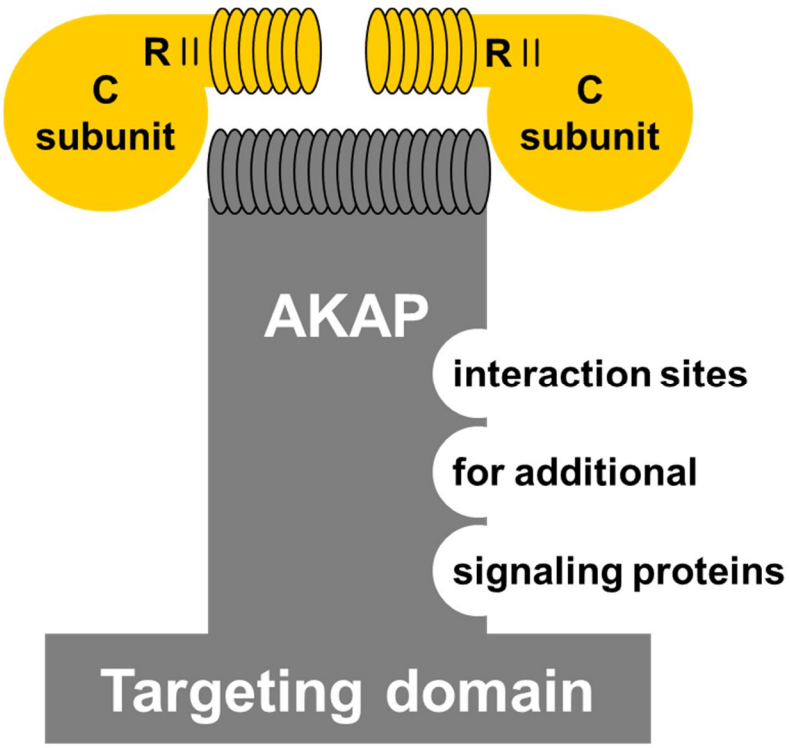


Figure 5. Properties of AKAPs. AKAPs bind to the regulatory subunit of PKA

AKAPs also bind additional signaling proteins in addition to PKA, including other protein kinases, protein phosphatases, phosphodiesterases, adenylyl cyclases and small G proteins. AKAPs mediate precise spatiotemporal control of target signaling complexes (Carnegie et al., 2009).

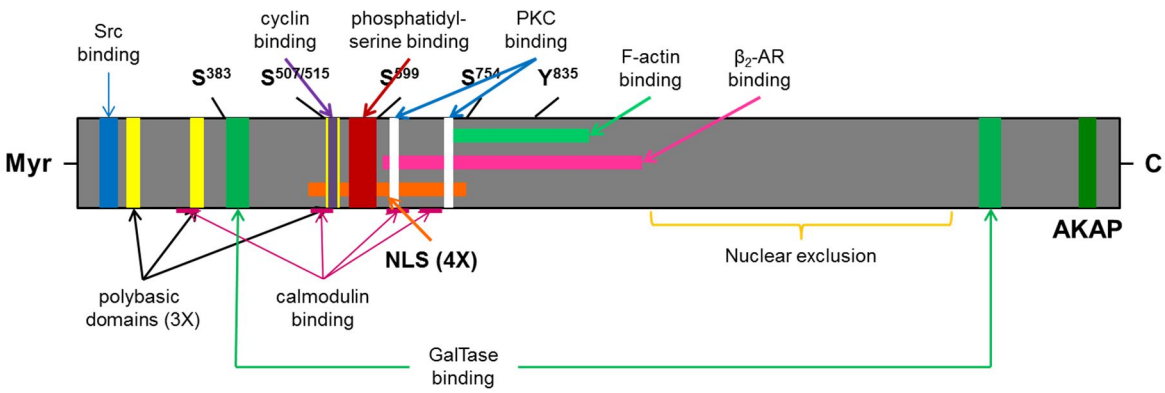


Figure 6. AKAP12 scaffolding domains

Binding domains on AKAP12 are shown for Src, cyclins, calmodulin, GalTase, phosphatidylserine, PKC, F-actin, β_2-adrenergic receptor, and PKA (Gelman, 2012).

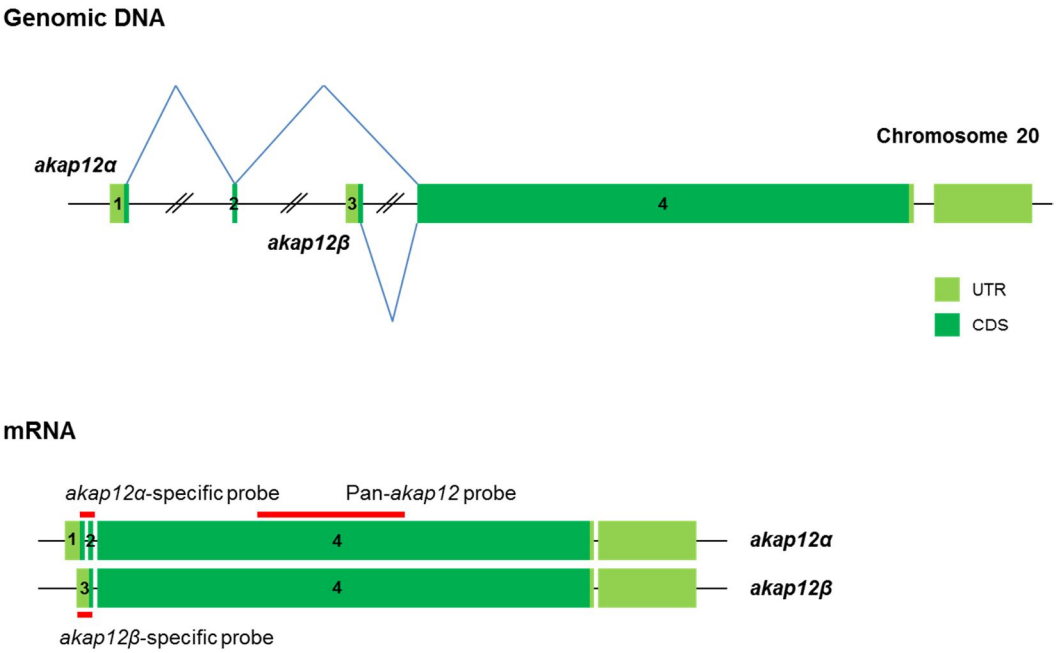


Figure 7. Genomic and transcript structure of zebrafish *akap12*

The arched lines connecting the exons represent the splicing of the two mRNAs encoded by *akap12*. The proteins encoded by the two *akap12* isoforms share >95% amino acid sequence identity but differ at their N termini. Two specific probes and a pan-*akap12* probe were designed based on the *akap12* genetic map (red bars).

4. Zebrafish as a model for behavioral studies

Over the past decade, the zebrafish has become an increasingly popular animal model for the study of vertebrate development. They can be obtained in large numbers and are easily removed from the chorion. Moreover, zebrafish embryos are transparent, which simplifies morphological studies, and their genetic manipulation is straightforward, the zebrafish has been used to facilitate the analysis of behavior throughout embryonic development (Chen et al., 2012; Downes and Granato, 2006; Muller and van Leeuwen, 2004; Naganawa and Hirata, 2011; Saint-Amant and Drapeau, 1998). Higher vertebrates pose several limitations to locomotion studies because systems are complex even at the early stages and the behaviors themselves are often difficult to characterize. Therefore, zebrafish embryos are well suited for studies of development of motility. In several larval fishes, including plaice (*pleuronectes platessa*), herring (*Clupea harengus*) and chinook salmon (*Oncorhynchus tshawytscha*), detailed kinematics have been reported for burst swimming (Budick and O'Malley, 2000). In larval and adult zebrafish, hydrodynamic flow patterns have been chariacterized (Muller et al., 2000). Furthermore, genetic screens

have revealed numerous mutations with selective effects on locomotion (Brockerhoff et al., 1995; Granato and Nusslein-Volhard, 1996; Granato et al., 1996; Nicolson et al., 1998) (Table 2).

Table 2. Mutants affecting embryonic motility

Gene	Abbreviation	Motility defect	Main defect(s)	Reference
spock	*spk*	Circling	Ears	a
backstroke	*bks*	Circling	Otoliths	a
what's up	*wup*	Circling	Otoliths	a
keinstein	*kei*	Circling	Otoliths	a
little ears	*lte*	Circling	Ears	a
u-boot	*ubo*	Reduced motility	Myoseptum	b
sonic you	*syu*	Reduced motility	Myoseptum	b
you-too	*yot*	Reduced motility	Myoseptum	b
chameleon	*con*	Reduced motility	Myoseptum	b,c
white snake	*wis*	Immotile	Brain	d
wirbel	*wir*	Immotile	Body shape	d
boxer	*box*	Reduced motility	Retinotectal projection, fins, jaw	e,f,g
noir	*nir*	Reduced motility	Pigment	h
zwart	*zwa*	Reduced motility	Pigment	h
submarine	*sum*	Balance	Pigment	h
polished	*pol*	Reduced motility	Pigment	h

touch down	*tdo*	Reduced motility	Pigment	h
blanched	*bch*	Reduced motility	Pigment	h
clorix	*clx*	Reduced motility	Pigment, ear	a,j
casanova	*cas*	Reduced motility	Heart	k
still heart	*sth*	Reduced motility	Heart	k, j

References: a, (Whitfield et al., 1996); b,(van Eeden et al., 1996a); c, (Brand et al., 1996); d, (Jiang et al., 1996); e, (Trowe et al., 1996); f, (van Eeden et al., 1996b); g, (Schilling et al., 1996); h, (Kelsh et al., 1996); j, (Odenthal et al., 1996); k, (Chen et al., 1996).

PURPOSE OF THIS STUDY

AKAP12 has been known to have activity as a tumor- and metastasis-suppressor. AKAP12 functions as a scaffolding protein, which controls mitogenic signaling and cytoskeletal remodeling by binding various signaling mediators in a spatiotemporal manner. Recently, it was reported that *akap12* plays a critical role in the migration of mesodermal cells during the gastrula period of zebrafish development. In our previous study, we revealed that Akap12 is required for the developmental angiogenesis that is accompanied with the vascular stability. However, we had continuously observed that *akap12* depleted zebrafish embryos showed impaired locomotor activity and the function of *akap12* on the development of embryonic and larval zebrafish locomotion is largely unknown. In current study, we aim at clarifying the cellular mechanism how *akap12* regulates the locomotor activity in development of zebrafish locomotion.

MATERIALS AND METHODS

1. Ethics statement

All work described was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Seoul National University.

2. Animals

Wild-type AB/TU zebrafish embryos were maintained and staged according to standard laboratory protocols (Westerfield, 2000). The *Tg(9.7kb smyhc1:GFP)*^{i104} transgenic line was obtained from Dr. Philip W. Ingham (Elworthy et al., 2008).

3. Morpholinos

Zebrafish embryos were injected with 2 ng of *akap12α* morpholino oligomer (MO), 4 ng of *akap12β* MO, and 4 ng of control oligos at the one-cell stage. All MOs have been described previously (Kwon et al., 2012) and were purchased from Gene Tools, LLC

(Philomath, OR, USA): standard control oligo (5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3'); *akap12α* splice- (5'-TAC CTT GCC ATC TGC GGT TTC TCC A-3') and translation- (5'-CAC GGA TGG TGT CGC TCC CAT GA-3') blocking MOs; and *akap12β* splice- (5'-TCT TAC CTG TTA GAG TTA TTG TCC C-3') and translation- (5'-CTG TTA GAG TTA TTG TCC CAA GCA T-3') blocking MOs (Table 3).

4. *In vitro* mRNA transcription

For the rescue experiment, *akap12α* and *β* mRNA were transcribed from linearized expression constructs using the mMessage mMachine Kit (Ambion) following the manufacturer's instructions. Fifty pg of zebrafish *akap12α* and *β* mRNA were co-injected with their splice-blocking MOs.

5. Reverse transcription (RT)-PCR

To measure the mRNA expression of *akap12*, RNA was isolated from zebrafish embryos at several stages (64- and 1,000-cell, sphere, shield, 75% and 90% epiboly, bud, 6-somite, and prim-5 stages) using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was

reverse transcribed into complementary DNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Two specific forward primers that correspond to the N-terminal region of the *α* and *β* isoforms and a reverse primer to the C-terminal region of the *akap12* isoforms were used (Table 3): *akap12α* (5'-ATG GGA GCG ACA CCA TCC G-3') and *akap12β* (5'-ATG CTT GGG ACA ATA ACT CTA ACA G-3') forward primers; *akap12* reverse primer (5'-TGA GGC CTG TCT TCT GTT GA-3'); and *β-actin2* forward (5'-GCA GAA GGA GAT CAC ATC CCT GGC-3') and reverse (5'-CAT TGC CGT CAC CTT CAC CGT TC-3') primers (Casadei et al., 2011). The reaction conditions were as follows: for *akap12α*, initial denaturation at 94°C for 5 min, followed by 27 cycles at 94°C for 45 sec, 60°C for 45 sec, 72°C for 5 min; for *akap12β*, initial denaturation at 94°C for 5 min, followed by 25 cycles at 94°C for 45 sec, 55°C for 45 sec, 72°C for 5 min; for *β-actin2*, initial denaturation at 94°C for 5 min, followed by 18 cycles at 94°C for 45 sec, 60°C for 45 sec, 72°C for 45 sec. PCR was carried out using 10 pmoles of each primer in a T3000 Thermocycler (Biometra).

6. Cryosectioning

For the fixation of samples, embryos were fixed by immersion in 4% v/v paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4 for 24 hours at 4°C. After fixation, paraformaldehyde was removed with five washes of 5 minutes in PBS with 0.1% Tween 20. The samples were then incubated in optimum cutting temperature (O.C.T.) Compound (Tissue-Tek) for 30 minutes and then in O.C.T. overnight at 4°C. Embryos were frozen at -80°C in cryomolds before sectioning. Embedded tissue was sectioned with a HM 525 Microm Cryostat (Thermo Scientific) into 10 μm transverse sections and adhered to Adhesion Microscope Slides (Marienfeld).

7. Hematoxylin and eosin (H&E) staining

Histological sections were stained with hematoxylin and eosin to observe possible morphological changes. Briefly, the technique involves immersing the sections in eosin for 1 minute, then washing with water every 30 minutes and further incubating for 1 minute in hematoxylin. Finally, the samples were dehydrated in ethanol of increasing concentration for 5 minutes each, ending with xylene. The

slides were mounted in Permount Mounting Media (Fisher Scientific) for analysis and storage. Images of hematoxylin and eosin staining were taken in a Leica DM5000B (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

8. *In situ* hybridization

Whole-mount *in situ* hybridization was performed as previously described (Thisse and Thisse, 2008). The 213-bp coding sequence for zebrafish *akap12α* (NCBI Reference Sequence: NM_001281788.1) was amplified by PCR using the following primers: Forward primer, 5′-ATG GGA GCG ACA CCA TCC GTG-3′; Reverse primer, 5′-TCA TTC AGC CGG TGG GTT CTC CT-3′. For *akap12β* (NCBI Reference Sequence: NM_001098184.2), 191 bp from the *akap12β* 5′ UTR region and the N terminus-specific coding sequence was amplified by PCR using the following primers (Table 3): Forward primer, 5′-ACA TCA TAG AGA AAG AGA GG-3′; Reverse primer, 5′-CTG TTA GAG TTA TTG TCC CA-3′. The amplified PCR products were cloned into the pGEM-T Easy vector (Promega). The anti-sense pan-*akap12* probe containing 2,256 bp of *akap12* conserved regions was used as previously described (Kwon et al., 2012). Probes for

myoD and *shha* were obtained from the Zebrafish Organogenesis Mutant Bank (ZOMB, Korea). All clones were linearized and synthesized using a DIG-RNA labeling mix (Roche Diagnostics, Indianapolis, IN, USA). Chromogenic hybridization was visualized by microscopy using a Zeiss Stemi 2000-c stereo microscope with an AxioCam ICc 1 digital camera (Zeiss, Bremen, Germany) and Leica DM5000B (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

9. Immunohistochemistry

For whole-mount immunohistochemistry, embryos were fixed in 4% paraformaldehyde overnight at 4°C and then washed several times in 1× PBS with 0.1% Tween 20. Embryos were blocked with 5% bovine serum albumin and 10% normal goat serum in PBDTT consisting of 1× PBS, 1% dimethyl sulfoxide, 0.5% Triton X-100, and 0.1% Tween 20, and incubated overnight at 4°C with primary antibodies. The antibodies used were anti-slow and fast myosin heavy chains, anti-engrailed [F59, F310, and 4D9 respectively; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA], and anti-Prox1 (ReliaTech GmbH). After the embryos were washed with PBDTT, they were incubated with species-matched Alexa 488-

or 546-conjugated secondary antibodies. The specimens were imaged using a Zeiss LSM 700 confocal microscope with ZEN 2011 software (Zeiss).

10. Behavioral analysis

Three types of behavior were analyzed in this study: spontaneous coiling, touch response, and free-swimming activity. To determine whether loss of *akap12* function affects spontaneous activity, embryos were monitored once every hour for 1 min under a stereo microscope with an AxioCam HSm high speed digital camera (Zeiss, Bremen, Germany) from 19 to 27 hpf (Saint-Amant and Drapeau, 1998). The results of three replicate experiments were analyzed, and 60 embryos in each injected group were counted.

For touch responses at 27, 36, and 48 hpf, embryos were dechorionated using fine forceps at 24 hpf (Downes and Granato, 2004), and a response was evoked by touching the caudal trunk region with a hair probe. The number of embryos showing bending or escape behavior after the touch was scored and recorded under the stereo microscope with a high-speed digital camera.

Free-swimming activity of larvae injected with each MO was

recorded over a 20-min period at 120 hpf. Larvae were individually placed in one well of a 24-well plate at 4 dpf, and their movements were measured using the DanioVision tracking system (Fig. 8) (Noldus Information Technology, Wageningen, the Netherlands), which included a high-resolution digital video camera. Locomotor activity was analyzed by calculating the total distance traveled and the mean velocity for each group using EthoVision XT 8 Locomotion Tracking software (Fig. 8) (Noldus Information Technology).

11. Statistical analysis

All experiments were repeated at least three times, and the data are shown as the mean ± SEM. Samples were compared using Student's t test to determine statistical significance. A $P < 0.05$ was considered statistically significant.

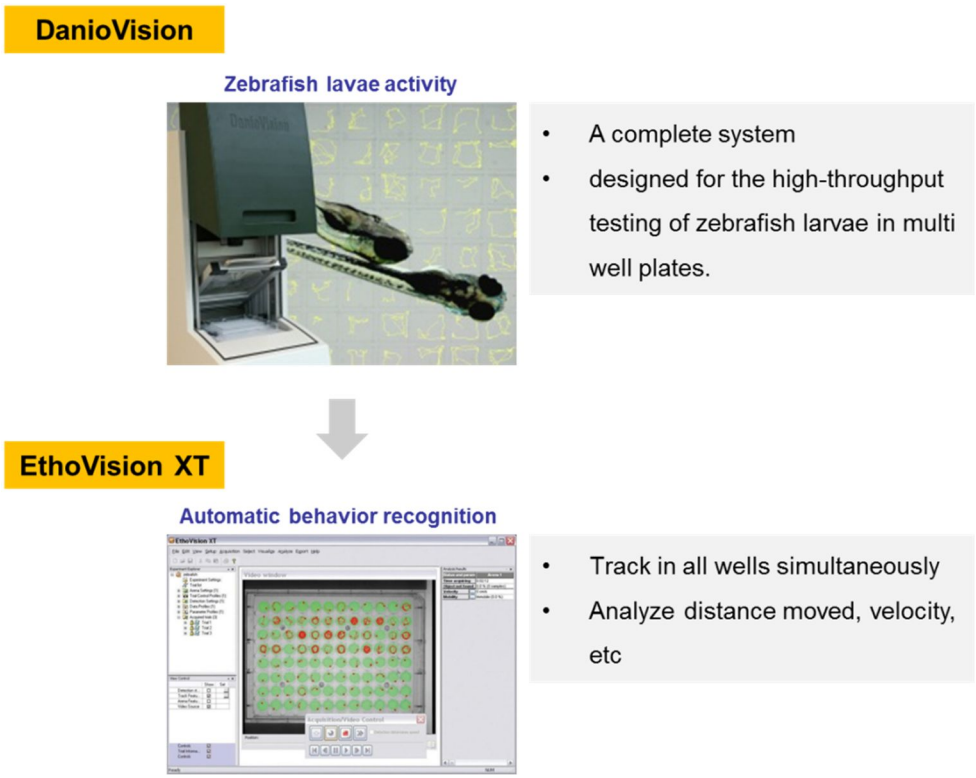


Figure 8. Zebrafish larvae tracking systems

DanioVision is an innovative system including an observation chamber, that allows for the tracking of up to 96 individuals simultaneously and EthoVision XT software on a computer.

Table 3. Primers and morpholino antisense olignucleotides

akap12α NCBI Reference Sequence: **NM_001281788.1**	
***akap12α* cloning primers**	Forward: 5'-ATG GGA GCG ACA CCA TCC G-3' Reverse: 5'-TGA GGC CTG TCT TCT GTT GA-3'
akap12β NCBI Reference Sequence: **NM_001098184.2**	
***akap12β* cloning primers**	Forward: 5'-ATG CTT GGG ACA ATA ACT CTA ACA G-3' Reverse: 5'-TGA GGC CTG TCT TCT GTT GA-3'
Probe primers	
NCBI Reference Sequence: **NM_001281788.1**	***akap12α*** Forward: 5′-ATG GGA GCG ACA CCA TCC GTG-3′ Reverse: 5′-TCA TTC AGC CGG TGG GTT CTC CT-3′
NCBI Reference Sequence: **NM_001098184.2**	***akap12β*** Forward: 5′-ACA TCA TAG AGA AAG AGA GG-3′ Reverse: 5′-CTG TTA GAG TTA TTG TCC CA-3′
Morpholinos *akap12*	
akap12α	Splice blocking MO: 5'-TAC CTT GCC ATC TGC GGT TTC TCC A-3'

akap12β	Translation blocking MO: 5'-CAC GGA TGG TGT CGC TCC CAT GA-3' Splice blocking MO: 5'-TCT TAC CTG TTA GAG TTA TTG TCC C-3' Translation blocking MO: 5'-CTG TTA GAG TTA TTG TCC CAA GCA T-3'

RESULTS

1. Depletion of *akap12* leads to defect in trunk morphology

Because two isoforms of *akap12*, *akap12α* and *akap12β*, are expressed in zebrafish, two different MOs (splice- and translation-blocking) with demonstrated efficacy were used to knock down each isoform (Kwon et al., 2012). Both types of MO produced the same phenotype, and the latter was used in these experiments except where otherwise noted.

Embryos injected with *akap12* MOs showed distinct morphological abnormalities. Only 52.7% and 62.0% of *akap12α* and *akap12β* morphants, respectively, had normal trunk morphology compared to 93.2% of control embryos, and many had short, curved body axes (43.1% and 34.9%, respectively) by 27 hpf (Fig. 9). The severity of the defect in trunk was significantly increased in both MOs treated embryos (Fig. 10).

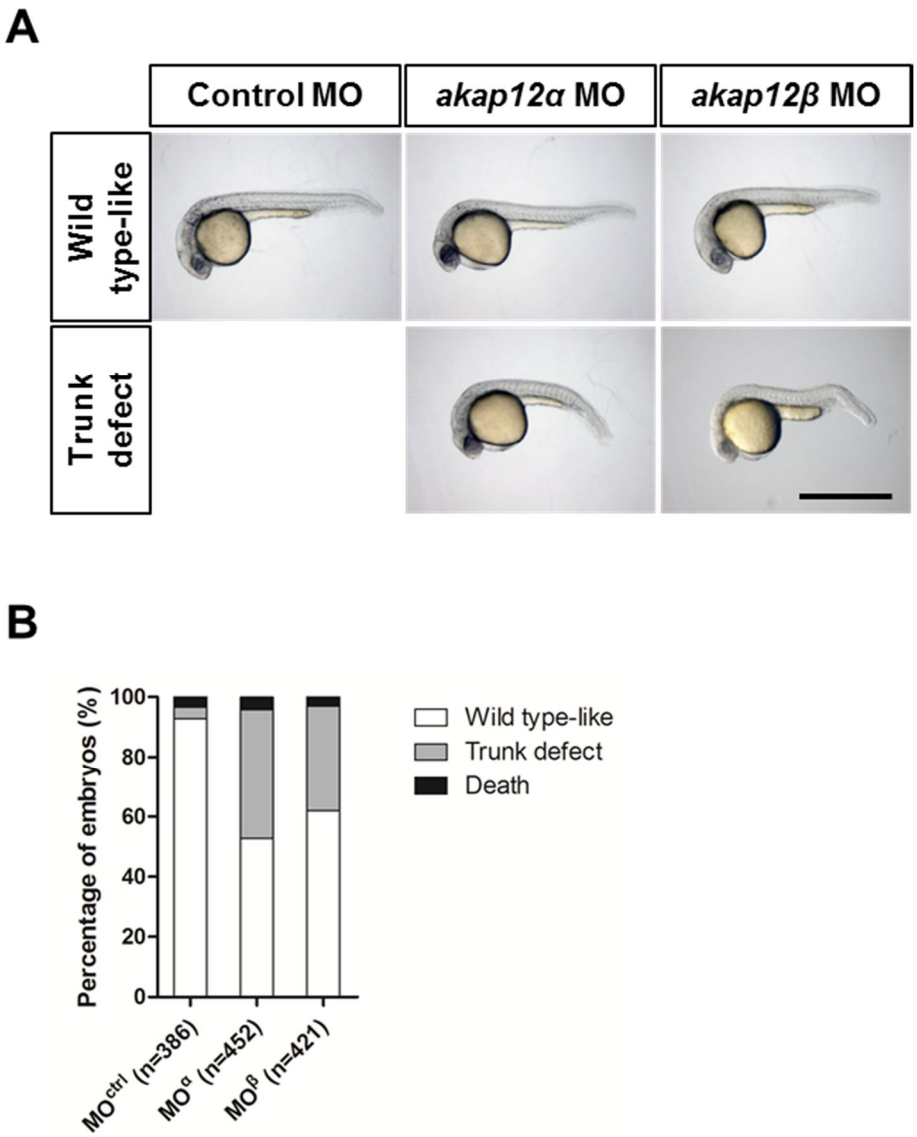


Figure 9. Embryos injected with *akap12* MOs showed distinct morphological abnormalities

(A) Lateral view of control embryos with normal development at 27 hpf. (Bottom) Lateral view of *akap12*-deficient embryos with abnormally curved trunks at 27 hpf. Scale bar = 0.5 mm. The right panel shows the percentage of abnormal embryos at 27 hpf.

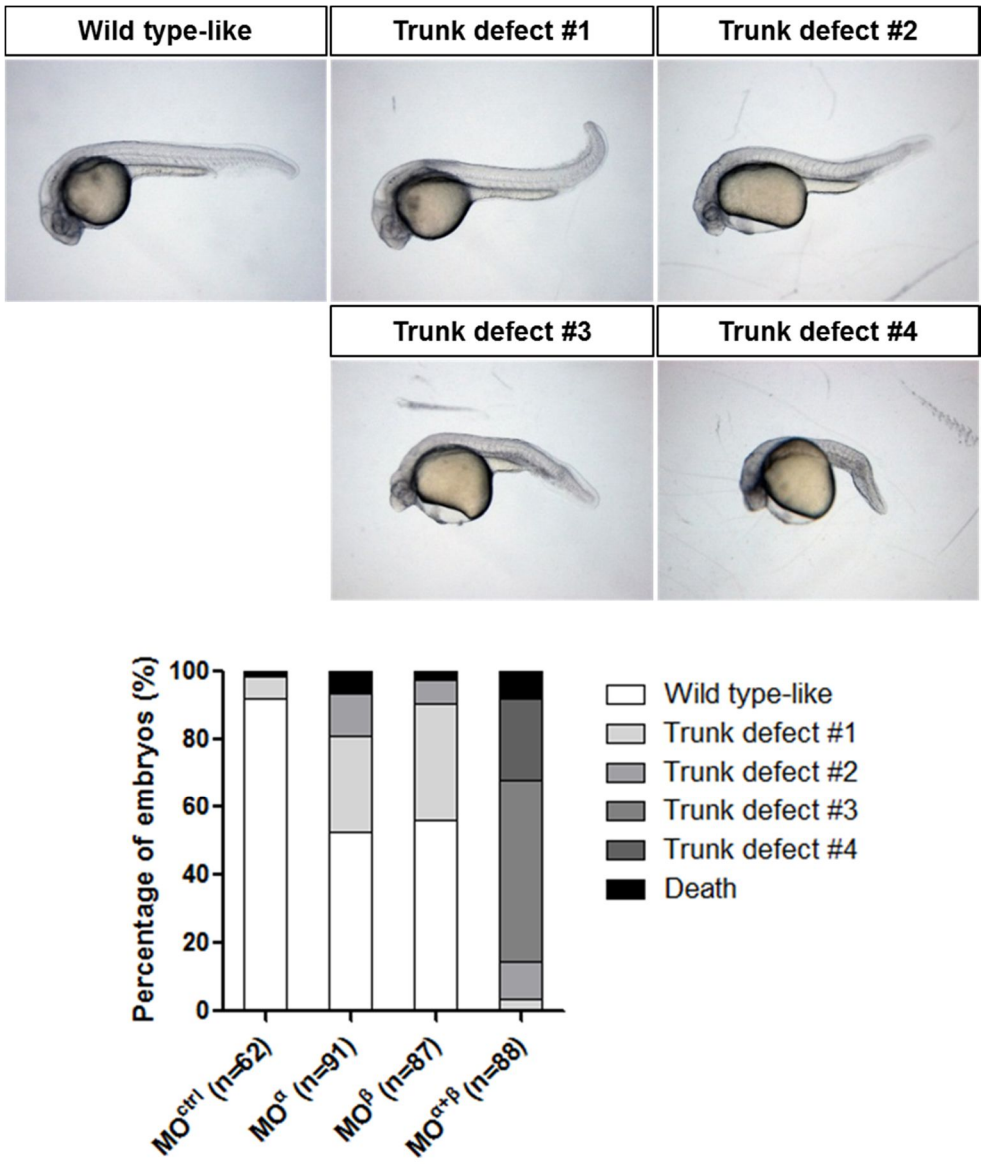


Figure 10. Trunk defects by knockdown of *akap12* using antisense MOs

(Top) Several types of trunk defects were induced in *akap12* morphant embryos by microinjection of *akap12* MOs (lateral view). All types of morphants showed the most defects in the body curvature and short body length. As the number for the type increases the defects become more severe. (Bottom) show a graphic depiction of the percentage of embryos with five types of phenotypes 27 hpf after *akap12* MOs injection.

2. *Akap12* depletion leads to abnormal locomotor behavior

Akap12 morphant embryos with normal trunks were no obvious morphological differences in the trunk region above the yolk sac extension (Fig. 11), and nonetheless showed decreased locomotor activity, as evidenced by delayed hatching and poor touch responsiveness. These results suggest that *akap12* is required for early locomotor behavior. Embryos with a normal trunk phenotype were selected for analysis in subsequent experiments to assess the effect of morphology, especially trunk musculature, on locomotion.

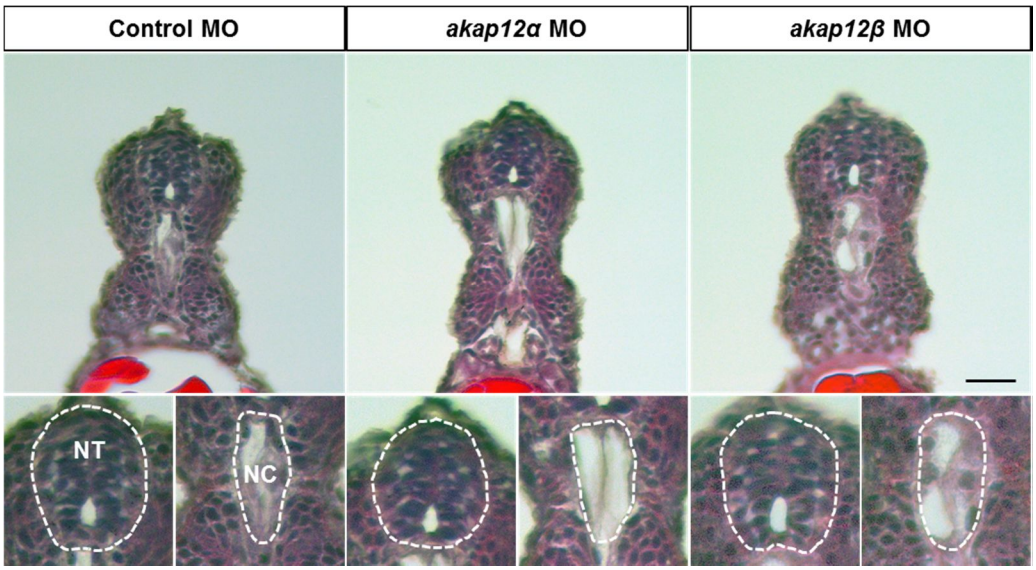


Figure 11. Examination of trunk tissues by hematoxylin and eosin (H&E) staining

Embryos at 24 hpf after control and *akap12* MO injection were sectioned at the trunk region above the yolk sac extension. Tissues were stained with H&E. NT, Neural Tube; NC, Notochord. Scale bar = 20 μm.

3. Spontaneous coiling contractions

Locomotion defects in *akap12* morphants were examined by quantifying the number of spontaneous coiling contractions (*i.e.*, alternating side-to-side tail contractions per minute) at different time points during early developmental stages. Spontaneous coiling was observed starting at 19 hpf, and it reached a peak at 22 hpf and decreased by 27 hpf (Fig. 12). *Akap12* morphants showed a nearly 2-fold reduction in activity by the time of onset of spontaneous coiling in controls and decreased frequency of contractions at all time points (19–27 hpf), with the peak frequency occurring at 23 hpf (Fig. 12). These results indicate that the reduced motility in a*kap12* morphants likely resulted from a primary defect (*i.e.*, structural or functional) in muscle development, rather than a progressive deterioration of normal musculature.

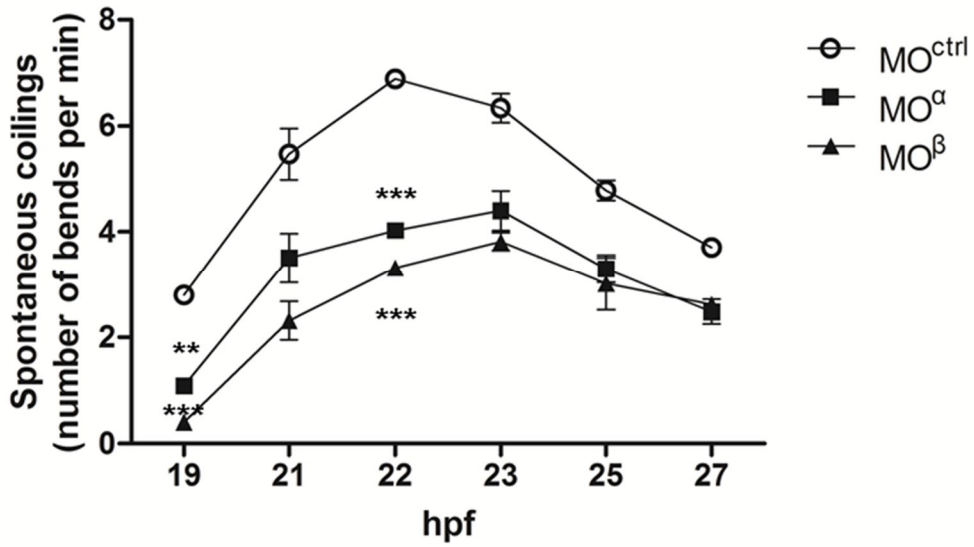


Figure 12. *Akap12* morphants exhibit the reduced spontaneous contractions

Spontaneous coiling contractions were recorded once every hour between 19 and 27 hpf. **P < 0.01, ***P < 0.001 compared to control embryos (n = 60).

4. Touch response

Given the abnormal locomotor activity exhibited by *akap12* morphants, two specific types of locomotion, touch response and free-swimming activity, were analyzed by recording the activity of injected embryos. To determine whether touch-induced response was affected, we video-recorded the activity of controls and *akap12* morphants at 27 hpf, when they responded to touch stimulus with vigorous coiling (Fig. 13). Similar to the effects on spontaneous coiling, *akap12* MO injection decreased tail flip duration (Fig. 13); control embryos responded to touch with continual tail flips that alternated between left and right, whereas *akap12* morphants reacted with prolonged, less coordinated muscle contractions. This reduction in responsiveness was also observed at later time points; at 36 and 48 hpf; *akap12*-depleted embryos had lower rates of escape response to touch stimuli than controls (Fig. 14). *Akap12* morphants also had slower swim speeds and swam over shorter distances than controls (data not shown).

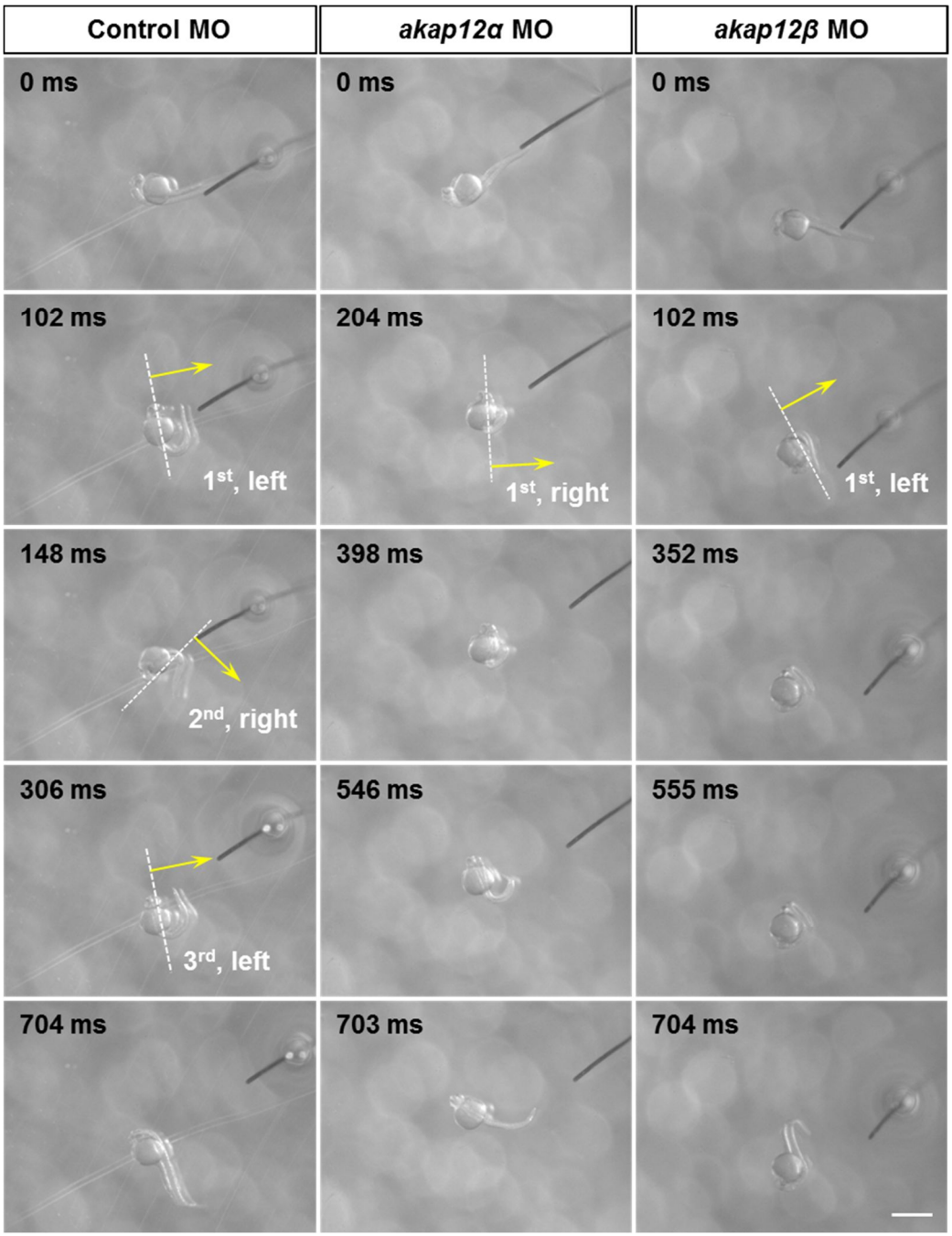

Control MO
akap12α MO
akap12β MO
0 ms
0 ms
0 ms
102 ms
1st, left
204 ms
1st, right
102 ms
1st, left
148 ms
2nd, right
398 ms
352 ms
306 ms
3rd, left
546 ms
555 ms
704 ms
703 ms
704 ms

Figure 13. *Akap12* depletion leads to reduced touch response at 27 hpf

Selected frames from high-speed video recordings of control- and *akap12* MO-injected embryos in response to a touch with a hair probe; *akap12* morphants showed a defect in motility characterized by prolonged muscle contractions. Time (ms) is indicated in each image. Scale bar = 200 µm.

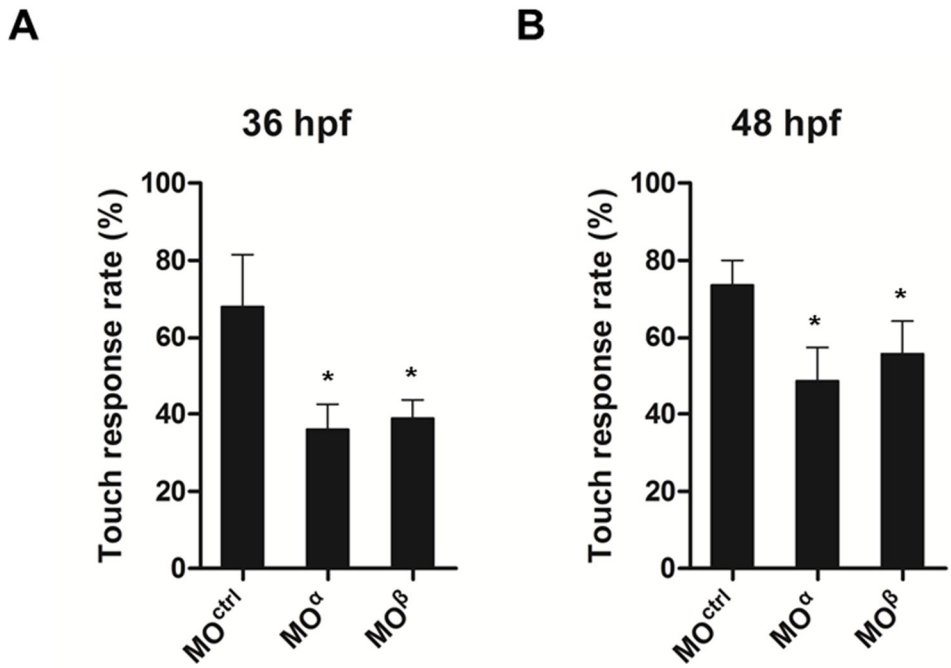


Figure 14. *Akap12* depletion leads to reduced touch response at 36 and 48 hpf

Touch response was analyzed at 36 (A) and 48 hpf (B) (n = 72). The number of embryos exhibiting body bending or swimming behavior after the initial touch was scored. ***P < 0.001 compared to control embryos.

5. Free-swimming activity

Free-swimming activity, which is independent of any touch stimuli, was also evaluated. Larvae in 24-well plates were subjected to a 20-min light period and their swimming tracks were recorded (Fig. 15). The swimming speed was decreased in morphants compared to controls (1.4 and 1.5 mm/s in *akap12α* and *akap12β* morphants, respectively, vs. 2.3 mm/s in controls) (Fig. 15). Moreover, the total distance traveled was also reduced in *akap12* MO-injected embryos (1,649 ± 136.9 and 1,748 ± 163.4 mm in *akap12α* and *akap12β* morphants, respectively vs. 2,697 ± 102.4 mm in controls) (Fig. 15). Together, these data demonstrate that *akap12* knockdown causes defects in locomotor activity in embryos, suggesting a critical role in muscle development.

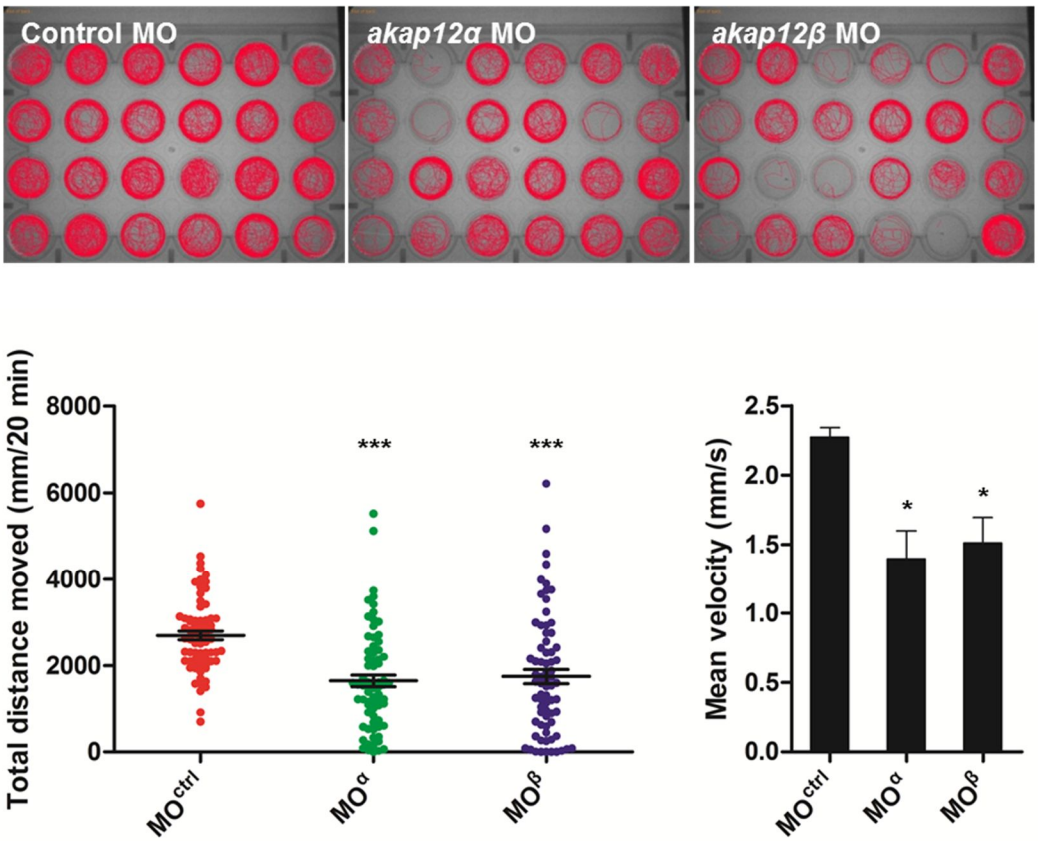


Figure 15. *Akap12* depletion leads to reduced free-swimming activity

(Top) Larvae swimming in a 24-well plate was monitored for 20 min at 120 hpf; a representative plot of their movement is shown. (Bottom) Free-swimming activity was measured by calculating the total distance traveled and mean velocity (n = 72). *P < 0.05, ***P < 0.001 compared to control embryos.

6. *Akap12* is expressed in adaxial cells during somitogenesis

The spatiotemporal expression pattern of *akap12* was examined in early embryos to determine its relationship with muscle development. *Akap12α* was initially expressed following gastrulation and the transcript levels increased during the segmentation period (Fig. 16). The *β* isoform was expressed earlier, first appearing at the sphere stage. The isoforms were expressed simultaneously during segmentation (Fig. 16).

Akap12 transcript expression was restricted to adaxial cells in the early segmentation period, as confirmed by *in situ* hybridization (Fig. 17). During later segmentation, *akap12* was expressed more diffusely throughout the myotomes (Fig. 17C); however, a cross-sectional view of an embryo at the same stage revealed that the *akap12* transcript was enriched in the most lateral surface of the trunk and horizontal myoseptum (Fig. 17D), which are adaxial cell derivatives. Probes specific to each isoform showed that *akap12α* and *akap12β* mRNA was specifically expressed in adaxial cells at the 12-somite stage (Fig. 18). The corresponding sense RNA probes produce no signal under the same hybridization and detection conditions of the antisense probes (Fig. 19). Moreover, *akap12*

mRNA was localized exclusively to F59-positive adaxial cells (Fig. 20). The expression of both isoforms in adaxial cells during segmentation provides further evidence that *akap12* is involved in muscle development.

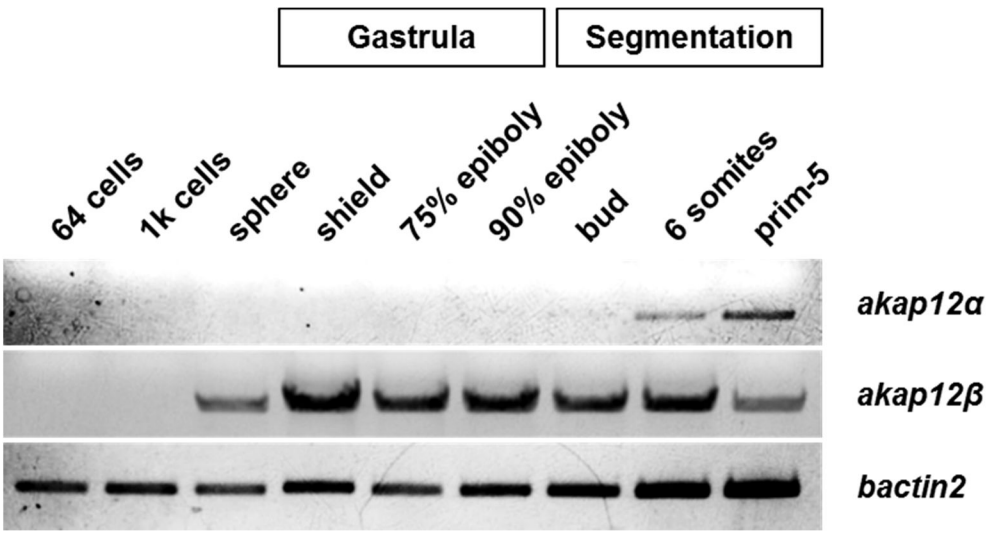


Figure 16. RT-PCR analysis of *akap12* mRNA during early development

Expression of *akap12* and *bactin2* was determined by reverse transcriptase PCR from 64-cell to 24 hpf embryos.

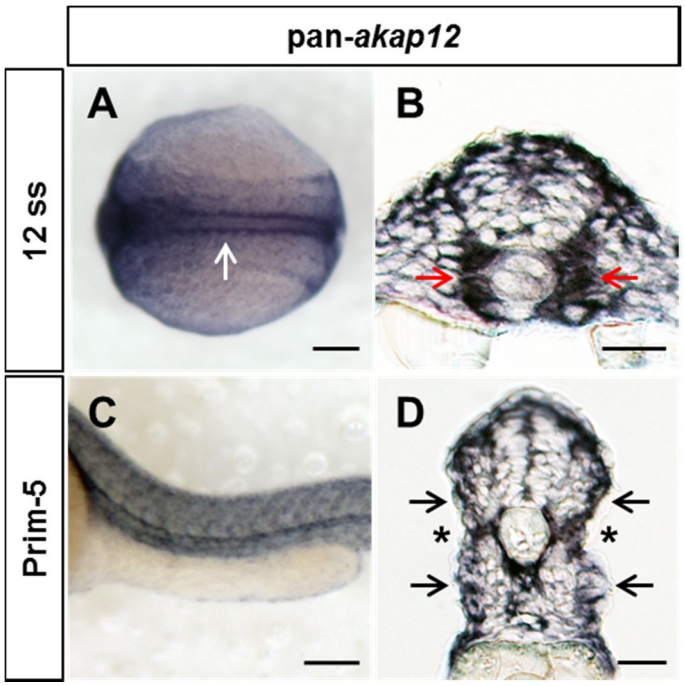


Figure 17*. In situ* hybridization using a pan-*akap12* antisense RNA probe

(A) At the 12-somite stage, *akap12* expression was restricted to adaxial cells (white arrow). (B) A cross-sectional view of a 12-somite stage embryo shows adaxial cells adjacent to the notochord (red arrows). (C) At 24 hpf, *akap12* was diffusely expressed in the myotome. (D) A cross-sectional view of a prim-5 stage embryo shows *akap12* expression in the outer layer of the myotome (black arrows), including the horizontal myoseptum (*). Scale bars = 100 μm (A, C)

and 20 μm (B, D).

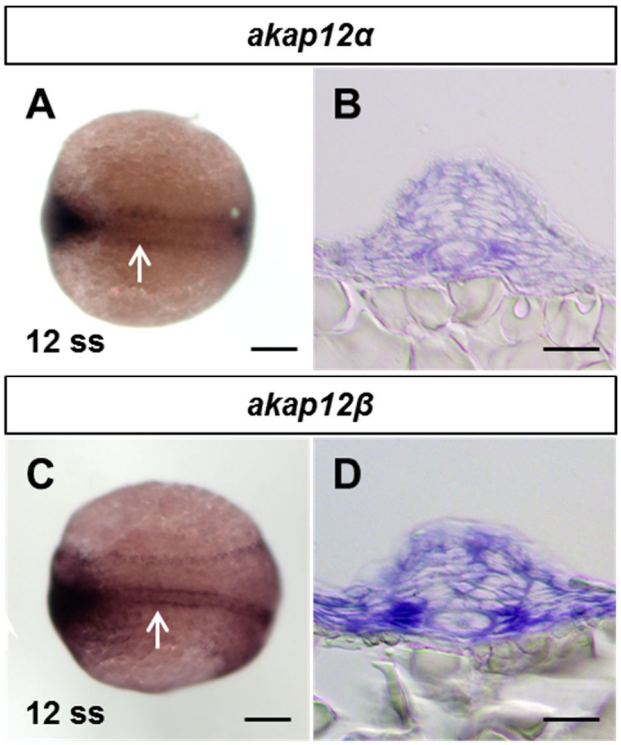


Figure 18. *In situ* hybridization using isoform specific RNA probes

(A, B) *Akap12α* and (C, D) *akap12β* isoforms were expressed in adaxial cells at the 12-somite stage (white arrows). Scale bars = 100 μm (A, C) and 20 μm (B, D).

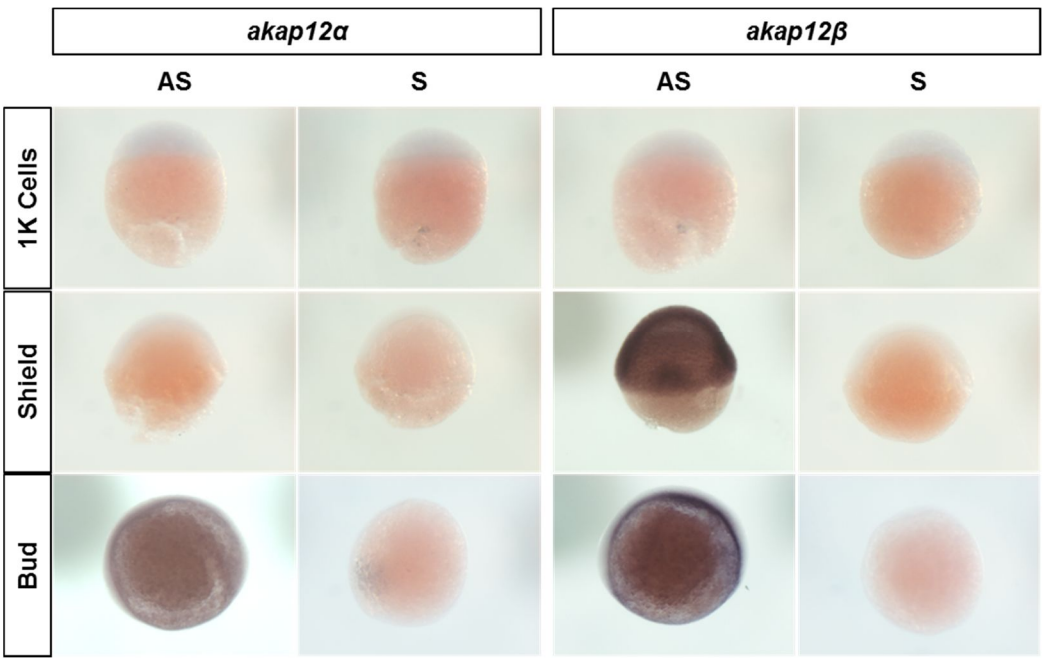


Figure 19. Whole-mount *in situ* hybridization analysis of zebrafish embryos using antisense and sense probes for two *akap12* isoforms

Spatial expression of zebrafish *akap12α* and *akap12β* at the 1,000-cell, shield, and bud stages. Embryos hybridized with the sense and antisense probes are shown.

Figure 20. Combined immunohistochemistry and *in situ* hybridization in trunk sections

Double labeling of cross section of the trunk with a pan-*akap12* riboprobe and F59 antibody shows that *akap12* was expressed in adaxial cells.

7. *Akap12* is required for slow muscle organization

To examine the role of *akap12* in skeletal muscle development, the distribution of slow muscle cells was analyzed at 27 hpf in the *Tg (9.7kb smyhc1:GFP)*^{i104} transgenic line, in which GFP is expressed under the control of the *slow myosin heavy chain 1* gene promoter (Elworthy et al., 2008). Slow muscle cells were grossly disarrayed and misaligned in *akap12* morphants at 27 hpf compared to control MO-injected embryos (Fig. 21). A rescue experiment was performed to confirm that the disorganized muscle phenotype was caused by knockdown of *akap12*. Co-injection of zebrafish *akap12* mRNA together with *akap12* splice-blocking MOs led to attenuation of the disorganized slow muscle phenotype; *akap12α* mRNA restored the muscle defects induced by *akap12β* depletion, and vice versa (Fig. 21), indicating that the two isoforms have complementary functions in slow muscle development. We observed that knockdown of both *akap12* isoforms gave a more severe phenotype than that observed in embryos individually injected with each MO (Fig. 22). Embryos that were co-injected with both MOs exhibited a highly penetrant, disarrayed and misaligned phenotype of slow muscle at the lateral extent of the myotome. Moreover, these embryos showed

significantly increased the number of gaps in the lateral palisade of slow muscle fibers (Fig. 22). These severe muscle defects in double-knockdown embryos may be dependent on the amount of Akap12 in the early developing zebrafish. Therefore, knockdown of each *akap12* isoform can result in a muscle defect with a less severe phenotype. These results confirm that the observed abnormal muscle development in morphants specifically results from a loss of *akap12* function.

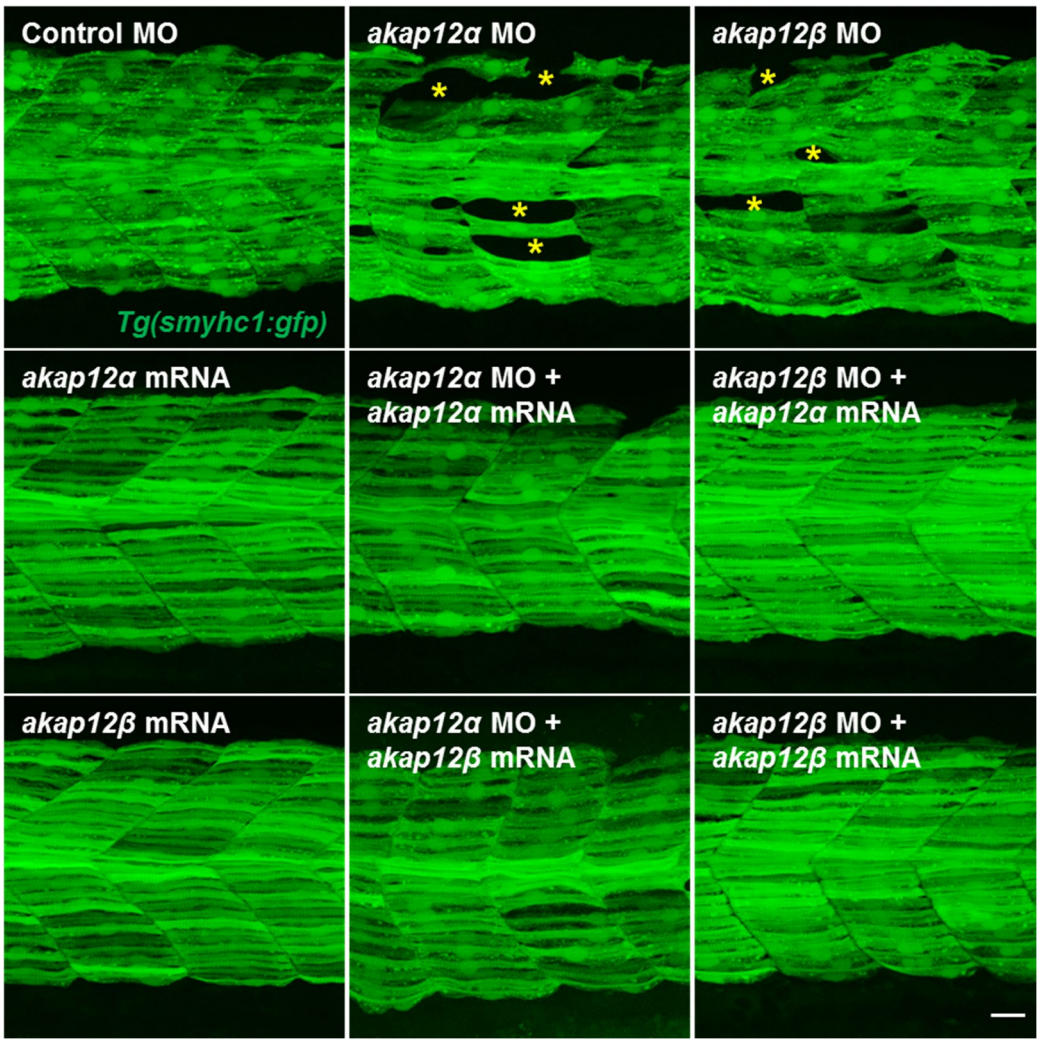


Figure 21. Loss of *akap12* disrupts slow muscle patterning

Lateral views of myotomal segments of *Tg(9.7kb smyhc1:gfp)*^{i104} embryos at 27 hpf. Slow muscle organization in *akap12* morphants is disrupted, with large gaps between fibrils (*). Injection of *akap12α*

and *akap12β* mRNA were showed in the left, middle and the left, bottom panel, respectively. There were no morphological differences between *akap12* mRNA-injected and control embryos. Co-injection of *akap12* mRNA rescued the phenotype caused by either splice-blocking morpholino.

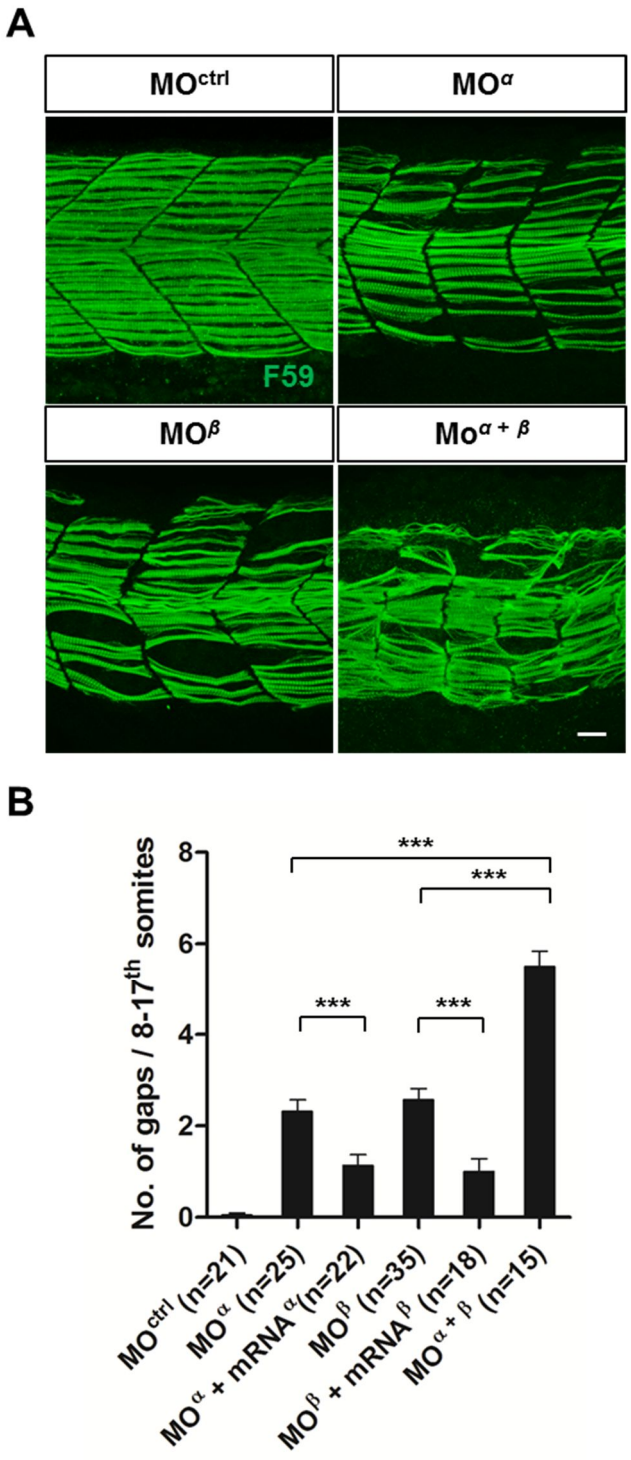

A
MO^ctrl
MO^α
F59
MO^β
Mo^α + β
B
No. of gaps / 8-17th somites
8
6
4
2
0
***
***
***
***
MO^ctrl (n=21)
MO^α (n=25)
MO^α + mRNA^α (n=22)
MO^β (n=35)
MO^β + mRNA^β (n=18)
MO^α + β (n=15)

Figure 22. Abnormal gaps in the lateral palisade of slow muscle fibers in *akap12* morphants

Knockdown of both *akap12* isoforms exhibit more severe phenotype compared to each MOs injected embryos. (A) Lateral views of 3D reconstructed myotomal segments of F59 stained embryos at 27 hpf. Co-injection of both MOs showed that the numbers of gaps are significantly increased compared to each MOs injected embryos. Scale bar = 20 μm. (B) Gap numbers were counted and their mean ± SEM values were presented. *** depicts a statistically significant difference ($p < 0.001$).

8. *akap12* morphants have normal fast muscle fibers

The effect of *akap12* on the differentiation of the fast muscle cells was assessed using antibody against fast muscle myosin fibers and phalloidin. However, there were no differences between morphants and controls in fast muscle fiber morphology, which had typical multinucleation, elongated form, and diagonal arrangement (Fig. 23). Thus, *akap12* is involved in slow but not fast muscle fiber development in zebrafish.

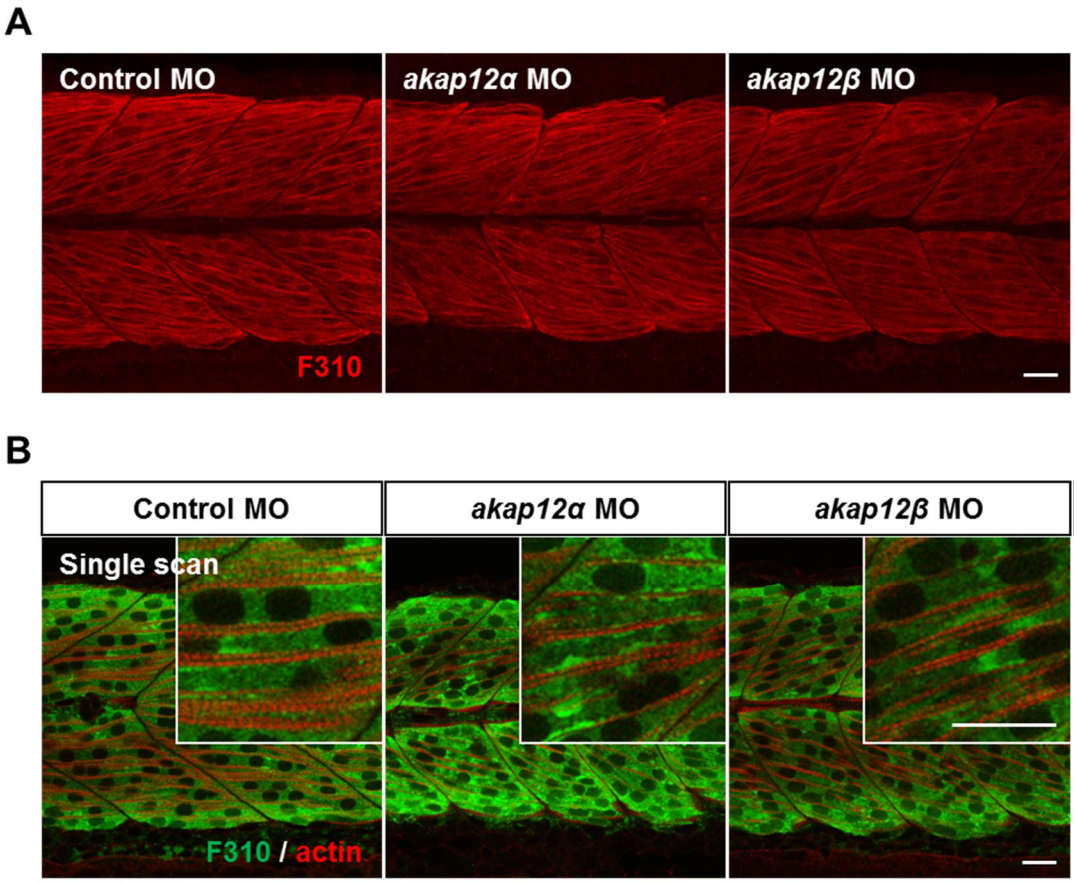


Figure 23. Immunostained images of control and *akap12* morphants at 27 hpf

(A) Confocal image stacks of fast myosin heavy chain immunoreactivity in 27 hpf embryos. (B) Single confocal scans of staining for fast muscle myosin filament (green), and actin (phalloidin; red). Scale bar = 20 μm.

9. *Akap12* is required for slow muscle cell migration

To determine whether the misalignment of slow fibers in *akap12* morphants resulted from the defective migration of slow muscle cells, the myotome was partially reconstructed from image stacks acquired by confocal microscopy. The three-dimensional rendering showed that many slow muscle cells failed to reach the lateral layer of the myotome in the *akap12*-depleted embryos and were located instead in the middle of the migratory trajectory, between somites 8 and 17 (Fig. 24). We also tested the expression of prox1, which is important for the terminal differentiation of the slow fibers, using an anti-prox1 antibody. The prox1-positive slow muscle nuclei were localized in the medial region of the myotome when compared with the control MO-injected embryos (Fig. 25, arrowheads); 31.6% and 43.6% of *akap12α* and *akap12β* morphants, respectively, showed this delayed migration, which was not seen in control embryos (Fig. 26). Moreover, the gaps which were seen in the lateral palisade of slow muscle fibers were generated by non-migratory slow muscle cells because they were in the medial focal plane of the same region (Fig. 27).

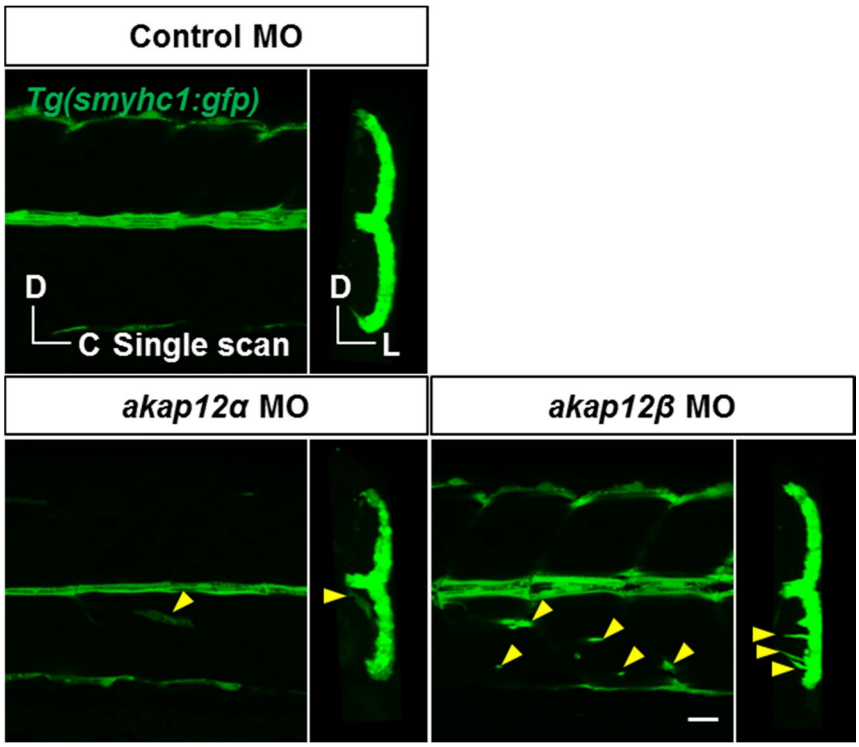


Figure 24. Loss of *akap12* function leads to decreased slow muscle cell migration

Three-dimensional reconstruction of single confocal image stacks showing lateral and cross-sectional views of 27 hpf embryos. Knockdown of *akap12* perturbed slow muscle cell migration (arrowheads). D, Dorsal; C, Caudal; L, Lateral. Scale bar = 20 μm.

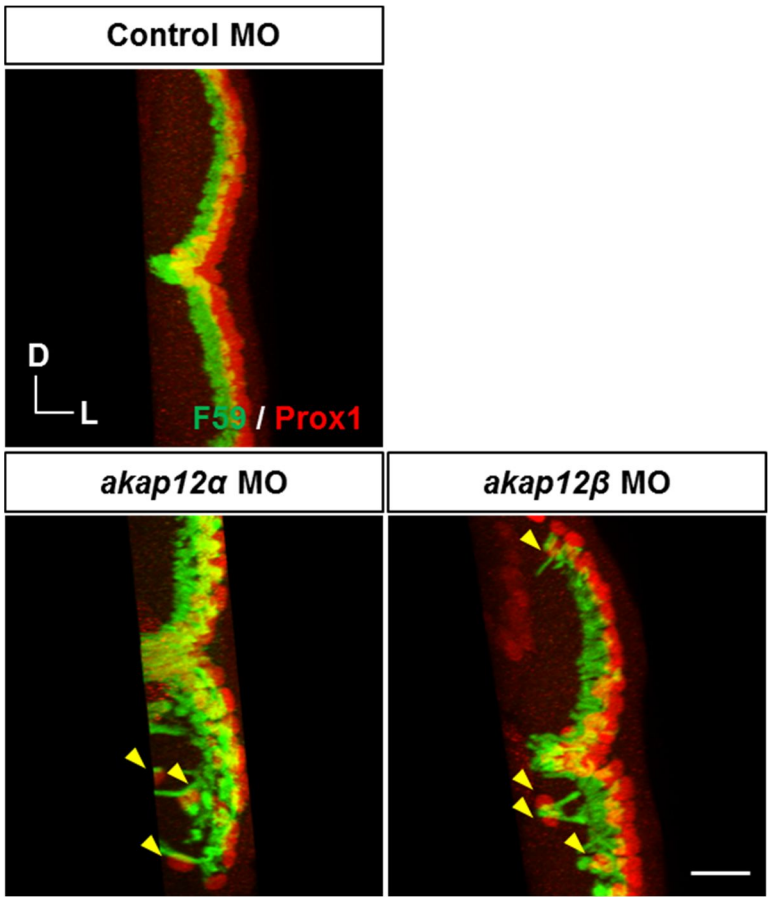


Figure 25. Whole-mount immunolabeling in control and *akap12* morphants of slow muscle myosin filament (F59, green) and nuclei of slow muscle (prox1, red)

Slow muscle cells fail to migrate to the lateral surface of the myotome as determined by the location of the nuclei (arrowheads). D, Dorsal; L, Lateral. Scale bar = 20 μm.

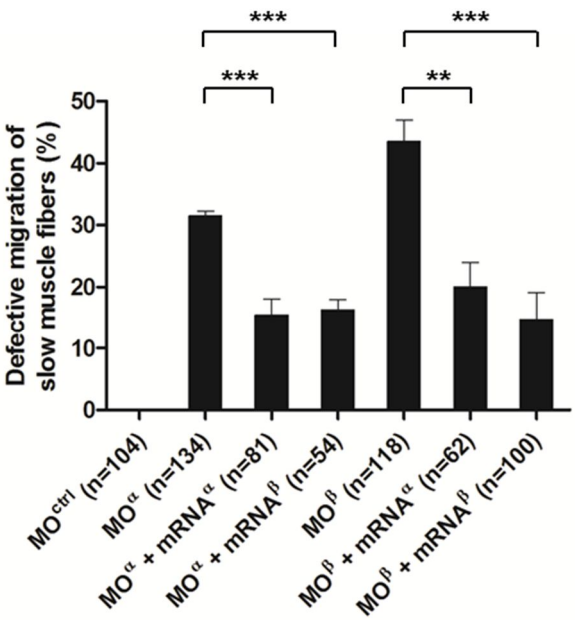


Figure 26. Percentage of 27 hpf embryos showing abnormal migration of slow muscle cells

**P < 0.01, ***P < 0.001 compared to the MO-treated embryos.

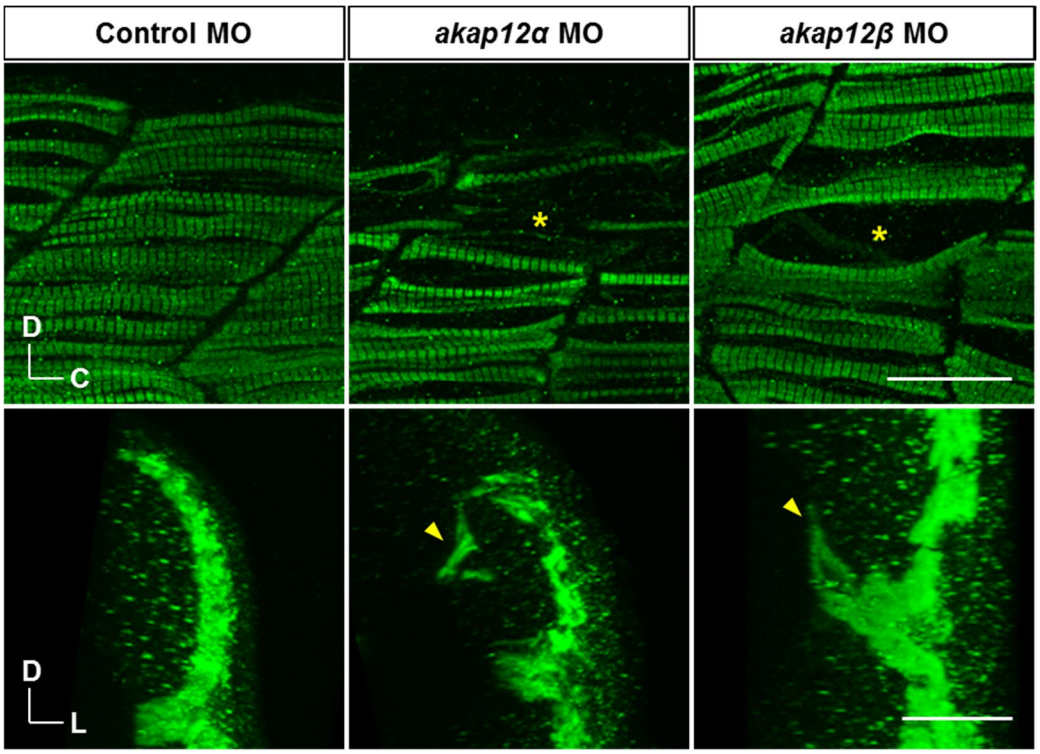


Figure 27. Slow fibers fail to migrate correctly, leading to gaps in the lateral surface of myotome

Embryos were incubated with an antibody that recognizes slow MyHC (F59) at 27 hpf. Upper panels are side views of 3D reconstructed slow muscle cells; lower panels are projections reconstructed 90° perpendicular to the upper data to generate a transverse view. Arrowheads indicate non-migratory slow cells. Scale bars = 20 μm. D, Dorsal; C, Caudal; L, Lateral.

10. *Akap12* is required for muscle pioneer cell migration

Some muscle pioneers were located distant from the notochord in the *akap12* morphants, indicating that they had aberrantly migrated laterally to the myotome (Fig. 28). To label muscle pioneer cells specifically, we used an anti-engrailed antibody in the transgenic embryos. We found that the nuclei of muscle pioneers (arrowheads and dotted line) in *akap12* morphants migrated obviously to the lateral region of the myotome when compared to control embryos (Fig. 29); 16.6% and 30.3% of *akap12α* and *akap12β* morphants, respectively, showed defective migration of muscle pioneers compared to 0% in controls (Fig. 30). Knockdown using the translation-blocking MO produced the same effects (Fig. 31).

The migration defects of slow muscle cells and muscle pioneers were rescued by co-injection of *akap12* mRNA with *akap12* MOs (Fig. 26 and Fig. 30), whereas the normal organization of the muscle was also restored. Thus, *akap12* is required for correct migration of muscle cells, which determines the normal morphology of muscle fibers.

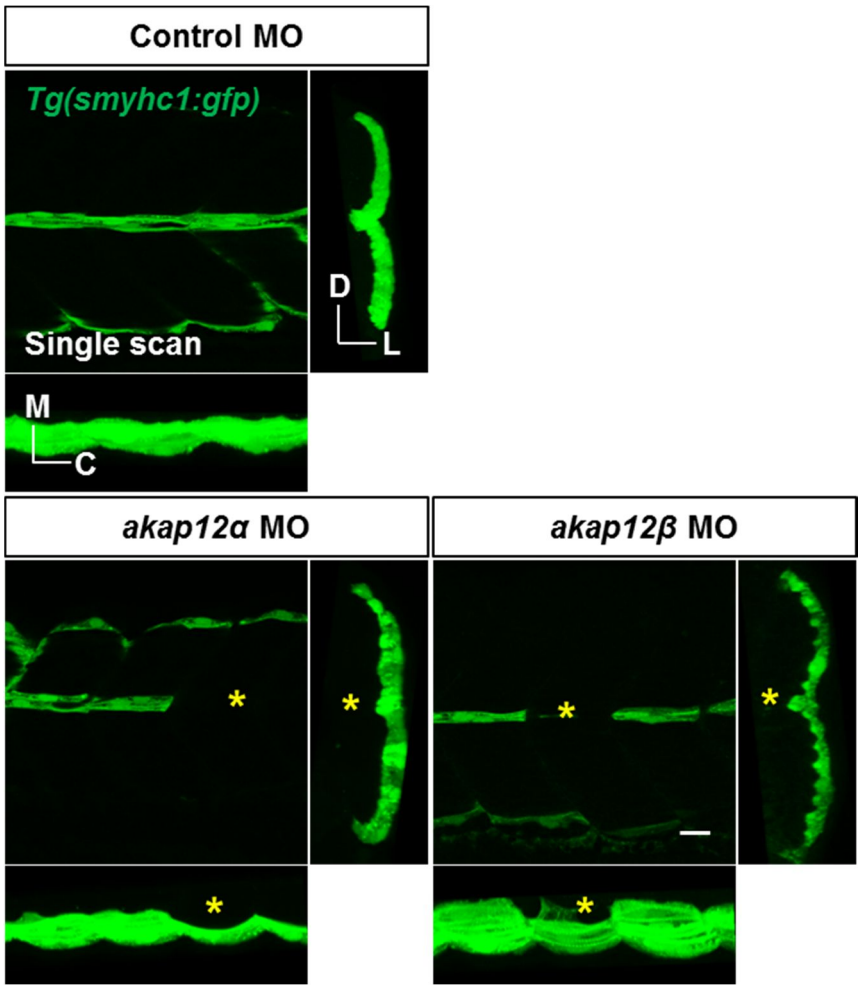


Figure 28. Loss of *akap12* function results in altered muscle pioneer migration

Knockdown of *akap12* promoted the migration of muscle pioneers to the surface of the myotome (*).D, Dorsal; C, Caudal; L, Lateral; M, Medial. Scale bar = 20 μm.

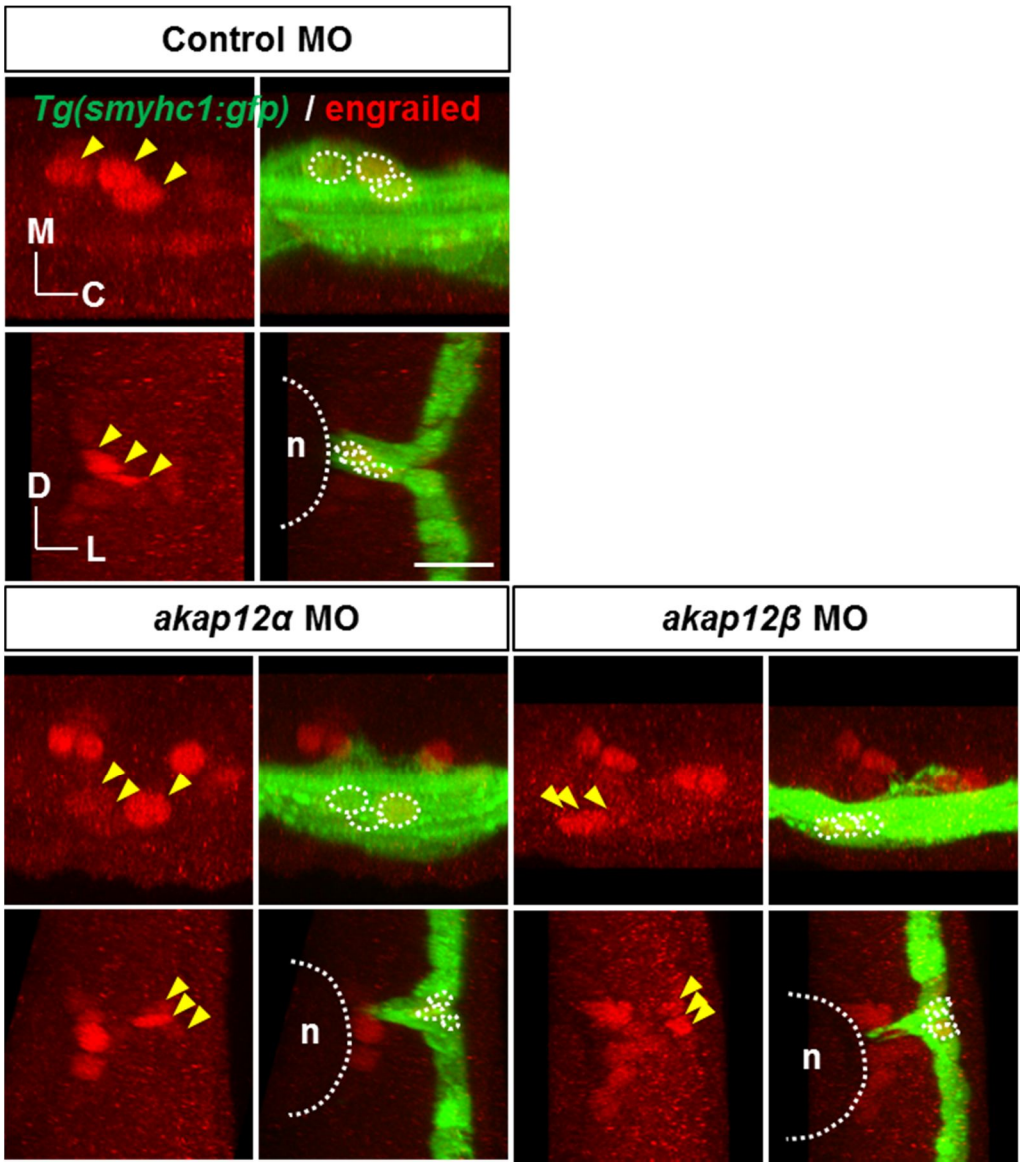


Figure 29. Whole-mount immunolabeling in control and *akap12* morphants of muscle pioneer cells (engrailed, red)

Embryos were incubated with an antibody that recognizes muscle pioneers (*engrailed*). The nuclei of muscle pioneers (dotted line and arrowheads) were adjacent to the lateral surface of the myotome in the *akap12* morphant embryos. D, Dorsal; C, Caudal; L, Lateral; M, Medial; n, notochord. Scale bar = 20 μm.

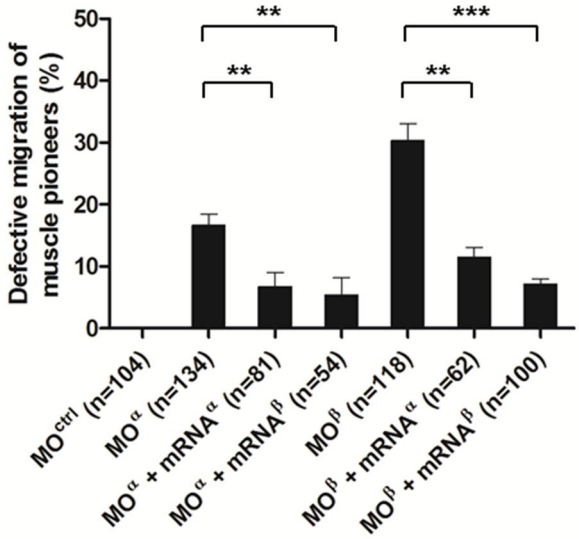


Figure 30. Percentage of 27 hpf embryos showing abnormal migration of muscle pioneers

**P < 0.01, ***P < 0.001 compared to the MO-treated embryos.

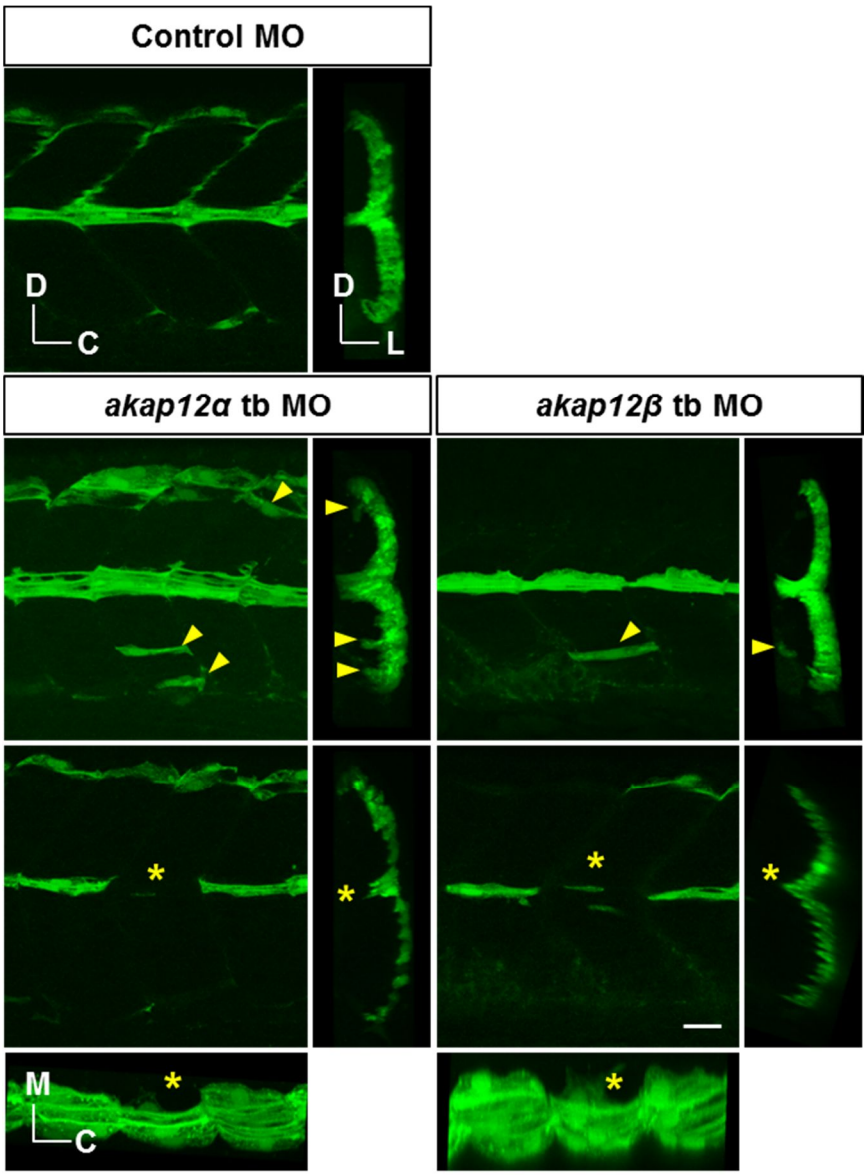


Figure 31. Injection of translation block (tb) morpholinos of *akap12* also results in abnormal migration of slow muscles and

muscle pioneers

Knockdown of *akap12* using tb-morpholinos perturbs slow muscles migration (arrow heads) and promotes the migration of muscle pioneers to the surface of the myotome (asterisks). Scale bar = 20 μm. D, Dorsal; C, Caudal; L, Lateral; M, Medial.

11. Hedgehog signaling is not altered in *akap12* morphants during early muscle differentiation period

We examined whether the differentiation of myofibers was normal in *akap12* morphants. The earliest event in the development of the slow fibers in zebrafish embryo is regulated by hedgehog signaling from the midline. To determine whether hedgehog signals were altered in *akap12* morphants, we examined the expression of *shha*, one of the major components in hedgehogs, in the *akap12* morphants. Labeling with a *shha* antisense RNA probe showed that expression of *shha* in *akap12* morphants is not affected at 18 ss (Fig. 32). Moreover, the expression of *myoD* was not also changed in *akap12* morphants (Fig. 33); the primal myogenic response to hedgehog signaling in adaxial cells is the induction of *myoD* expression.

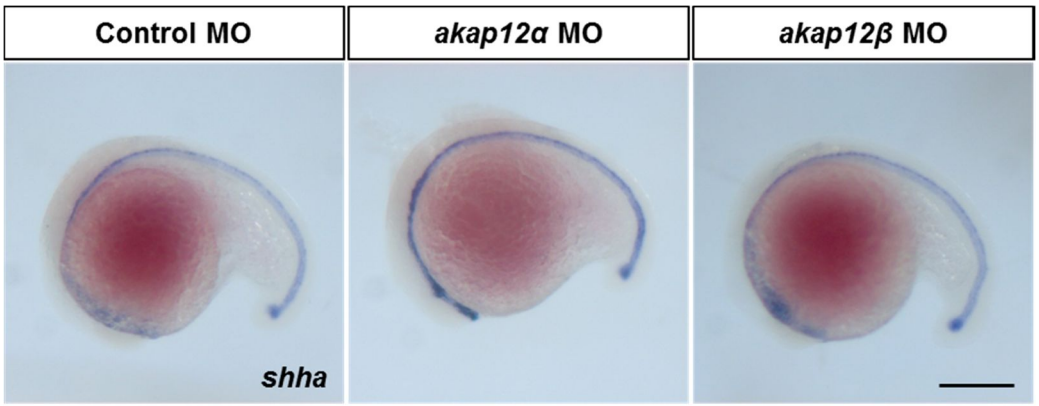


Figure 32. *In situ* hybridization using a *shha* antisense RNA probe

Expression of *shha* in *akap12* morphants are not affected at 18 ss.

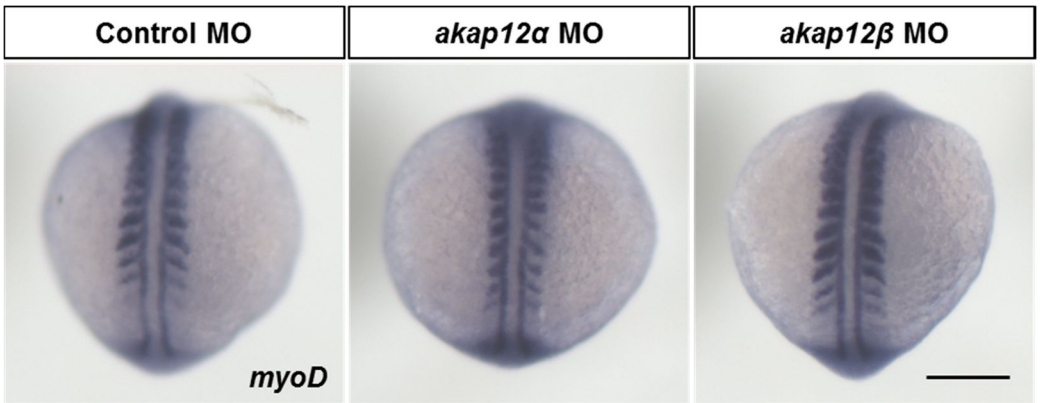


Figure 33. *In situ* hybridization using a *myoD* antisense RNA probe

Expression of *myoD* in *akap12* morphants are not affected at 14 ss.

12. Hedgehog signaling is not altered in *akap12* morphants during later muscle differentiation period

In a separate inductive pathway, hedgehog signaling acts to induce engrailed expression in the muscle pioneers and medial fast fibers. Thus we counted the number of engrailed positive cells and muscle pioneer cells; both are engrailed-positive, and muscle pioneer cells are double-positive for engrailed and prox1. We found that the distribution and number of engrailed positive cells were similar in *akap12* morphants (Fig. 34), and the number of cells double-positive for prox1 and engrailed was not changed (Fig. 35). Furthermore, slow muscle cells failed to reach the lateral surface of the myotome in the *akap12*-depleted embryos well expressed prox1 and didn't fuse with fast muscle fibers, indicating that their fate of the slow muscle fibers was not changed (Fig. 36). Collectively, our data suggest that knockdown of *akap12* causes muscle defects resulting from a direct effect within muscle cells and not an indirect effect from the notochord. Therefore, *akap12* regulates the correct migration of slow muscles and muscle pioneers in a cell-autonomous manner.

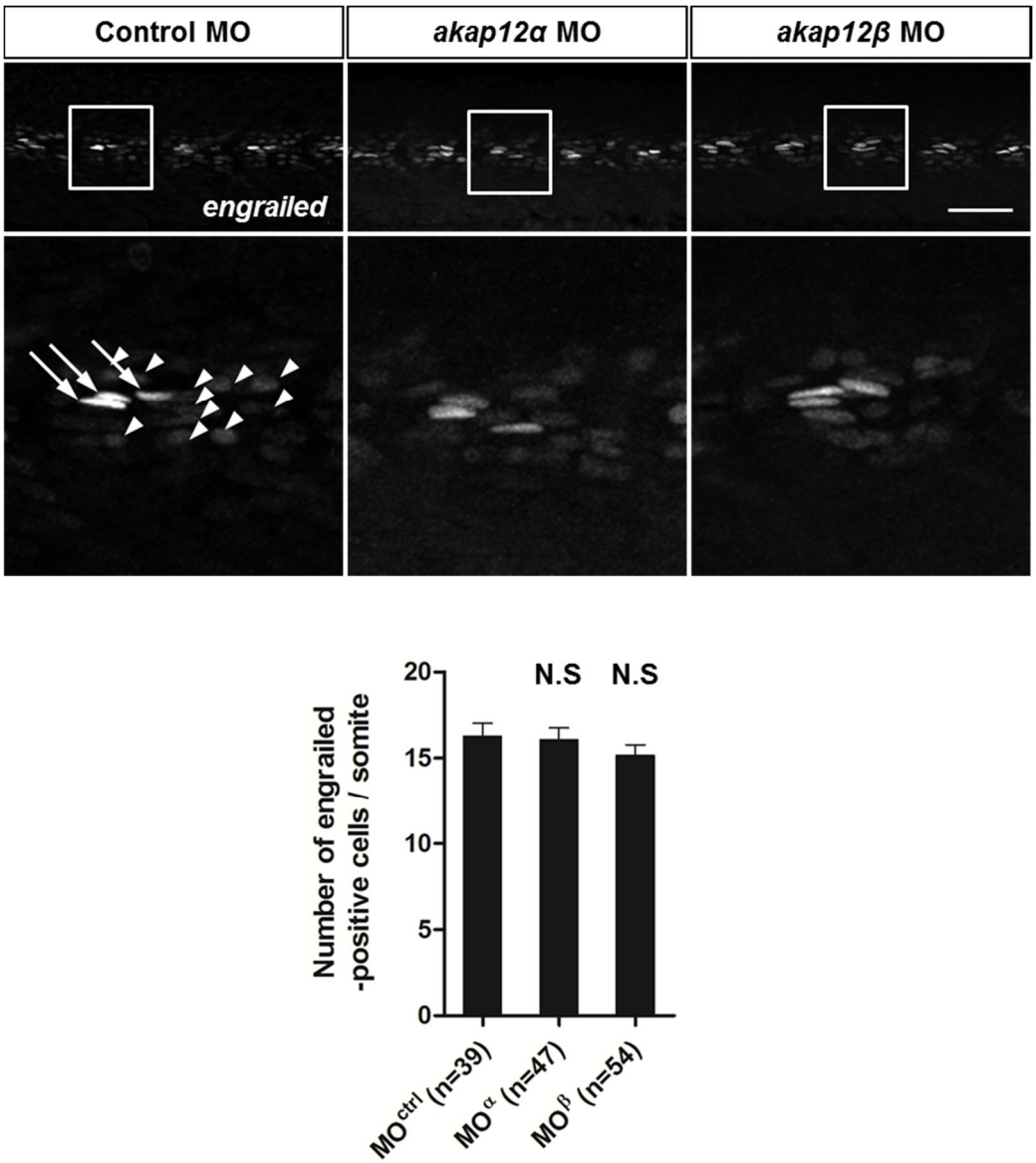
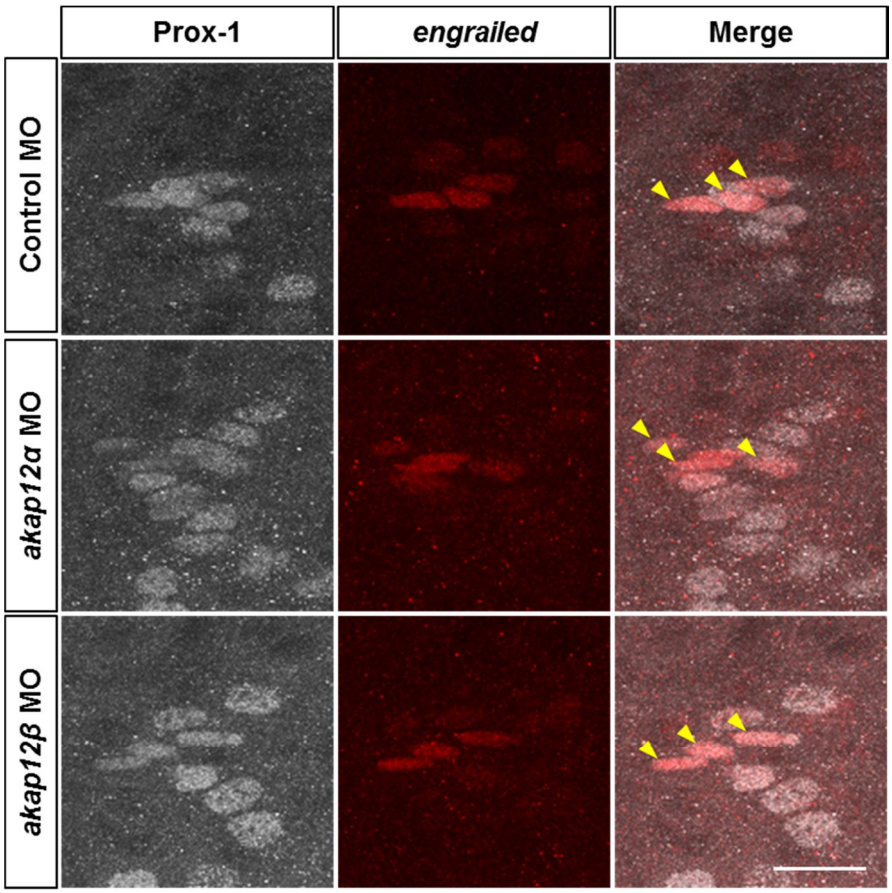


Figure 34. Confocal images of the engrailed-positive cells in the control or *akap12* morphants

(Top) Labeling with anti-engrailed at 27 hpf showed that the distribution and the number of engrailed-positive cells (arrows and arrowheads) in *akap12* morphants were similar to those in the control. (Bottom) Engrailed-positive cells were counted and the mean ± SEM values are presented. N.S = not significant. Scale bar = 20 μm.


Prox-1
engrailed
Merge
Control MO
akap12α MO
akap12β MO

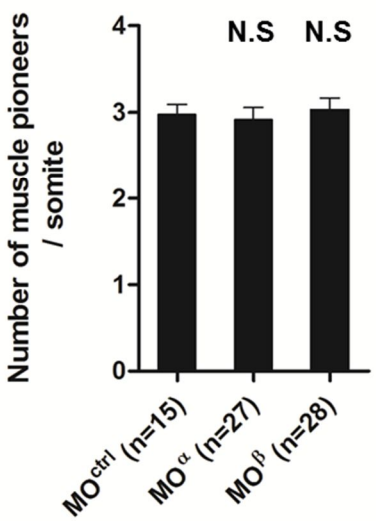

Number of muscle pioneers / somite
4
3
2
1
0
N.S
N.S
MOctrl (n=15)
MOα (n=27)
MOβ (n=28)

Figure 35. Confocal images of the muscle pioneer cells in the control or *akap12* morphants

(Top) The number of cells double-positive for Prox1 and engrailed did not change in *akap12* morphants. (Bottom) Muscle pioneer cells in control and *akap12* morphants were visually counted, and the mean ± SEM values are presented. N.S = not significant. Scale bar = 20 μm.

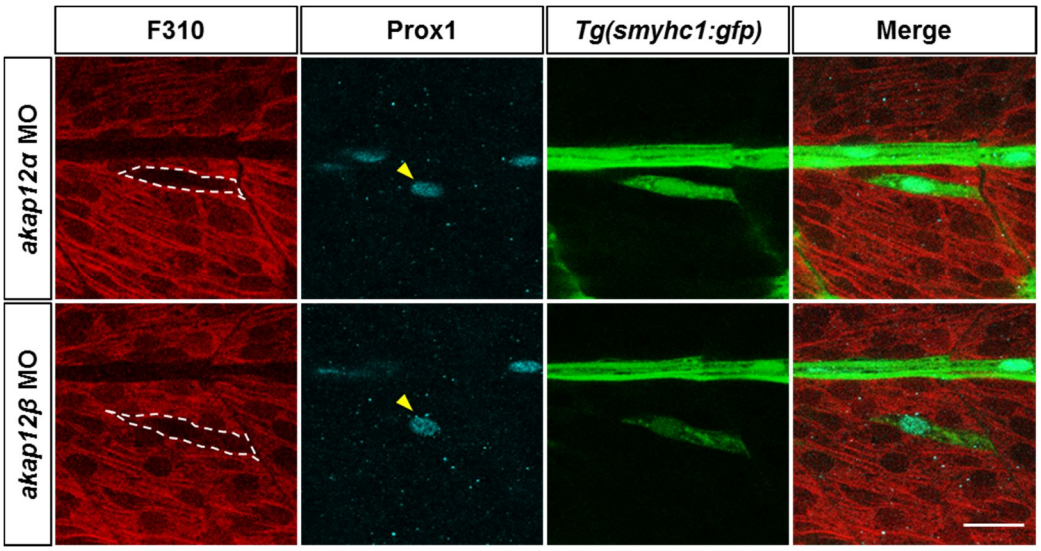


Figure 36. Fate switch of muscle cells did not occur in non-migratory slow muscles in *akap12* morphants

Slow muscle cells failed to reach the lateral surface of the myotome in the *akap12*-depleted embryos well expressed prox1 (arrowheads) and didn't fuse with fast muscle fibers. Therefore, the prox1 positive, gfp expressed slow fibers are not merged with F310 (dotted lines). Scale bar = 20 μm.

DISCUSSION

Akap12 was initially identified in patients with myasthenia gravis (Gordon et al., 1992) and is a known tumor suppressor. Although previous studies have demonstrated that *akap12* in zebrafish regulates mesodermal cell behavior during gastrulation (Weiser et al., 2007) and vascular integrity (Kwon et al., 2012), its function in muscle development is largely unknown. The dynamic patterns of *akap12* expression during early vertebrate development (Gelman et al., 2000; Klingbeil et al., 2001; Weiser et al., 2007) imply that it controls diverse developmental processes. In the present study, *akap12* was found to be critical for the development of locomotor behavior in zebrafish through regulation of muscle cell morphogenesis and migration.

1. Locomotor defects resulting from loss of *akap12* function

Spontaneous coiling is the first movement observed during early zebrafish development and occurs independently of brain input (Downes and Granato, 2006; Saint-Amant and Drapeau, 1998). The

present data demonstrated that the onset of spontaneous coiling was delayed in *akap12* morphants and remained at a low frequency (Fig. 12), indicating that *akap12* is required for this activity. Zebrafish embryos initially respond to mechanosensory stimuli with touch-evoked tail coiling (Brustein et al., 2003; Granato et al., 1996), which involves the activation of spinal sensory neurons followed by neural integration (Brustein et al., 2003). A significant reduction in the rate of touch response was observed in *akap12* morphants (Fig. 13 and Fig. 14). Correspondingly, in these embryos, the total distance traveled and swimming speed were decreased compared to control embryos (Fig. 15), indicating that the behavioral defects were caused by abnormal development of the muscle and not of the mechanosensory modality. It was recently reported that coiling behavior at 24 hpf is mediated solely by slow muscle, whereas burst swimming at 48 hpf is executed primarily by fast muscle (Naganawa and Hirata, 2011). In this study, *akap12* morphants showed severe defects in slow muscle cells and muscle pioneers but not in fast muscle cells, although the burst swimming response to touch stimulation was affected. This discrepancy may be explained by the finding that muscle pioneers in *akap12* morphants were also mispatterned; although the role of

these cells in the generation of swimming behavior is not well understood, it can be speculated that the ultrastructural defects caused by the failure of muscle pioneers to migrate normally are sufficient to compromise overall locomotor activity.

2. Role of *akap12* in zebrafish muscle morphogenesis

In the zebrafish embryo, muscle morphogenesis is initiated in adaxial cells expressing the slow myosin heavy chain. After their specification, these cells migrate from their medial position to the surface of the myotome to form slow muscle fibers (Blagden et al., 1997; Devoto et al., 1996), some of which remain next to the notochord and develop into muscle pioneers (Ekker et al., 1992; Halpern et al., 1993; Hatta et al., 1991). In contrast, fast muscle cells differentiate from the rest of the myotome below the slow fibers and around the muscle pioneers (Blagden et al., 1997; Devoto et al., 1996; Henry and Amacher, 2004; Moore et al., 2007). Both the α and β isoforms of *akap12* are expressed in adaxial cells during segmentation; indeed, *akap12* has been shown to be expressed at high levels in pathfinding cells, but at low levels in stationary cell types in mice (Gelman et al., 2000). Correspondingly, loss of *akap12*

function in zebrafish affects the migratory cell types, *i.e.*, slow muscle cells and muscle pioneers, but not fast muscle cells.

Previous studies have shown that *akap12*, a scaffolding protein, regulates cytoskeletal remodeling and cell migration in several cancer cell types and in rodents (Gelman et al., 2000; Lin and Gelman, 1997; Lin et al., 1996; Nelson et al., 1999). The results of the current study show that in zebrafish, muscle morphogenesis (Fig. 21, 22) and slow muscle cell and muscle pioneer migration (Fig. 24, 25, 27, 28, and 29), which are processes that require a dynamic cytoskeleton, are *akap12*-dependent, and ultimately dictate motility. Thus, Akap12 in nascent myoblasts could control various inputs to coordinate migration.

3. Function of two *akap12* isoforms in zebrafish development

Depletion of *akap12* results in defects in convergent extension during gastrulation, producing a short, curved body axis in zebrafish (Weiser et al., 2007); thus, in this study only embryos with normal trunk morphology were analyzed to specifically examine the role of *akap12* in muscle development. Previously, only the function of the β isoform of *akap12* had been described. Here, it was found that

akap12α mRNA is initially expressed at the tailbud stage, at the end of convergent extension (Fig. 16, 19); accordingly, *akap12α* morphants showed no defects in convergent extension. Given that most tissues and cell types co-express both isoforms in mice, they are presumed to have the same function (Akakura et al., 2008; Gelman et al., 2000), which is supported by their similar roles in vascular integrity in zebrafish (Kwon et al., 2012). Likewise, the similar musculature defects were observed upon injection of the each *akap12* MOs and the severity was significantly increased in double depleted embryos (Fig. 22). Because the two isoforms contain scaffolding domains in a common exon 4, which encodes nearly 1,525 amino acids (representing 95.6% of the *α* isoform or 99.5% of the *β* isoform) and is important for *akap12* function (Fig. 7), it seems plausible that one isoform could recover the defective phenotypes that resulted from depletion of the other (Fig. 21, 26 and 30). Therefore, we suggest that two isoforms of *akap12* may be functionally identical during muscle development.

4. Regulation of cell migration by AKAP12

Appropriate cell migration requires cells to interpret their

extracellular inputs through various receptors in order to regulate and rearrange distinct cytoskeletal and adhesive events in intracellular space. Thus, the cell migration machinery must be regulated by signaling intermediates that can be activated by diverse stimuli and can exert control over a large number of downstream targets in a spatiotemporal manner. In this regard, scaffolding proteins such as AKAP12 are thought to control cell migration by regulating key mediators.

AKAP12 fulfills typical scaffolding functions required to coordinate signal transduction pathways (Burack and Shaw, 2000; Johnson, 2002). AKAP12 crosslinks several cellular compartments, the actin cytoskeleton and the plasma membrane, thereby facilitating signal transduction from the cell surface to distinct subcellular compartments. This is mediated by F-actin-binding domain (Xia and Gelman, 2002) and by N-terminal myristoylation (Lin et al., 1996) and phospholipid binding domains (Weiser et al., 2007; Yan et al., 2009b). Although AKAP12 localizes at the plasma membrane under basal conditions, it is known to translocate to alternative subcellular compartments after mitogenic stimulation (Lin et al., 1996). Redistribution of AKAP12 from the cell periphery to the cytosol has

been demonstrated to occur in response to PKC activation by phorbol ester treatment (Nelson et al., 1999; Piontek and Brandt, 2003). PKC activity directs AKAP12 to a vesicular compartment near the nucleus, and that this translocation also causes similar redistribution of PKA (Yan et al., 2009b). Mitogen- induced and FAK-dependent phosphorylation of AKAP12 in or near scaffolding sites for PKC or F-actin correlates with decreased binding activity, resulting in increased PKC-induced cytoskeletal remodeling, and cell motility (Guo et al., 2011; Xia and Gelman, 2002). In addition, elevation of intracellular calcium concentration has been shown to cause AKAP12 redistribution through a presumed mechanism involving Ca^{2+}/calmodulin binding to AKAP12's membrane-associated polybasic domains (Tao et al., 2006). AKAP12 redistribution regulates cross-talk between PKA-dependent signaling and receptor-mediated events involving Ca^{2+} and PKC (Schott and Grove, 2013). Finally, AKAP12 multimerizes as a homomultimer or as a heteromultimer with other scaffolding proteins such as AKAP5, a function thought to amplify scaffolding activity at specific cellular sites (Gao et al., 2011a, b).

Especially, it has been reported that Akap12 acts through

Rho/ROCK/Myosin Ⅱ pathway to form blebs in zebrafish. Unlike lamellipodial protrusions, which are driven by actin polymerization, blebs are formed by myosin-based contraction of cortical actin (Charras et al., 2005). The authors showed that precise regulation of Rho family GTPases and downstream effectors may be an essential aspect of Akap12's role. They found that inhibition of Rho kinase or Myosin Ⅱ, which is regulated by Rho kinase, blocked all ectopic blebbing in *akap12* morphants, suggesting that a key role for Akap12 is to transiently shut down Rho kinase activity and myosin-based contration in order to make the transition to the extension phase (Weiser et al., 2007). In addition, AKAP12 can block activation of Rho family GTPases by Src and prevent Rho-dependent formation of invasive protrusions, such as podosomes, in a cultured cell system (Gelman and Gao, 2006). Moreover, the expression of p21-activated kinase (PAK) 2, an actin cytoskeletal regulator was reduced in AKAP12-depleted endothelial cells (Kwon et al., 2012). PAKs, downstream effectors of GTP-bound Cdc42 or Rac, are well known to be involved in the reorganization of actin fibers by regulating actin-myosin coupling (Bokoch, 2003; Coniglio et al., 2008) (Fig. 37).

Calcium and the cAMP-dependent PKA are pleiotropic cellular

regulators and both exert powerful, diverse effects on cytoskeletal dynamics, cell adhesion and cell migration (Berridge, 1975; Howe, 2011). The most direct route connecting Ca^{2+} to PKA is through Ca^{2+}-mediated regulation of adenylyl cyclases (AC) activity and thus cAMP production (Hanoune and Defer, 2001; Willoughby et al., 2010). Ca^{2+} may also regulate PKA signaling by affecting AKAP function. Ca^{2+}/calmodulin binding inhibits the interaction of AKAP79 and AKAP12 with membrane phospholipids (Gorski et al., 2005; Tao et al., 2006). The role of PKA in cytoskeletal organization and cell migration is to exert both negative and positive effects. Negative effects of PKA on migration have been reported for endothelial cell migration (Kim et al., 2000). Conversely, elevation of cAMP and activation of PKA have been shown to be required for efficient cell migration (Grieshaber et al., 2000; O'Connor and Mercurio, 2001). Thus, it appears that a balance of cAMP/PKA actibity (e.g. extent, space, time) is crucial for successful cell movement (Edin et al., 2001; Ydrenius et al., 1997) (Fig. 38). Therefore, Akap12 is not a simple promoter or inhibitor of directed cell migration but is required for proper control of complex cell movements (Weiser et al., 2007). In the absence of Akap12, slow muscle cells display less migratory phenotypes and muscle pioneer

cells exhibit pro-migratory phenotypes. These cells are therefore unable to form the proper muscle organization required for normal locomotor activity.

In zebrafish, it has been reported that dynamic and reciprocal waves of N-cadherin and M-cadherin expression within the myotome correlate precisely with slow muscle cell migration (Cortes et al., 2003). Altering cadherin expression, either in migrating slow muscle cells themselves or within cells through which slow muscle cells must migrate, results in aberrant migration, suggesting a possible role for differential cell adhesion in the control of migration. Akap12 can possibly regulates the cell adhesion by its scaffolding function, however, the molecular mechanism underlying the defective migration of slow muscle and muscle pioneer cells should be elucidated in future study.

In summary, in the absence of *akap12*, zebrafish embryos had reduced locomotor activity, which included reduced spontaneous coiling, touch response, and free swimming. These defects were associated with and likely caused by a severe disorganization in slow muscle cells and muscle pioneer alignment and a corresponding

failure of these cells to migrate. These results provide novel insight into the role of *akap12* in the regulation of muscle morphogenesis and the development of locomotor behavior in a simple vertebrate.

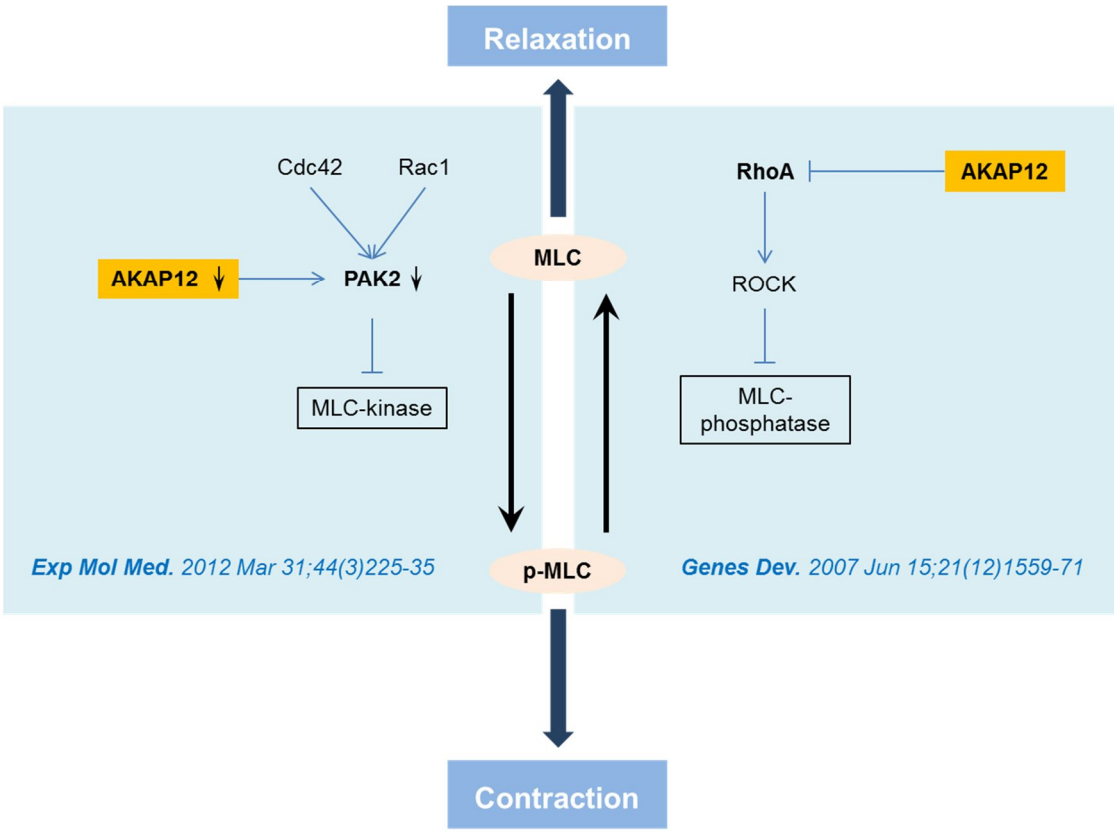


Figure 37. Schematic molecular mechanisms of Akap12 function in zebrafish

AKAP12 depletion decreases PAK2 expression, resulting in stress fiber formation (Kwon et al., 2012). Akap12 inhibits Rho/ROCK/Myosin Ⅱ pathway, resulting in cells with non-migratory phase (Weiser et al., 2007). PAK, p21-activated kinase; MLC, myosin

light chain; ROCK, Rho-associated protein kinase.

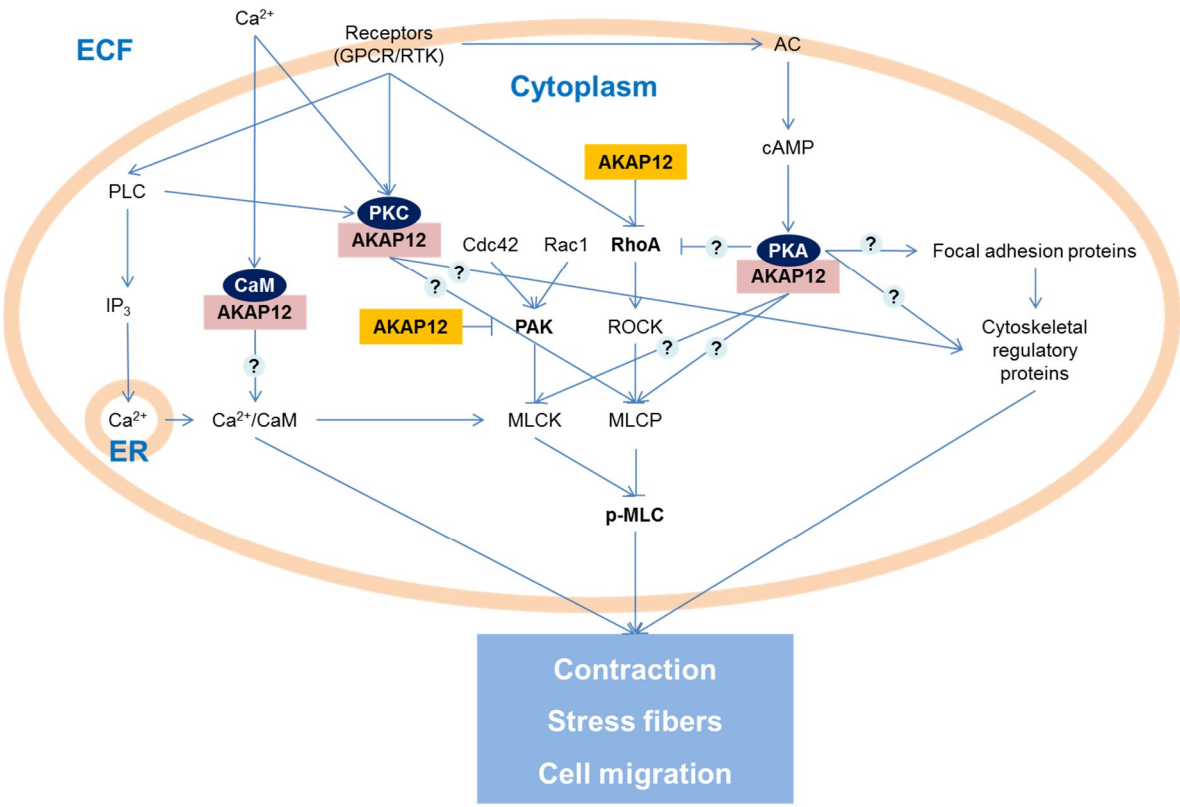


Figure 38. Schematic connections between AKAP12 and cell migration

PKC activity and elevation of intracellular calcium concentration has been shown to cause AKAP12 redistribution. AKAP12 redistribution regulates cross-talk between PKA-dependent signaling and receptor-mediated events involving Ca^{2+} and PKC. Calcium concentration and

a balance of cAMP/PKA actibity (e.g. extent, space, time) is crucial for successful cell movement. ECF, extracellular fluid; ER, endoplasmic reticulum; AC, adenylyl cyclase; GPCR, G-protein coupled receptor; RTK, receptor tyrosine kinase; CaM, calmodulin; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PLC, phospholipase C; IP_3, inositol 1,4,5-triphosphate; cAMP, cyclic adenosine monophosphate.

REFERENCES

Akakura, S., Huang, C., Nelson, P.J., Foster, B., Gelman, I.H., 2008. Loss of the SSeCKS/Gravin/AKAP12 gene results in prostatic hyperplasia. Cancer research 68, 5096-5103.

Arnold, H.H., Braun, T., 2000. Genetics of muscle determination and development. Current topics in developmental biology 48, 129-164.

Barresi, M.J., D'Angelo, J.A., Hernandez, L.P., Devoto, S.H., 2001. Distinct mechanisms regulate slow-muscle development. Current biology : CB 11, 1432-1438.

Berridge, M.J., 1975. The interaction of cyclic nucleotides and calcium in the control of cellular activity. Advances in cyclic nucleotide research 6, 1-98.

Blagden, C.S., Currie, P.D., Ingham, P.W., Hughes, S.M., 1997. Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog. Genes Dev 11, 2163-2175.

Bokoch, G.M., 2003. Biology of the p21-activated kinases. Annual review of biochemistry 72, 743-781.

Brand, M., Heisenberg, C.P., Warga, R.M., Pelegri, F., Karlstrom,

R.O., Beuchle, D., Picker, A., Jiang, Y.J., Furutani-Seiki, M., van Eeden, F.J., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D.A., Kelsh, R.N., Mullins, M.C., Odenthal, J., Nusslein-Volhard, C., 1996. Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. Development (Cambridge, England) 123, 129-142.

Brockerhoff, S.E., Hurley, J.B., Janssen-Bienhold, U., Neuhauss, S.C., Driever, W., Dowling, J.E., 1995. A behavioral screen for isolating zebrafish mutants with visual system defects. Proceedings of the National Academy of Sciences of the United States of America 92, 10545-10549.

Brustein, E., Saint-Amant, L., Buss, R.R., Chong, M., McDearmid, J.R., Drapeau, P., 2003. Steps during the development of the zebrafish locomotor network. Journal of physiology, Paris 97, 77-86.

Budick, S.A., O'Malley, D.M., 2000. Locomotor repertoire of the larval zebrafish: swimming, turning and prey capture. The Journal of experimental biology 203, 2565-2579.

Burack, W.R., Shaw, A.S., 2000. Signal transduction: hanging on a scaffold. Current opinion in cell biology 12, 211-216.

Carnegie, G.K., Means, C.K., Scott, J.D., 2009. A-kinase anchoring proteins: from protein complexes to physiology and disease. IUBMB life 61, 394-406.

Casadei, R., Pelleri, M.C., Vitale, L., Facchin, F., Lenzi, L., Canaider, S., Strippoli, P., Frabetti, F., 2011. Identification of housekeeping genes suitable for gene expression analysis in the zebrafish. Gene expression patterns : GEP 11, 271-276.

Charras, G.T., Yarrow, J.C., Horton, M.A., Mahadevan, L., Mitchison, T.J., 2005. Non-equilibration of hydrostatic pressure in blebbing cells. Nature 435, 365-369.

Chen, J.N., Haffter, P., Odenthal, J., Vogelsang, E., Brand, M., van Eeden, F.J., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh, R.N., Mullins, M.C., Nusslein-Volhard, C., 1996. Mutations affecting the cardiovascular system and other internal organs in zebrafish. Development (Cambridge, England) 123, 293-302.

Chen, X., Huang, C., Wang, X., Chen, J., Bai, C., Chen, Y., Chen, X., Dong, Q., Yang, D., 2012. BDE-47 disrupts axonal growth and motor behavior in developing zebrafish. Aquatic toxicology (Amsterdam, Netherlands) 120-121, 35-44.

Cheng, C., Liu, H., Ge, H., Qian, J., Qin, J., Sun, L., Shen, A., 2007. Essential role of Src suppressed C kinase substrates in endothelial cell adhesion and spreading. Biochemical and biophysical research communications 358, 342-348.

Cho, M., Webster, S.G., Blau, H.M., 1993. Evidence for myoblast-extrinsic regulation of slow myosin heavy chain expression during muscle fiber formation in embryonic development. The Journal of cell biology 121, 795-810.

Close, R.I., 1972. Dynamic properties of mammalian skeletal muscles. Physiological reviews 52, 129-197.

Colledge, M., Scott, J.D., 1999. AKAPs: from structure to function. Trends in cell biology 9, 216-221.

Coniglio, S.J., Zavarella, S., Symons, M.H., 2008. Pak1 and Pak2 mediate tumor cell invasion through distinct signaling mechanisms. Molecular and cellular biology 28, 4162-4172.

Cortes, F., Daggett, D., Bryson-Richardson, R.J., Neyt, C., Maule, J., Gautier, P., Hollway, G.E., Keenan, D., Currie, P.D., 2003. Cadherin-mediated differential cell adhesion controls slow muscle cell migration in the developing zebrafish myotome. Developmental cell 5, 865-876.

Coutelle, O., Blagden, C.S., Hampson, R., Halai, C., Rigby, P.W., Hughes, S.M., 2001. Hedgehog signalling is required for maintenance of myf5 and myoD expression and timely terminal differentiation in zebrafish adaxial myogenesis. Developmental biology 236, 136-150.

Currie, P.D., Ingham, P.W., 1996. Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. Nature 382, 452-455.

Currie, P.D., Ingham, P.W., 1998. The generation and interpretation of positional information within the vertebrate myotome. Mechanisms of development 73, 3-21.

Devoto, S.H., Melancon, E., Eisen, J.S., Westerfield, M., 1996. Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. Development (Cambridge, England) 122, 3371-3380.

Diviani, D., Scott, J.D., 2001. AKAP signaling complexes at the cytoskeleton. Journal of cell science 114, 1431-1437.

Dodge, K., Scott, J.D., 2000. AKAP79 and the evolution of the AKAP model. FEBS letters 476, 58-61.

Downes, G.B., Granato, M., 2004. Acetylcholinesterase function is dispensable for sensory neurite growth but is critical for neuromuscular synapse stability. Developmental biology 270, 232-245.

Downes, G.B., Granato, M., 2006. Supraspinal input is dispensable to generate glycine-mediated locomotive behaviors in the zebrafish embryo. Journal of neurobiology 66, 437-451.

Du, S.J., Devoto, S.H., Westerfield, M., Moon, R.T., 1997. Positive and negative regulation of muscle cell identity by members of the hedgehog and TGF-beta gene families. The Journal of cell biology 139, 145-156.

Edin, M.L., Howe, A.K., Juliano, R.L., 2001. Inhibition of PKA blocks fibroblast migration in response to growth factors. Experimental cell research 270, 214-222.

Ekker, M., Wegner, J., Akimenko, M.A., Westerfield, M., 1992. Coordinate embryonic expression of three zebrafish engrailed genes. Development (Cambridge, England) 116, 1001-1010.

Elworthy, S., Hargrave, M., Knight, R., Mebus, K., Ingham, P.W., 2008. Expression of multiple slow myosin heavy chain genes reveals a diversity of zebrafish slow twitch muscle fibres with differing requirements for Hedgehog and Prdm1 activity. Development (Cambridge, England) 135, 2115-2126.

Fan, G., Shumay, E., Wang, H., Malbon, C.C., 2001. The scaffold protein gravin (cAMP-dependent protein kinase-anchoring protein 250) binds the beta 2-adrenergic receptor via the receptor cytoplasmic Arg-329 to Leu-413 domain and provides a mobile scaffold during desensitization. The Journal of biological chemistry 276, 24005-24014.

Felsenfeld, A.L., Walker, C., Westerfield, M., Kimmel, C., Streisinger,

G., 1990. Mutations affecting skeletal muscle myofibril structure in the zebrafish. Development (Cambridge, England) 108, 443-459.

Gao, S., Wang, H.Y., Malbon, C.C., 2011a. AKAP5 and AKAP12 Form Homo-oligomers. Journal of molecular signaling 6, 3.

Gao, S., Wang, H.Y., Malbon, C.C., 2011b. AKAP12 and AKAP5 form higher-order hetero-oligomers. Journal of molecular signaling 6, 8.

Gelman, I.H., 2012. Suppression of tumor and metastasis progression through the scaffolding functions of SSeCKS/Gravin/AKAP12. Cancer metastasis reviews 31, 493-500.

Gelman, I.H., Gao, L., 2006. SSeCKS/Gravin/AKAP12 metastasis suppressor inhibits podosome formation via RhoA- and Cdc42-dependent pathways. Molecular cancer research : MCR 4, 151-158.

Gelman, I.H., Lee, K., Tombler, E., Gordon, R., Lin, X., 1998. Control of cytoskeletal architecture by the src-suppressed C kinase substrate, SSeCKS. Cell motility and the cytoskeleton 41, 1-17.

Gelman, I.H., Tombler, E., Vargas, J., Jr., 2000. A role for SSeCKS, a major protein kinase C substrate with tumour suppressor activity, in cytoskeletal architecture, formation of migratory processes, and cell migration during embryogenesis. The Histochemical journal 32, 13-26.

Gordon, T., Grove, B., Loftus, J.C., O'Toole, T., McMillan, R., Lindstrom, J., Ginsberg, M.H., 1992. Molecular cloning and preliminary characterization of a novel cytoplasmic antigen recognized by myasthenia gravis sera. The Journal of clinical investigation 90, 992-999.

Gorski, J.A., Gomez, L.L., Scott, J.D., Dell'Acqua, M.L., 2005. Association of an A-kinase-anchoring protein signaling scaffold with cadherin adhesion molecules in neurons and epithelial cells. Molecular biology of the cell 16, 3574-3590.

Granato, M., Nusslein-Volhard, C., 1996. Fishing for genes controlling development. Current opinion in genetics & development 6, 461-468.

Granato, M., van Eeden, F.J., Schach, U., Trowe, T., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh, R.N., Mullins, M.C., Odenthal, J., Nusslein-Volhard, C., 1996. Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. Development (Cambridge, England) 123, 399-413.

Grieshaber, N.A., Boitano, S., Ji, I., Mather, J.P., Ji, T.H., 2000. Differentiation of granulosa cell line: follicle-stimulating hormone induces formation of lamellipodia and filopodia via the adenylyl cyclase/cyclic adenosine monophosphate signal. Endocrinology 141, 3461-3470.

Grunwald, D.J., Kimmel, C.B., Westerfield, M., Walker, C., Streisinger, G., 1988. A neural degeneration mutation that spares primary neurons in the zebrafish. Developmental biology 126, 115-128.

Guo, L.W., Gao, L., Rothschild, J., Su, B., Gelman, I.H., 2011. Control of protein kinase C activity, phorbol ester-induced cytoskeletal remodeling, and cell survival signals by the scaffolding protein SSeCKS/GRAVIN/AKAP12. The Journal of biological chemistry 286, 38356-38366.

Halpern, M.E., Ho, R.K., Walker, C., Kimmel, C.B., 1993. Induction of muscle pioneers and floor plate is distinguished by the zebrafish no tail mutation. Cell 75, 99-111.

Hanoune, J., Defer, N., 2001. Regulation and role of adenylyl cyclase isoforms. Annual review of pharmacology and toxicology 41, 145-174.

Harris, A.J., Fitzsimons, R.B., McEwan, J.C., 1989. Neural control of the sequence of expression of myosin heavy chain isoforms in foetal mammalian muscles. Development (Cambridge, England) 107, 751-769.

Hatta, K., Bremiller, R., Westerfield, M., Kimmel, C.B., 1991. Diversity of expression of engrailed-like antigens in zebrafish. Development (Cambridge, England) 112, 821-832.

Hausken, Z.E., Scott, J.D., 1996. Properties of A-kinase anchoring proteins. Biochemical Society transactions 24, 986-991.

Henry, C.A., Amacher, S.L., 2004. Zebrafish slow muscle cell migration induces a wave of fast muscle morphogenesis. Developmental cell 7, 917-923.

Howe, A.K., 2011. Cross-talk between calcium and protein kinase A in the regulation of cell migration. Current opinion in cell biology 23, 554-561.

Jackson, H.E., Ingham, P.W., 2013. Control of muscle fibre-type diversity during embryonic development: the zebrafish paradigm. Mechanisms of development 130, 447-457.

Jiang, Y.J., Brand, M., Heisenberg, C.P., Beuchle, D., Furutani-Seiki, M., Kelsh, R.N., Warga, R.M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D.A., Mullins, M.C., Odenthal, J., van Eeden, F.J., Nusslein-Volhard, C., 1996. Mutations affecting neurogenesis and brain morphology in the zebrafish, Danio rerio. Development (Cambridge, England) 123, 205-216.

Johnson, G., 2002. Signal transduction. Scaffolding proteins--more than meets the eye. Science 295, 1249-1250.

Kahn, J.A., Roberts, A., 1982. The central nervous origin of the swimming motor pattern in embryos of Xenopus laevis. The Journal

of experimental biology 99, 185-196.

Kelsh, R.N., Brand, M., Jiang, Y.J., Heisenberg, C.P., Lin, S., Haffter, P., Odenthal, J., Mullins, M.C., van Eeden, F.J., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Kane, D.A., Warga, R.M., Beuchle, D., Vogelsang, L., Nusslein-Volhard, C., 1996. Zebrafish pigmentation mutations and the processes of neural crest development. Development (Cambridge, England) 123, 369-389.

Kim, S., Harris, M., Varner, J.A., 2000. Regulation of integrin alpha vbeta 3-mediated endothelial cell migration and angiogenesis by integrin alpha5beta1 and protein kinase A. The Journal of biological chemistry 275, 33920-33928.

Kimmel, C.B., Patterson, J., Kimmel, R.O., 1974. The development and behavioral characteristics of the startle response in the zebra fish. Developmental psychobiology 7, 47-60.

Klingbeil, P., Frazzetto, G., Bouwmeester, T., 2001. Xgravin-like (Xgl), a novel putative a-kinase anchoring protein (AKAP) expressed during embryonic development in Xenopus. Mechanisms of development 100, 323-326.

Kudo, N., Yamada, T., 1987. N-methyl-D,L-aspartate-induced locomotor activity in a spinal cord-hindlimb muscles preparation of the newborn rat studied in vitro. Neuroscience letters 75, 43-48.

Kwon, H.B., Choi, Y.K., Lim, J.J., Kwon, S.H., Her, S., Kim, H.J., Lim, K.J., Ahn, J.C., Kim, Y.M., Bae, M.K., Park, J.A., Jeong, C.H., Mochizuki, N., Kim, K.W., 2012. AKAP12 regulates vascular integrity in zebrafish. Exp Mol Med 44, 225-235.

Lee, S.W., Jung, K.H., Jeong, C.H., Seo, J.H., Yoon, D.K., Suh, J.K., Kim, K.W., Kim, W.J., 2011. Inhibition of endothelial cell migration through the downregulation of MMP-9 by A-kinase anchoring protein 12. Molecular medicine reports 4, 145-149.

Lewis, K.E., Currie, P.D., Roy, S., Schauerte, H., Haffter, P., Ingham, P.W., 1999. Control of muscle cell-type specification in the zebrafish embryo by Hedgehog signalling. Developmental biology 216, 469-480.

Lin, F., Wang, H., Malbon, C.C., 2000. Gravin-mediated formation of signaling complexes in beta 2-adrenergic receptor desensitization and resensitization. The Journal of biological chemistry 275, 19025-19034.

Lin, X., Gelman, I.H., 1997. Reexpression of the major protein kinase C substrate, SSeCKS, suppresses v-src-induced morphological transformation and tumorigenesis. Cancer research 57, 2304-2312.

Lin, X., Tombler, E., Nelson, P.J., Ross, M., Gelman, I.H., 1996. A novel src- and ras-suppressed protein kinase C substrate associated with cytoskeletal architecture. The Journal of biological chemistry 271,

28430-28438.

Moore, C.A., Parkin, C.A., Bidet, Y., Ingham, P.W., 2007. A role for the Myoblast city homologues Dock1 and Dock5 and the adaptor proteins Crk and Crk-like in zebrafish myoblast fusion. Development (Cambridge, England) 134, 3145-3153.

Muller, U.K., Stamhuis, E.J., Videler, J.J., 2000. Hydrodynamics of unsteady fish swimming and the effects of body size: comparing the flow fields of fish larvae and adults. The Journal of experimental biology 203, 193-206.

Muller, U.K., van Leeuwen, J.L., 2004. Swimming of larval zebrafish: ontogeny of body waves and implications for locomotory development. The Journal of experimental biology 207, 853-868.

Naganawa, Y., Hirata, H., 2011. Developmental transition of touch response from slow muscle-mediated coilings to fast muscle-mediated burst swimming in zebrafish. Developmental biology 355, 194-204.

Nelson, P.J., Moissoglu, K., Vargas, J., Jr., Klotman, P.E., Gelman, I.H., 1999. Involvement of the protein kinase C substrate, SSeCKS, in the actin-based stellate morphology of mesangial cells. Journal of cell science 112 (Pt 3), 361-370.

Nicolson, T., Rusch, A., Friedrich, R.W., Granato, M., Ruppersberg,

J.P., Nusslein-Volhard, C., 1998. Genetic analysis of vertebrate sensory hair cell mechanosensation: the zebrafish circler mutants. Neuron 20, 271-283.

O'Connor, K.L., Mercurio, A.M., 2001. Protein kinase A regulates Rac and is required for the growth factor-stimulated migration of carcinoma cells. The Journal of biological chemistry 276, 47895-47900.

O'Donovan, M.J., 1989. Motor activity in the isolated spinal cord of the chick embryo: synaptic drive and firing pattern of single motoneurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 9, 943-958.

Ochi, H., Westerfield, M., 2007. Signaling networks that regulate muscle development: lessons from zebrafish. Development, growth & differentiation 49, 1-11.

Odenthal, J., Rossnagel, K., Haffter, P., Kelsh, R.N., Vogelsang, E., Brand, M., van Eeden, F.J., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Mullins, M.C., Nusslein-Volhard, C., 1996. Mutations affecting xanthophore pigmentation in the zebrafish, Danio rerio. Development (Cambridge, England) 123, 391-398.

Piontek, J., Brandt, R., 2003. Differential and regulated binding of cAMP-dependent protein kinase and protein kinase C isoenzymes to

gravin in human model neurons: Evidence that gravin provides a dynamic platform for the localization for kinases during neuronal development. The Journal of biological chemistry 278, 38970-38979.

Roy, S., Wolff, C., Ingham, P.W., 2001. The u-boot mutation identifies a Hedgehog-regulated myogenic switch for fiber-type diversification in the zebrafish embryo. Genes Dev 15, 1563-1576.

Saint-Amant, L., Drapeau, P., 1998. Time course of the development of motor behaviors in the zebrafish embryo. Journal of neurobiology 37, 622-632.

Saint-Amant, L., Drapeau, P., 2000. Motoneuron activity patterns related to the earliest behavior of the zebrafish embryo. The Journal of neuroscience : the official journal of the Society for Neuroscience 20, 3964-3972.

Saint-Amant, L., Drapeau, P., 2001. Synchronization of an embryonic network of identified spinal interneurons solely by electrical coupling. Neuron 31, 1035-1046.

Schiaffino, S., Reggiani, C., 2011. Fiber types in mammalian skeletal muscles. Physiological reviews 91, 1447-1531.

Schilling, T.F., Piotrowski, T., Grandel, H., Brand, M., Heisenberg, C.P., Jiang, Y.J., Beuchle, D., Hammerschmidt, M., Kane, D.A., Mullins, M.C., van Eeden, F.J., Kelsh, R.N., Furutani-Seiki, M.,

Granato, M., Haffter, P., Odenthal, J., Warga, R.M., Trowe, T., Nusslein-Volhard, C., 1996. Jaw and branchial arch mutants in zebrafish I: branchial arches. Development (Cambridge, England) 123, 329-344.

Schott, M.B., Grove, B., 2013. Receptor-mediated Ca2+ and PKC signaling triggers the loss of cortical PKA compartmentalization through the redistribution of gravin. Cellular signalling 25, 2125-2135.

Su, B., Bu, Y., Engelberg, D., Gelman, I.H., 2010. SSeCKS/Gravin/AKAP12 inhibits cancer cell invasiveness and chemotaxis by suppressing a protein kinase C- Raf/MEK/ERK pathway. The Journal of biological chemistry 285, 4578-4586.

Tao, J., Shumay, E., McLaughlin, S., Wang, H.Y., Malbon, C.C., 2006. Regulation of AKAP-membrane interactions by calcium. The Journal of biological chemistry 281, 23932-23944.

Thisse, C., Thisse, B., 2008. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nature protocols 3, 59-69.

Trowe, T., Klostermann, S., Baier, H., Granato, M., Crawford, A.D., Grunewald, B., Hoffmann, H., Karlstrom, R.O., Meyer, S.U., Muller, B., Richter, S., Nusslein-Volhard, C., Bonhoeffer, F., 1996. Mutations disrupting the ordering and topographic mapping of axons in the retinotectal projection of the zebrafish, Danio rerio. Development (Cambridge, England) 123, 439-450.

van Eeden, F.J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh, R.N., Mullins, M.C., Odenthal, J., Warga, R.M., Allende, M.L., Weinberg, E.S., Nusslein-Volhard, C., 1996a. Mutations affecting somite formation and patterning in the zebrafish, Danio rerio. Development (Cambridge, England) 123, 153-164.

van Eeden, F.J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh, R.N., Mullins, M.C., Odenthal, J., Warga, R.M., Nusslein-Volhard, C., 1996b. Genetic analysis of fin formation in the zebrafish, Danio rerio. Development (Cambridge, England) 123, 255-262.

van Mier, P., 1988. Reticulospinal neurons, locomotor control and the development of tailswimming in Xenopus. Acta biologica Hungarica 39, 161-177.

van Mier, P., Armstrong, J., Roberts, A., 1989. Development of early swimming in Xenopus laevis embryos: myotomal musculature, its innervation and activation. Neuroscience 32, 113-126.

Weiser, D.C., Pyati, U.J., Kimelman, D., 2007. Gravin regulates mesodermal cell behavior changes required for axis elongation during zebrafish gastrulation. Gene Dev 21, 1559-1571.

Weiser, D.C., St Julien, K.R., Lang, J.S., Kimelman, D., 2008. Cell shape regulation by Gravin requires N-terminal membrane effector domains. Biochemical and biophysical research communications 375, 512-516.

Westerfield, M., 2000. The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio). 4th ed. Univ. of Oregon Press, Eugene.

Whitfield, T.T., Granato, M., van Eeden, F.J., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh, R.N., Mullins, M.C., Odenthal, J., Nusslein-Volhard, C., 1996. Mutations affecting development of the zebrafish inner ear and lateral line. Development (Cambridge, England) 123, 241-254.

Willoughby, D., Wachten, S., Masada, N., Cooper, D.M., 2010. Direct demonstration of discrete Ca2+ microdomains associated with different isoforms of adenylyl cyclase. Journal of cell science 123, 107-117.

Wolff, C., Roy, S., Ingham, P.W., 2003. Multiple muscle cell identities induced by distinct levels and timing of hedgehog activity in the zebrafish embryo. Current biology : CB 13, 1169-1181.

Wong, W., Scott, J.D., 2004. AKAP signalling complexes: focal points in space and time. Nature reviews. Molecular cell biology 5, 959-970.

Xia, W., Gelman, I.H., 2002. Mitogen-induced, FAK-dependent tyrosine phosphorylation of the SSeCKS scaffolding protein. Experimental cell research 277, 139-151.

Yan, M., Cheng, C., Jiang, J., Liu, Y., Gao, Y., Guo, Z., Liu, H., Shen, A., 2009a. Essential role of SRC suppressed C kinase substrates in Schwann cells adhesion, spreading and migration. Neurochemical research 34, 1002-1010.

Yan, X., Walkiewicz, M., Carlson, J., Leiphon, L., Grove, B., 2009b. Gravin dynamics regulates the subcellular distribution of PKA. Experimental cell research 315, 1247-1259.

Ydrenius, L., Molony, L., Ng-Sikorski, J., Andersson, T., 1997. Dual action of cAMP-dependent protein kinase on granulocyte movement. Biochemical and biophysical research communications 235, 445-450.

요약 (국문초록)

어류의 유영 행동 (swimming behavior)은 여러 종류의 근육섬유가 조화롭게 수축하여 이루어진다. 다른 척추동물에서와 같이, 제브라피쉬 (zebrafish)의 골격근은 생리적 기능에 따라 지근섬유 (slow muscle fibers)와 속근섬유 (fast muscle fibers)로 나뉘어 진다. 제브라피쉬에서 지근세포의 이동은 정상적인 근육 네트워크의 형성에 중요하다고 알려져 있다. 현재까지 근육세포의 운명을 결정하는 분자적 기전은 많이 알려져 있음에도 불구하고 근육모세포 (myoblast)의 정상적인 형태형성 및 이동이 초기 배아의 운동성을 조절하는지에 대해서는 자세히 알려지지 않았다. 본 논문은 발생과정 중 근육발달과 배아의 운동성 유지에 있어 *akap12*의 기능을 조사한 것이다. 모폴리노를 이용하여 *akap12*의 발현을 억제한 제브라피쉬 배아의 경우 자발적 근수축 (spontaneous muscle contractions), 접촉자극에 대한 몸통 감기 (touch-evoked coiling), 자유유영 (swimming)의 운동성이 모두 저하되는 현상을 보였다. *In situ* hybridization을 통하여 *akap12*의

발현을 조사한 결과, *akap12* 유전자는 제브라피쉬 발생 초기단계에서 adaxial cell과 이 세포로부터 분화되는 지근세포와 muscle pioneer cell에서 많이 발현됨을 확인하였고, *akap12* morphant에서 지근세포와 muscle pioneer cell의 이동에 결함을 초래하여 근육세포 배열에 간극이 나타나는 현상을 관찰하였다. 또한 *akap12* 발현을 저하시킨 배아에 *akap12* 유전자를 다시 발현시켰을 때 이러한 결함이 회복되는 현상을 확인하였으므로, 근육세포의 결함이 *akap12* 발현의 저하로 인한 결과이고, 이것이 운동성의 저하와 직접적으로 관련이 있음을 규명하였다. 그러나 근육발생과정에서 중요하다고 알려진 *hedgehog* 신호전달체계 중 *shha, myoD, engrailed*와 같은 유전자의 발현이 정상배아와 비교하였을 때 큰 변화를 보이지 않았으므로 결과적으로 *akap12*의 결손으로 인한 근육의 결함은 척삭을 포함한 다른 조직으로부터 조절되는 간접적인 결과가 아니라 근육세포 자체적인 이동 결함에 의한 것으로 예상이 된다. 이러한 결과들을 통해 adaxial cell에서 발현하는 *akap12*가 지근세포와 muscle pioneer cell에 직접적으로 작용하여 근육의 형태형성에 필수적인 세포의 이동을 조절하고 초기 배아의 이동성을 유지하는데 중요한

역할을 한다는 새로운 사실을 규명하였다.

주 요 어 : *akap12*; locomotion; muscle; morphogenesis; development; zebrafish

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