Developmental Biology xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish

Cheng-Yung Lin, Wei-Ta Chen, Hung-Chieh Lee, Ping-Hsi Yang, Hsin-Jung Yang, Huai-Jen Tsai *

Institute of Molecular and Cellular Biology, National Taiwan University, Room 307, Fisheries Science Building, No. 1, Section 4, Roosevelt Road, Taipei, 106, Taiwan

ARTICLE INFO

Article history:
Received for publication 14 November 2008
Revised 15 April 2009
Accepted 24 April 2009
Available online xxxx

Keywords:
Six1a
Myf5
Myod
Cranial myogenesis
Zebrafish

ABSTRACT

Transcription factor Six1a plays important roles in morphogenesis, organogenesis, and cell differentiation. However, the role of Six1a during zebrafish cranial muscle development is still unclear. Here, we demonstrated that Six1a was required for sternohyoideus, medial rectus, inferior rectus, and all pharyngeal arch muscle development. Although Six1a was also necessary for *myod* and *myogenin* expression in head muscles, it did not affect *myf5* expression in cranial muscles that originate from head mesoderm. Overexpression of *myod* enabled embryos to rescue all the defects in cranial muscles induced by injection of *six1a*-morpholino (MO), suggesting that *myod* is directly downstream of *six1a* in controlling craniofacial myogenesis. However, overexpression of *six1a* was unable to rescue arch muscle defects in the *tbx1*- and *myf5*-morphants, suggesting that *six1a* is only involved in myogenic maintenance, not its initiation, during arch muscle myogenesis. Although the craniofacial muscle defects caused by *pax3*-MO phenocopied those induced by *six1a*-MO, injection of *six1a*, *myod* or *myf5* mRNA did not rescue the cranial muscle defects in *pax3* morphants, suggesting that *six1a* and *pax3* do not function in the same regulatory network. Therefore, we proposed four putative regulatory pathways to understand how *six1a* distinctly interacts with either *myf5* or *myod* during zebrafish craniofacial muscle development.

Introduction

During embryogenesis, the trunk and limb muscles originate from somites, which are epithelial structures in the mesoderm flanking the neural tube, whereas head muscles mostly originate from cranial mesoderm (Noden and Francis-West, 2006). Three groups contribute to the craniofacial skeletal muscles: (1) branchial arch muscles, which are derived from the unsegmented head mesoderm and operate the jaw, facial expression, pharyngeal, laryngeal and gill function; (2) most extraocular muscles, which develop from the prechordal mesoderm and control eye movement; and (3) those muscles derived from progenitor cells in the occipital somites of trunk, which move into the head and give rise to muscles of the tongue and neck (Noden and Francis-West, 2006; Chai and Maxson, 2006; Shih et al., 2007).

Despite the varied origins of these muscles, all of them are controlled by myogenic regulatory factors (MRFs) (Buckingham, 2006). Proliferative myoblasts, which have undergone initial myogenic commitment, are marked by the expressions of Myf5 and MyoD, while later myogenic differentiation is marked by Myogenin and MRF4. However, because of the different origins of these muscle cells, MRFs are regulated differently in the head and trunk. For example, mice lacking Myf5 and Pax3 do not develop skeletal muscle in the trunk and limb, whereas they do develop normal head muscles (Tajbakhsh et al., 1997). Taking another example, Lbx1/Pax7/Paraxis in chick are necessary for trunk myogenesis, but they are not necessary for head myogenesis (Mootoosamy and Dietrich, 2002). In fact, the Wnt signals, which promote trunk myogenesis, have been proven to block head myogenesis in chick (Tzahor et al., 2003). In zebrafish, we have clearly defined the distinct functions of Myf5 and Myod that regulate head muscle development, and we have demonstrated that they exhibit their own regulatory pathways (Lin et al., 2006). Although myogenic progression is similar in all developing muscle groups, it seems, therefore, that the specification of cells just before myoblast differs significantly between head and trunk (Rawls and Olson, 1997; Mootoosamy and Dietrich, 2002).

Only a few factors have been reported to play roles in head myogenesis, and we enumerate them here. Mice lacking Capsulin and *myoR* fail to express *myf5* in the first arch and lose a subset of mandibular arch-derived muscle (Lu et al., 2002). It has been found that *tbx1*, which is expressed in the premyoblast mesoderm in the first and second branch arch, is required for the development of some head muscles (Kelly et al., 2004; Dastjerdi et al., 2007). Although *bmp4* promotes cardiac differentiation, it also inhibits head skeletal muscle differentiation (Tirosh-Finkel et al., 2006). Similarly, *fgf8* is shown to promote branchiomeric muscle development, but it inhibits extraocular muscle development (von Scheven et al., 2006). Finally, *pitx2* is a paired-related homeobox gene, which is required for the expression of the premyoblast specification markers *tbx1*, *tcf21* (Capsulin), and *msc* (MyoR) to set up the premyoblast in the first branch arch (Dong et al.;

* Corresponding author. Fax: +886 2 2363 8483.
E-mail address: hjtsai@ntu.edu.tw (H.-J. Tsai).

0012-1606/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.ydbio.2009.04.029

2006; Shih et al., 2007; L'Honore et al., 2007). However, it remains unclear what additional factors may be involved in head myogenesis.

The *six* gene is a vertebrate homolog of the *Drosophila* homeobox gene *sine oculis* (*so*) and plays important roles in morphogenesis, organogenesis, and cell differentiation (Kawakami et al., 2000). Six protein is a transcription factor that contains two conserved domains, the Six domain (SD) and the homeodomain (HD). Both domains are required for specification through binding DNA and cooperative interaction with cofactors (Kawakami et al., 1996; Chen et al., 1997; Pignoni et al., 1997). For example, *Drosophila so* is required for eye formation through binding the synergistic regulatory network, such as *eyeless* (Pax), *eyes absent* (Eya) and *dachshund* (Dach) (Chen et al., 1997; Pignoni et al., 1997).

In vertebrates, Six protein displays a similar regulatory network during myogenesis and development of the metanephric kidney and inner ear (Heanue et al., 1999; Xu et al., 1999; Xu et al., 2003; Li et al., 2003). Moreover, Six protein is reported to directly control the expressions of *myf5* and *myogenin* through binding at the MEF3 in their promoters (Spitz et al., 1998; Giordani et al., 2007). There are six *Six* genes (*Six1* to *Six6*) in mouse and human genomes (Kawakami et al., 2000). *Six1* is expressed from E8 stage and throughout skeletal muscle development in mouse embryos (Grifone et al., 2004). *Six1* and *Six4* are expressed as overlapping in muscle territories, such as dermomyotome, myotome, limb bud and migrating muscle precursors (Ozaki et al., 2001; Laclef et al., 2003). *Six1*-knockout fetuses suffer from muscle hypoplasia (Laclef et al., 2003), whereas *Six1* and *Six4* double knockout embryos appear to have more severe muscle defects, especially in leg muscles (Grifone et al., 2005), suggesting that *Six4* shares a common function with *Six1* during myogenesis. Furthermore, in both $Six1^{-/-}Six4^{-/-}$ and $Eya1^{-/-}Eya2^{-/-}$ double mutants, *pax3* fails to express in the hypaxial dermomyotome, which then causes cell death and reduces muscle progenitor cells in the limbs (Grifone et al., 2005; Grifone et al., 2007). In zebrafish, six members of the *six* gene have been defined: *six1a–1b*, *six2–2.1*, *six3a–3b*, *six4.1–4.3*, *six7* and *six9* (Kobayashi et al., 1998, 2000, 2001; Drivenes et al., 2000; Wargelius et al., 2003; Bessarab et al., 2004, 2008). Both *six1a* and *six4.2* are expressed in the presomitic mesoderm, somites and pectoral fin bud. Moreover, Bessarab et al. (2004) reported that *six1a* expression is regulated by the Notch pathway during trunk muscle differentiation. Knockdown of *six1a* causes *myogenin* expression to be reduced in somites, resulting in abnormal differentiation of trunk fast muscles (Bessarab et al., 2008). They also demonstrated that the *six1a* transcript is expressed in craniofacial muscle. More importantly, it has been reported that the *six1a* gene is involved in branchio-oto-renal syndrome (Ruf et al., 2004). Therefore, detailed knowledge about the mechanisms controlling molecular interaction among genes involved in head muscle development should not only give insight into craniofacial morphogenesis but also help in the development of therapies designed to treat clinical syndromes affecting head and facial development. However, the function that Six1a plays in head muscle development is still unknown.

In this study, we focus on the role of Six1a in head myogenesis. When Six1a is absent by injection of *six1a*-specific morpholino (MO), we show that *myf5* fails to express in the cranial muscles that originate from trunk paraxial mesoderm, whereas *myf5* continues to be normally expressed in cranial muscles that originate from head mesoderm. In contrast, *myod* is lost in the cranial muscles that originate both from trunk and head mesoderm. We also demonstrate that injection of *myod* mRNA can rescue the *six1a*-MO-induced defect, but that injection of *myf5* mRNA could only rescue the muscle defects that originate from trunk paraxial mesoderm. We prove that the function of Six1a is equivalent to Pax3 and that Six1a is not involved in the Tbx1 pathway. Furthermore, we propose four putative regulatory pathways to demonstrate that *six1a* interacts separately with either *myf5* or *myod* to modulate the development of craniofacial muscles in zebrafish.

Materials and methods

Fish embryos

The wild-type AB strain (University of Oregon, Eugene, OR) and the transgenic line Tg(α-actin:RFP) (Lin et al., 2006) of zebrafish (*Danio rerio*) were used. The culture condition, embryo stage, egg production and collection were described previously (Lin et al., 2006). Fluorescent signal in embryos was observed under a fluorescent stereomicroscope (MZ FLIII, Leica) equipped with 583 nm (emission) filters.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization has been described previously (Lee et al., 2006), except that the following genes were used as probes: *six1a* (Bessarab et al., 2004); *myf5*, *myod*, *myogenin*, *et1* (Miller et al., 2000); *fgf3*, *dlx2* (Akimenko et al., 1994); *tbx1* (Piotrowski et al., 2003); *pax3* (Seo et al., 1998) and *eya1* (Sahly et al., 1999) cDNAs (GenBank Accession Nos. NM207095, NM131576, NM131262, NM131006, AF281858, NM131291, U03875, NM183339, AF014366, and BC154187, respectively).

MOs for blocking translation and mRNAs for rescue experiments

MOs designed specifically as translational inhibitors of *six1a* were (1) *six1a*-MO (Nica et al., 2006), 5′-CGAAAGAAGGCAACATTGACATGAC-3′, which is complementary to nucleotides (nt) 142–166 of zebrafish *six1a* cDNA (GenBank Accession No. NM207095) and was injected at the concentration of 8, 6, 4, 2, or 1 ng per embryo; (2) UMMO (Bessarab et al., 2008), 5′-TCTCCTCTGGATGCTA-CGAAGGAAG-3′, which is complementary to nt 93–117 of zebrafish *six1a* cDNA (GenBank Accession No. NM207095) and was injected at 8 ng per embryo; and (3) SM-MO (Bessarab et al., 2008), 5′-CGCTTAATTACC*TTTCTTTCGCCTC*-3′, which is complementary to nt 87073–87097 (intron sequence is underlined) of the clone DKEY-225H23 (GenBank Accession No. BX649231), binding the splice donor site of *six1a* pre-mRNA, and was injected at 8 ng per embryo. Regarding MOs that were designed specifically as translational inhibitors of MRFs, they were (1) *myf5*-MO, 5′-TCTGGGATGTGGAGAATACGTCCAT-3′, which is complementary to nt 44–68 of zebrafish *myf5* cDNA (GenBank Accession No. NM131576) and was injected at 4 ng per embryo; and (2) *myod*-MO (Lin et al., 2006), 5′-ATATCCGAC-AACTCCATCTTTTTTG-3′, which is complementary to nt 172–196 of zebrafish *myod* cDNA (GenBank Accession No. NM131262) and was injected at 4 ng per embryo. Regarding MOs that were designed specifically as translational inhibitors of *tbx1*, *pax3* and *eya1*, they were (1) *tbx1*-MO, 5′-GGGCTTGATATTGCTGAAA-TCATTC-3′, which is complementary to nt 359–383 of zebrafish *tbx1* cDNA (GenBank Accession No. NM183339) and was injected at 10 ng per embryo; (2) *pax3*-MO (Lee et al., 2006), 5′-ACGAAAAAAGGATGCACGAAGCACT-3′, which is complementary to nt 241–265 of zebrafish *pax3* cDNA (GenBank Accession No. AF014366) and was injected at 3 ng per embryo; and (3) *eya1*-MO (Bricaud and Collazo, 2006), 5′-AGCTAGATCCTGCATTTCCATAGAC-3′, which is complementary to nt 274–298 of zebrafish *eya1* cDNA (GenBank Accession No. AF014366) and was injected at 10 ng per embryo. All MOs were prepared at a stock concentration of 1 mM and were diluted to the desired concentrations for microinjection.

In order to further prove the specific effectiveness of *six1a*-, *myod*- and *myf5*-MO, we designed the following synthetic mRNA: (1) *six1a-egfp* mRNA, in which the *six1a* cDNA, including *six1a*-MO target sequence, is fused in frame with *egfp* cDNA; (2) *myod-egfp* mRNA, in which the *myod* cDNA, including *myod*-MO target sequence, is fused in frame with *egfp* cDNA; and (3) *myf5*-MO-*target-egfp* mRNA, in which the *myf5*-MO target sequence is fused in frame with *egfp* cDNA. Regarding that the introduced *six1a* mRNA is not bound by *six1a*-MO during the rescue experiment, we designed (1) a wobble *six1a* mRNA,

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029

in which we changed the nt 145–166 of zebrafish *six1a* cDNA (GenBank Accession NO. NM207095) from 5′-ATGTCAATGTTGCCTTCTTTCG-3′ to 5′-ATGAGTATGCTCCCGAGCTTCG-3′, but without altering the amino acid residues; and (2) a wobble *six1a-egfp* mRNA, in which the wobble *six1a* cDNA was fused in frame with *egfp* cDNA. Capped mRNA of wobble *six1a* was synthesized according to the protocols of the manufacturer (Epicentre). The mRNAs of *myf5* and *myod* were also synthesized. The generated mRNAs were diluted with distilled water to 110 ng/μl and 66 ng/μl for *six1a* mRNA, to 44 ng/μl and 22 ng/μl for *myf5* mRNA and to 22 ng/μl for *myod* mRNA (Lin et al., 2006). Each time, approximately 2.3 nl of solution was injected into the one-cell stage of zebrafish embryos.

Western blot analysis

The Western blot was performed after the total proteins were analyzed on a 12% SDS-PAGE by following the procedures described previously (Lee et al., 2007), except that the yolk was removed, and the antibodies of anti-Six1a (abcan, ab22072) and anti-Glyceraldehyde 3-phosphate dehydrogenase (ABBIOTEC, 250504) were used at the dilution of 1:1000.

Results

Expression patterns of six1a, myf5 and myod in zebrafish head muscle development

To study the roles of Six1a during cranial muscle development, we first analyzed the spatiotemporal expression of *six1* from 24 to 72 hpf and made a comparison with the expression patterns of some MRFs, particularly *myf5* and *myod*. At 24 hpf, *six1a* was detected in the olfactory placode (olp), otic vesicle (ov), anterior lateral line and vestibular ganglia (allg) (Fig. 1A), which was consistent with what was reported by Bessarab et al. (2004). We also noted that *six1a* was only expressed in the neural ectoderm, but not in the cranial mesoderm, during 24 hpf. However, *myf5*, but not *myod*, were detected in the cranial muscle precursors during 24 hpf (Figs. 1B, C). At 32 hpf, *six1a* initiated expression in branchial arch and in extraocular muscle primordial of the medial rectus (mr) and inferior rectus (ir) (Fig. 1D), while *myf5* started to gradually reduce its expression in the first branchial arch, but began to express in the extraocular muscle primordial inferior oblique (io) and superior oblique (so) (Fig. 1E). At the same time, *myod* transcripts were initially detected in the head muscle primordia of the mr, ir, lateral rectus (lr), and first branchial arch mesoderm core (Fig. 1F), which was similar to the expression pattern of *six1a*. At 36 hpf, *six1a* was strongly expressed in the branchial arch (Fig. 1G), while the *myf5* transcripts were gradually decreased in the arch region (Fig. 1H). However, *myod* was now detected in head muscles derived from the first (masticatory plate, MP; intermandibularis, IM) and the second arch mesoderm cores (constrictor hyoideus dorsalis, CHD; constrictor hyoideus ventralis, CHV) (Fig. 1I). This result was consistent with the report of Schilling and Kimmel (1994). At 72 hpf, all cranial muscles were *six1a*- (Figs. 1J, L) and *myod*-positive (Figs. 1K, M).

Comparing the expression patterns of *six1a*, *myf5* and *myod* during the head muscle development of zebrafish, we concluded that *six1a* was expressed in all cranial muscles. The expression stage of *six1a* in hyoid (1st), mandibular (2nd), and branchial arch (3rd) was later than that of *myf5*, but obviously earlier than that of *myod*. In extraocular muscle, *six1a* started to express in mr and ir primordia and sustained its expression to the later stage of 72 hpf, which was similar to *myod*, but different from *myf5*, which was expressed in io and so primordia. Thus, we can further conclude that the expression of *myf5* is earlier than that of *six1a* in the cranial mesoderm, whereas the expression of *six1a* is earlier than that of *myod* in the arches.

Six1a is involved in zebrafish cranial muscle development

To understand whether *six1a* plays roles in craniofacial muscle development, we used a transgenic line, Tg(*α-actin:RFP*), in which the RFP reporter is labeled in all craniofacial muscles (Figs. 2A, B), as previously reported (Lin et al., 2006). When the embryos derived from this line were injected with *six1a*-MO, we observed that the muscle primordia of extraocular muscles mr and ir, all arch muscles and sh were missing at 72 hpf (Figs. 2C, D), whereas the muscle primordial of so, io, sr, and lr, and some remnants of arch muscle cells, were all normal and presented as RFP-positive (Figs. 2A, B). Furthermore, besides *six1a*-MO, we also designed two other types of morpholinos, UM-MO and SM-MO, to specifically knockdown the translation of *six1a* mRNA. The defective phenotype induced by injection of either UM-MO or SM-MO was similar to that of injection of *six1a*-MO (Supplemental Fig. S1). Western blot analysis proved that the Six1a expression level was greatly reduced in the *six1a*-MO-injected embryos (Supplemental Fig. S2). In addition, we co-injected a wobble *six1a-egfp* mRNA with a *six1a*-MO, and the Six1a-GFP fusion protein was detected in embryos (Supplemental Fig. S3), indicating that the injected *six1a*-MO cannot inhibit the translation of the introduced wobble *six1a-egfp* mRNA. Furthermore, co-injection of *six1a*-MO with wobble *six1a*-mRNA, but not *egfp* mRNA, enabled embryos to rescue the defective phenotypes induced by *six1a*-MO and resulted in the normal development of all head muscles (Figs. 2E, F; and Table 2). We also noticed that the degree of defective phenotype induced by *six1a*-MO was dose-dependent (Table 1). Overall, evidence indicates that the defects induced by *six1a*-MO are specific and we therefore concluded that *six1a* is necessary for the development of extraocular muscles mr and ir, all arch muscles and sh, which migrate from trunk and contribute to head muscle.

Six1a functions with Myf5 and Myod in cranial muscle development, but in different modulations

Lin et al. (2006) categorized all zebrafish cranial muscles into three groups and defined three regulatory pathways involved in cranial muscle development. Among them, the extraocular muscles so and io, the dorsal pharyngeal arch muscles lap, do, am, ah and ao, and the trunk migratory head muscle sh, are categorized as Group I, whose primordial cells require Myf5 to activate their downstream MRFs, such as *myod* and *myogenin*. In this study, we found that *myf5* was normally expressed in pharyngeal arch muscle precursors of the *six1a*-MO-injected embryos during 36–48 hpf (Figs. 2I vs. M and J vs. N). In addition, compared to the wild-type embryos, the expressions of *myf5*, *myod* and *myogenin* remained unchanged in the extraocular muscles so and io of *six1a* morphants (Figs. 2J vs. N, K vs. O, and L vs. P). However, the expressions of *myod* and *myogenin* were greatly reduced in the pharyngeal arch muscle precursors, lap, do, am, ah, and ao, of *six1a*-MO-injected embryos at 48 hpf (Figs. 2K vs. O and L vs. P), suggesting that Six1a is required for the normal expressions of *myod* and *myogenin* in the precursors of dorsal pharyngeal arch muscles. On the other hand, the primordial muscle sh, which originates from trunk, lost both *myf5* and *myod* expression in the *six1a* morphants (Figs. 5B vs. E and C vs. F), suggesting that Six1a is required for the expressions of *myf5* and *myod* in sh primordial muscle.

The extraocular muscle lr and the ventral pharyngeal arch muscles ima, imp, ih and hh are categorized as Group II, whose primordial cells are *myf5*-expressed precursors and require *myod* to play a major role in myogenesis. Both *myf5* and *myod* are necessary for the development of Group II precursors. Here, we revealed that *myf5* was normally expressed in the precursor of pharyngeal arch muscles of *six1a* morphants at 36–48 hpf, compared to the wild-type embryos. In addition, the *myod* and *myogenin* expressions remained unchanged in the extraocular muscle lr of *six1a* morphants at 48 hpf (Figs. 2K vs. O and L vs. P). However, the *myod* and *myogenin* expressions were totally lost in the ventral

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029

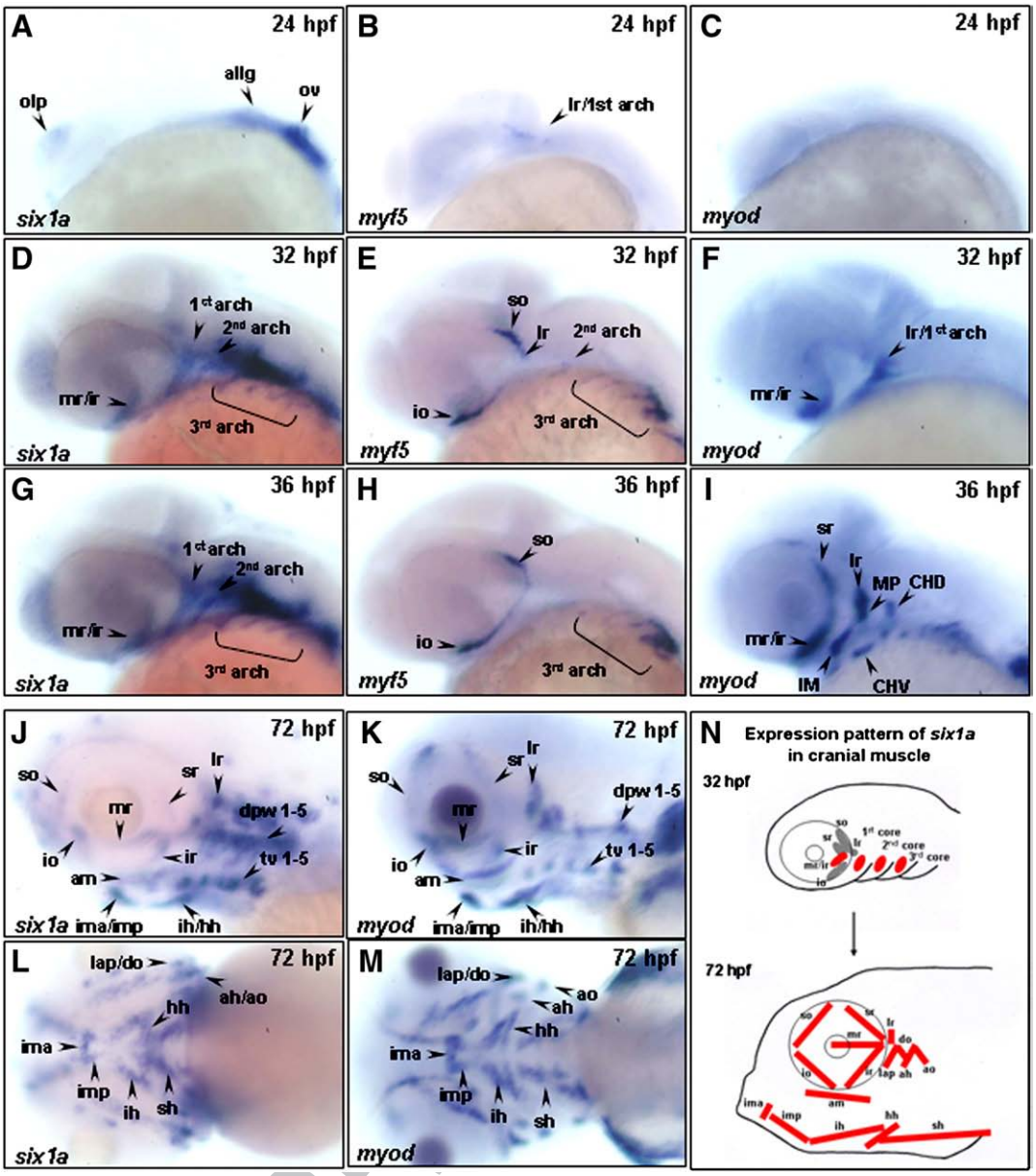

Fig. 1. The temporal expressions of *six1a*, *myf5* and *myod* during cranial muscle development of zebrafish. The temporal expressions of *six1a* (A, D, G, J, L), *myf5* (B, E, H) and *myod* (C, F, I, K, M) transcripts of zebrafish were analyzed by whole-mount *in situ* hybridization in embryos from lateral view (A–K) and ventral view (L–M). The transcript of *six1a* in the olp, ov, and allg at 24 hpf (A); in the mr, ir, 1st, 2nd, and 3rd arches at 32 and 36 hpf (D, G); and in all the cranial muscles at 72 hpf (J, L). The *myf5* transcript was detected in the craniofacial region at 24 hpf (B, arrow); in the so, io, 2nd and 3rd arches at 32 hpf (E); and in the so, io, and 3rd arches at 36 hpf (H). Although the *myod* was not expressed in the craniofacial muscles at 24 hpf (C), it was detected in the mr/ir, lr, and 1st arch at 32 hpf (F); in the mr, ir, sr, lr, MP, IM, CHD, and CHV at 36 hpf (I); and in all the cranial muscles at 72 hpf (K, M). The schematic diagram illustrates the expression of *six1a* in the cranial muscles during 32–72 hpf (N). ah, adductor hyoideus; allg, anterior lateral line and vestibular ganglia; am, adductor mandibulae; ao, adductor operculi; do, dilator operculi; dpw1–5, dorsal pharyngeal wall 1–5; hh, hyohyoideus; ih, interhyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; io, inferior oblique; ir, inferior rectus; lap, levator arcus palatini; lr, lateral rectus; mr, medial rectus; olp, olfactory placode; ov, otic vesicle; sh, sternohyoideus; so, superior oblique; sr, superior rectus and tv 1–5, transversus ventralis 1–5. CHD: the constrictor hyoideus dorsalis, which differentiates to ah and ao; CHV: the constrictor hyoideus ventralis, which differentiates to ih and hh; IM: the intermandibularis, which differentiates to ima and imp; MP: the masticatory plate, which differentiates to CD and am. CD: the constrictor dorsalis, which differentiates to lap and do.

pharyngeal arch muscles ima, imp, ih and hh in the *six1a* morphants at 48 hpf (Figs. 2K vs. O and L vs. P), suggesting that Six1a affects *myod* expression in the ventral pharyngeal arch muscle of zebrafish.

The extraocular muscles sr, mr and ir belong to Group III, whose primordial cells require *myod*, but not *myf5*, as a major factor in muscle development. The expressions of *myod* and *myogenin* in the extraocular muscle sr of the *six1a*-MO-injected embryos appeared the same as the extraocular muscle sr of the wild-type embryos (Figs. 2K vs. O and L vs. P). However, the extraocular muscles mr and ir of the *six1a*-MO-injected embryos were completely lost when observed at 48 hpf (Figs. 2K vs. O and L vs. P). This evidence suggests that Six1a modulates *myod* expression in extraocular muscles mr and ir.

Taken together, during craniofacial muscle development of zebrafish, we conclude that Six1a is required for (1) *myf5* expression in trunk migratory head muscle sh and (2) *myod* expression in the extraocular muscles mr and ir in all pharyngeal arch muscles and the trunk migratory head muscle sh.

The defective pharyngeal arch muscles are induced specifically by loss of Six1a

Pharyngeal arch is developed from three germ layers: the mesoderm core, the endoderm pharyngeal pouch and the ectoderm neural crest cells (Graham and Smith, 2001). It was necessary to Q1

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029

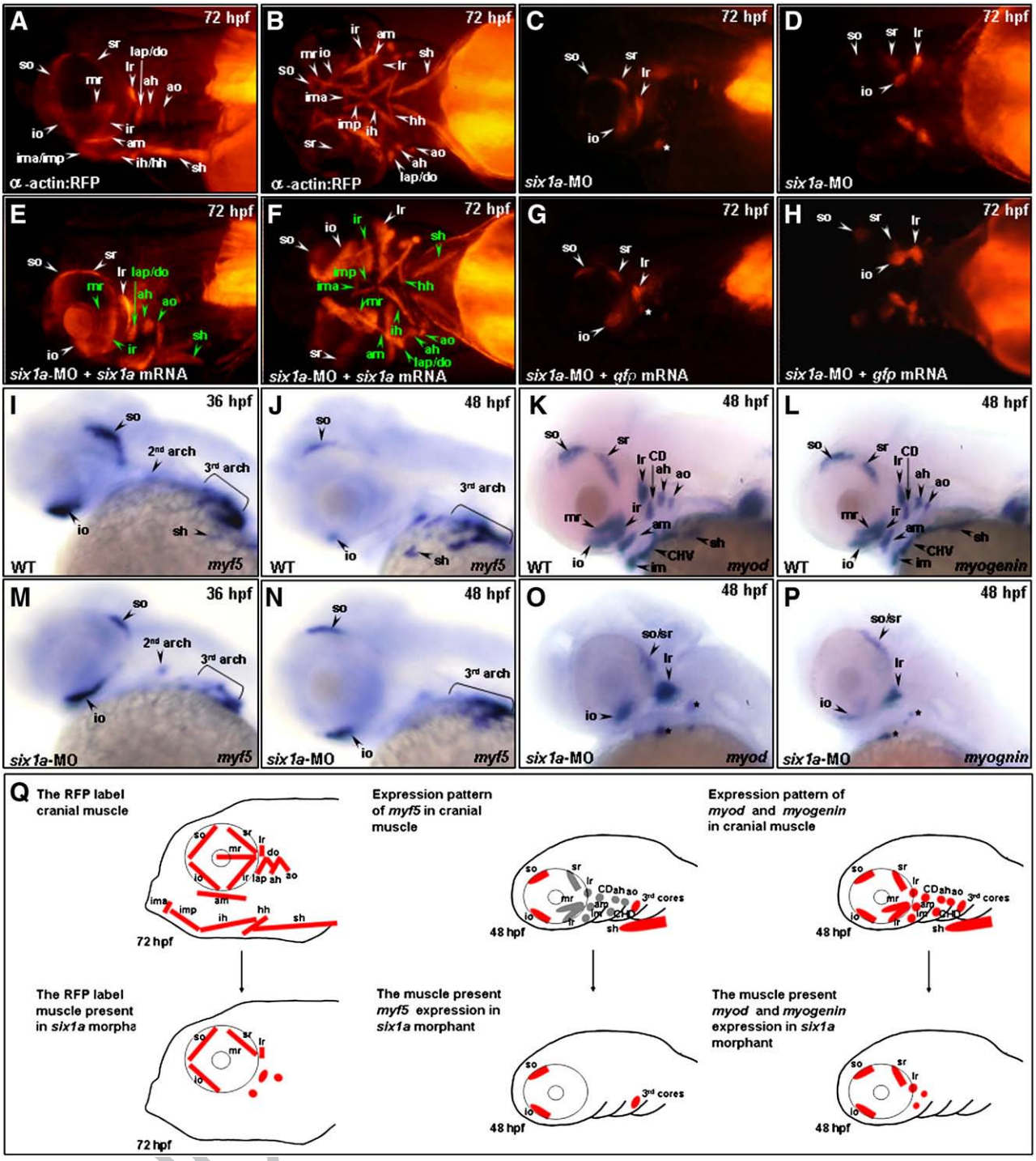

Fig. 2. Six1a is required for the development of mr, ir, sh and all pharyngeal muscles. Embryos derived from the transgenic line Tg(α-*actin*:RFP) (A–H), all of whose skeletal muscles appear as red fluorescent protein (RFP), were injected with 8 ng of *six1a*-morpholino oligonucleotide (MO) to specifically inhibit *six1a* mRNA translation. RFP signal was detected only in the so, io, sr, lr, and remnant dorsal branchial arch muscle (white star) primordia in the *six1a*-MO-injected embryos (A vs. C and B vs. D). When embryos were injected with 8 ng of *six1*-MO together with 150 pg of *six1a* mRNA, results showed that the defective muscle primordia induced by *six1a*-MO were rescued and appeared as RFP-labeled muscles (E, F; the rescued muscles are marked in green typeface). In contrast, the rescue experiment failed when embryos were injected with 8 ng of *six1a*-MO with 200 pg of *gfp* mRNA (G, H), suggesting that the defects of *six1a* morphants were specific. The expressions of *myf5* (I, J, M, N), *myod* (K, O), and *myogenin* (L, P) were also observed at the stages indicated. When wild-type embryos were injected with *six1a*-MO, *myf5* was expressed normally in the *six1a* morphants, both at 36- (I vs. M) and at 48-hpf (J vs. N), except sh. On the other hand, the expressions of *myod* (K vs. O) and *myogenin* (L vs. P) were decreased in the extraocular io, so, sr and lr in the *six1* morphants at 48 hpf. Weak signals of *myod* and *myogenin* were also noticed in the remnant dorsal branchial muscles (black stars) of *six1a* morphants. The schematic diagram illustrates the cranial muscle defects in *six1a* morphants and compares the expressions of *myf5*, *myod* and *myogenin* between wild-type (upper row, Q) and *six1a* morphants (lower row, Q). Lateral view: A, C, E, G and I–P; and ventral view: B, D, F and H. ah, adductor hyoideus; am, adductor mandibulae; ao, adductor operculi; do, dilator operculi; hh, hyohyoideus; ih, interhyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; io, inferior oblique; ir, inferior rectus; lap, levator arcus palatini; lr, lateral rectus; mr, medial rectus; sh, sternohyoideus; so, superior oblique and sr, superior rectus.

confirm whether the defects in pharyngeal muscles in the *six1a* morphants were exclusively the result of Six1a loss or the result of defective mesoderm core, defective endoderm pharyngeal pouch, or neural crest cells. To accomplish this, we detected the expressions of *et1*, *fgf3* and *dlx2*, which are the gene markers of the ventral mesoderm core (Miller et al., 2000), the endoderm pouch (David et al., 2002) and

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029

Table 1
Six1 is required for the developments of medial rectus (mr), inferior rectus (ir), sternohyoideus (sh) and all arch muscles.

six1-MO-injected concentration	Defect (%)		
	Absent-muscle	Reduced-muscle	Wild-type like
Uninjected	0 (0/107)	0 (0/107)	100 (107/107)
1 ng	3.1 (3/98)	91.8 (90/98)	5.1 (5/98)
2 ng	19.0 (20/105)	76.2 (80/105)	4.8 (5/108)
4 ng	42.1 (48/114)	55.2 (63/114)	2.7 (3/114)
6 ng	65.1 (58/89)	34.9 (31/89)	0 (0/89)
8 ng	81.8 (54/66)	18.2 (12/66)	0 (0/66)

The morphological defects were observed at 72 hpf. Absent-muscle defect indicated that mr, ir, sh and ventral arch muscles were completely lost but remnants of dorsal arch muscles still remained. Reduced-muscle defect indicated that mr, ir, sh and all arch muscles were partially lost. Wild-type like phenotype indicated that the head muscles were not lost.

neural crest cells (Akimenko et al., 1994), respectively. Results showed that both *et1* (Figs. 3A vs. B), *fgf3* (Figs. 3C vs. D) and *dlx2* (Figs. 3E vs. F) were normally expressed in the *six1a*-MO-injected embryos at 36 hpf, indicating that mesoderm core, endoderm pouch and neural crest cells develop normally. The loss of pharyngeal arch muscle in *six1a* morphants does not arise from the lost structures of mesoderm core, pharyngeal pouch and neural crest cells. Therefore, we proposed that Six1a is directly involved in pharyngeal arch myogenesis.

six1a links with either myf5 or myod to modulate the development of craniofacial muscles

Based on the expression patterns and the muscle defects which occurred in the *six1a* morphants, we hypothesized the plausibility of a *myf5-six1a-myod* regulatory pathway in craniofacial muscle development. After we confirmed the specific activities of *myf5*-MO and *myod*-MO (Supplemental Fig. S3), we microinjected either *myf5*-MO or *myod*-MO together with *six1a* mRNA to determine which embryo would be rescued by *six1a* mRNA from MO-induced defect. Results showed that both *myf5*- (Figs. 4E–H) and *myod*-morphants (Figs. 4I–K) failed to be rescued from their muscle defects through the addition of exogenous *six1a* mRNA (Wild type control, Figs. 4A, B). Therefore, we next microinjected *six1a*-MO with either *myf5* mRNA or *myod* mRNA to determine which mRNA enabled rescue of the embryos from the defects induced by *six1a*-MO (*six1a*-MO phenotype control, Figs. 4C, D). By co-injection of *six1a*-MO with *myf5* mRNA, results showed that, while the loss of mr, ir and the remnants of pharyngeal arch muscle were observed, muscle sh had been rescued (Figs. 4M, N; and Table 2). This evidence suggests that *myf5* may not be an upstream modulator of *six1a*. Instead, *myf5* and *six1a* may independently regulate the craniofacial muscles derived from the head paraxial mesoderm, mr, ir and the pharyngeal arch muscles. In contrast, co-injection of *myod* mRNA enabled embryos to rescue all head muscle defects induced by *six1a*-MO, suggesting that *six1a* was the upstream regulatory gene of *myod* (Figs. 4O, P; and Table 2).

It is noteworthy that the muscle sh, which originates from the trunk, could be rescued by *myf5* mRNA in the *six1a* morphants. Based on this evidence, we hypothesized that Six1a plays roles in different regulatory pathways between cranial and trunk myogenesis. To demonstrate this hypothesis, we analyzed the development of the sh, fin bud (fb) and posterior hypoaxial muscle (phm) that come from the dermomyotome in the anterior somites. Using whole-mount *in situ* hybridization, we observed that *six1a*, *myf5* and *myod* were all expressed in the muscle primordia of wild-type at 36 hpf (Figs. 5B, C;

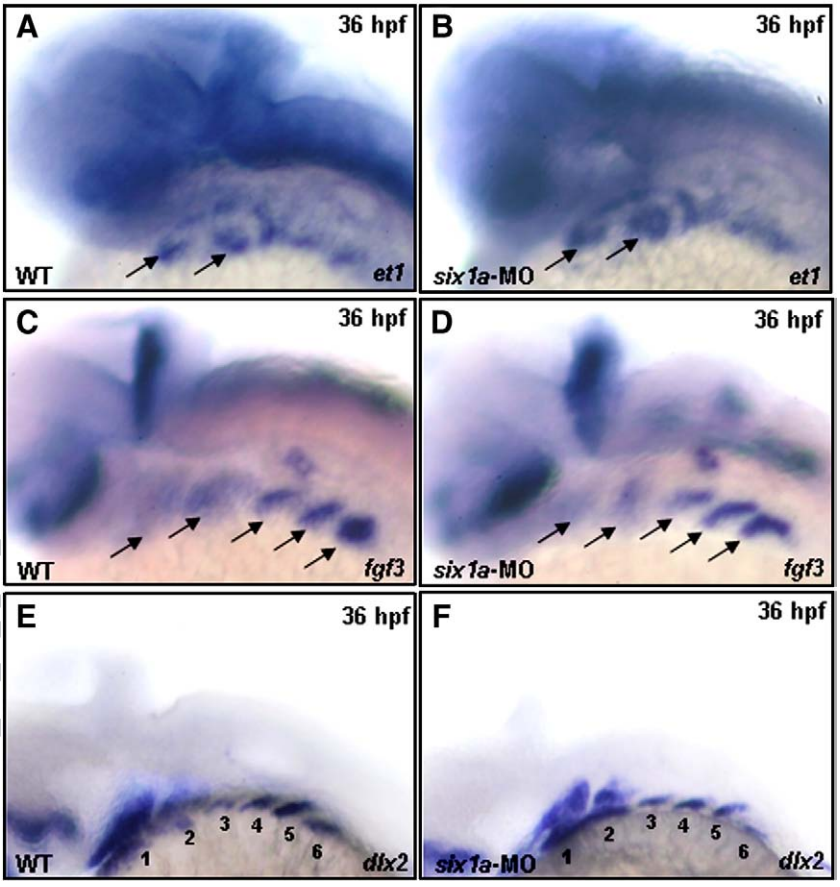

Fig. 3. Loss of Six1a function does not impede the normal development of mesoderm core and pharyngeal pouch. The expression patterns of *et1*, *fgf3* and *dlx2* were examined in the wild-type and in the *six1a*-MO-injected embryos at 36 hpf. Results showed that the transcripts of *et1*, *fgf3* and *dlx2* exhibited similarly in ventral mesoderm cores (A vs. B, arrows), pharyngeal pouches (C vs. D, arrows), and neural crest cells (E vs. F, six arches), respectively, between wild-type embryos and *six1a* morphants.

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029

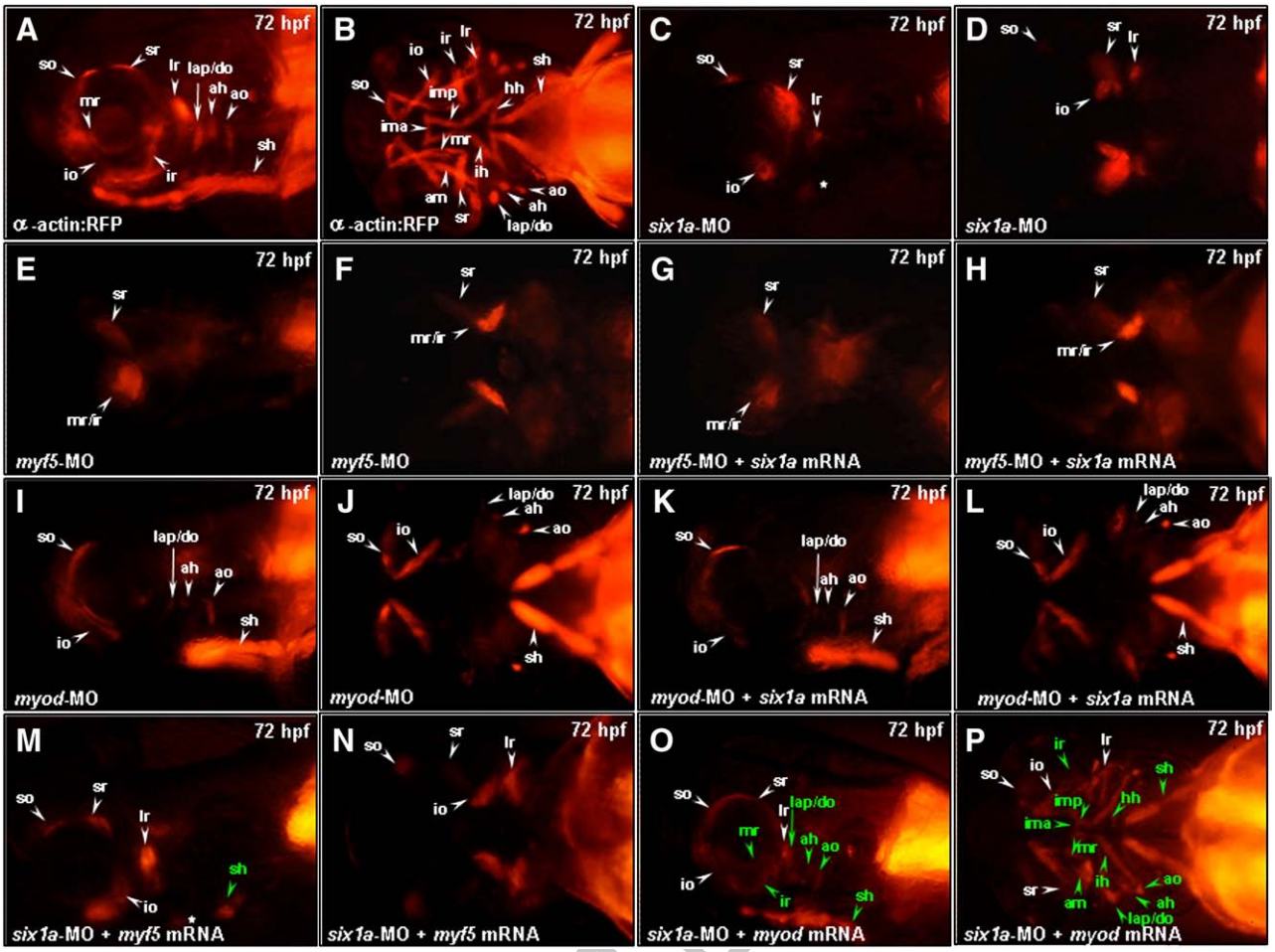

Fig. 4. Injection of *myod* mRNA enables embryos to rescue the defective muscle derived from cranial mesoderm in the *six1a* morphants. Embryos derived from Tg (α-*actin*:RFP) (A, B as non-treated) were injected with 8 ng of *six1a*-MO (C, D) and either 4 ng of *myf5*-MO (E, F) or *myod*-MO (I, J) to serve as control groups. Embryos injected with 4 ng of *myf5*-MO and 150 pg of *six1a* mRNA (G, H), 4 ng of *myod*-MO and 150 pg of *six1a* mRNA (K, L), 8 ng of *six1a*-MO and 100 pg of *myf5* mRNA (M, N), and 8 ng of *six1a*-MO and 50 pg of *myod* mRNA (O, P) were used to examine the appearance of RFP-labeled muscles. Results showed that only sr and mr/ir muscles exhibited in the *myf5*-MO-injected embryos and in the *myf5*-MO-*six1a*-mRNA-injected embryos (E vs. G and F vs. H). In contrast, only so, io, lap/do, ah, ao, and sh muscles exhibited in the *myod*-MO-injected embryos and in the *myod*-MO-*six1a*-mRNA-injected embryos (I vs. K and J vs. L). Similar to *six1a* morphants, embryos co-injected with *six1a*-MO and *myf5* mRNA exhibited the so, io, sr, lr and remnant dorsal branchial arch muscles. However, injection of *myf5* mRNA enabled embryos to rescue only sh primordia muscle among defects induced by *six1a*-MO (M, N), while injection of *myod* mRNA enabled embryos to rescue all the defective muscle primordia induced by *six1a*-MO (O, P). The rescued muscles are marked in green typeface. Lateral view: A, C, E, G, I, K, M and O; Ventral view: B, D, F, H, J, L, N and P. ah, adductor hyoideus; am, adductor mandibulae; ao, adductor operculi; do, dilator operculi; hh, hyohyoideus; ih, interhyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; io, inferior oblique; ir, inferior rectus; lap, levator arcus palatini; lr, lateral rectus; mr, medial rectus; sh, sternohyoideus; so, superior oblique and sr, superior rectus.

and Supplemental Fig. S4). However, we also observed that the sh, fb and phm were absent in the *six1a*-MO-injected embryos derived from the transgenic line Tg (α-actin:RFP) at 72 hpf (Fig. 5D). In addition, *myf5* and *myod* lost their expressions in these muscles at 36 hpf (Figs. 5E, F). Thus, we reasoned that Six1a is required for the trunk migratory muscles. When *six1a*-MO was co-injected with *six1a* mRNA into embryos, the sh, fb and phm primordia appeared normally at 72 hpf (Fig. 5G). Furthermore, both *myf5* and *myod* were detected at 48 hpf (Figs. 5 H, I). Interestingly, co-injection of either *myf5* or *myod* mRNA could rescue the defective development of sh, fb and phm primordia induced by *six1*-MO at 72 hpf (Figs. 5J, M). The expressions of *myod* and *myogenin* were partially restored in the embryos co-injected with *myf5* mRNA and *six1a*-MO when observed at 48 hpf (Figs. 5K, L). Similarly, the expressions of *myf5* and *myogenin* were also partially rescued in the embryos co-injected with *myod* mRNA and *six1a*-MO (Figs. 5N, O). Thus, we concluded that *six1a* is required for the expressions of *myf5* and *myod* in sh and trunk myogenesis, but *six1a* activates *myf5* and *myod* through the *six1a*-*myf5* pathway and *six1a*-*myod* pathway, respectively.

Table 2
The loss of mr, ir and arch muscles, which originate from cranial mesoderm, in the *six1*-morphants was rescued by *myod* mRNA, but not *myf5* mRNA.

six1-MO-injected concentration	Defect[a] (%)		
	Absent-muscle	Reduced-muscle	Wild-type like
8 ng	85.7 (66/77)	13.0 (10/77)	1.3 (1/77)
8 ng + 150 pg *six1* mRNA	20.5 (14/68)	44.1 (30/68)	35.4 (24/68)
8 ng + 250 pg *six1* mRNA	23.4 (11/47)	36.2 (17/47)	40.4 (19/47)
8 ng + 50 pg *myf5* mRNA	86.0 (49/57)	14.0 (8/57)	0 (0/57)
8 ng + 100 pg *myf5* mRNA	84.1 (53/63)	14.3 (9/63)	1.6 (1/63)
8 ng + 50 pg *myod* mRNA	21.3 (16/75)	42.7 (32/75)	36.0 (27/75)

The morphological defects were observed at 72 hpf. Absent-muscle defect indicated that mr, ir and ventral arch muscles were completely lost but the remnants of dorsal arch muscles still remained. Reduced-muscle defect indicated that mr, ir and all arch muscles were partially lost. Wild-type like phenotype indicated that the head muscles were not lost.

[a] The sh muscle was not included because it originates from the trunk.

Regulatory pathways that control six1a expression

Six1a plays important roles in pharyngeal arch muscle, mr and ir development. It has been reported that mouse T-box gene, *tbx1*, is an early cranial mesoderm inducer which regulates arch muscle

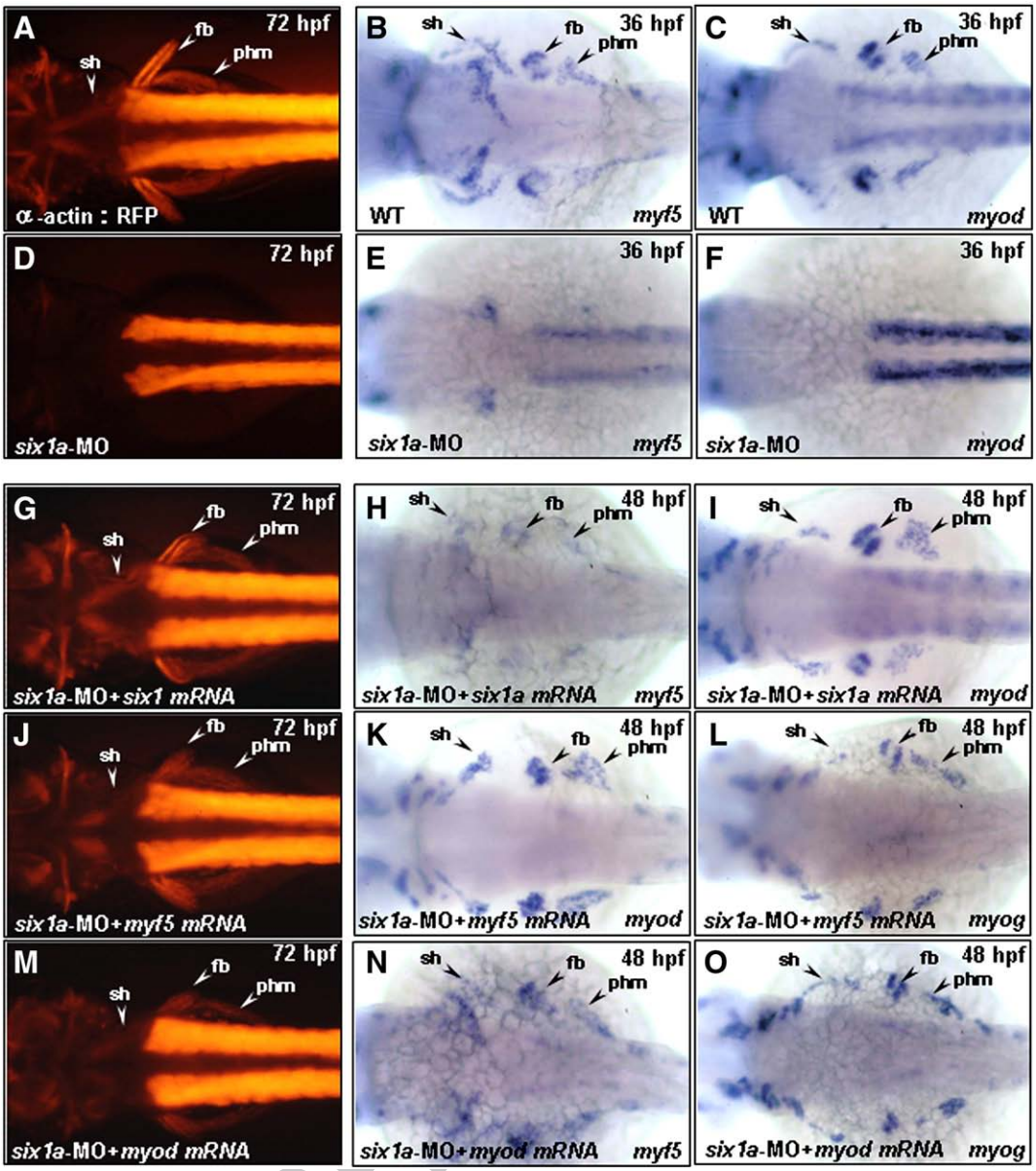

Fig. 5. Injection of *six1a*-, *myf5*-and *myod* mRNA enables embryos to rescue trunk migratory head muscle sternohyoideus (sh) defect in *six1a* morphants. Dorsal views of embryos derived either from the transgenic line Tg(α-*actin*:RFP) (A, D, G, J, M) or from the wild-type (B, C, E, F, H, I, K, L). The RFP expression in the embryos derived from the transgenic line at 72 hpf (A) and the detection of *myf5* and *myod* by whole-mount *in situ* hybridization at 36 hpf (B, C) served as control groups. Injection of embryos with either 8 ng of *six1a*-MO alone (D–F) or co-injection with 8 ng of *six1a*-MO and 150 pg of *six1a* mRNA (G–I), 100 pg of *myf5* mRNA (J–L) or 50 pg of *myod* mRNA (M–O) were examined. RFP, *myf5* and *myod* were not detected in sh, fb, or phm primordia in the *six1a* morphants (D–F); however, co-injection of *six1a* mRNA enabled embryos to rescue the defective expressions of RFP, *myf5* and *myod* in sh, fb and phm primordia induced by *six1a*-MO at 48 hpf (H, I) and at 72 hpf (G). Meanwhile, injection of *myf5* mRNA enabled embryos to rescue the defective expressions of RFP, *myod* and *myogenin* in sh, fb and phm primordia induced by *six1a*-MO at 48 hpf (K, L) and at 72 hpf (J). Injection of *myod* mRNA enabled embryos to rescue the defective expressions of RFP, *myf5* and *myogenin* in sh, fb and phm primordia induced by *six1a*-MO at 48 hpf (N, O) and at 72 hpf (M). fb, fin bud; phm, posterior hypoaxial muscle; and sh, sternohyoideus.

development (Grifone and Kelly, 2007; Kelly et al., 2004; Piotrowski et al., 2003). To understand whether *tbx1* is upstream of *six1a*, we detected the expression of *tbx1* and found that *tbx1* was expressed in zebrafish pharyngeal arch region at 36 hpf (Fig. 6A). In *tbx1*-MO-injected embryos derived from the transgenic line *Tg(alpha-actin-RFP)*, all the pharyngeal arch muscles were lost when observed at 72 hpf under fluorescent microscopy. However, the *six* extraocular muscles were normally developed (Fig. 6E). We also detected *six1a*, *myf5* and *myod* expressions in the *tbx1*-MO-injected embryos. Results showed that the expressions of *six1a*, *myf5* and *myod* were lost in pharyngeal arch muscles, but not the extraocular muscles (Figs. 6B–D), suggesting that *tbx1* is required for the expressions of *six1a*, *myf5* and *myod* in pharyngeal arch muscle. Interestingly, when *six1a*-, *myf5*- or *myod* mRNA was co-injected with *tbx1*-MO, we found that *myf5*-mRNA (Fig. 6G), but not *six1a*-mRNA or *myod* mRNA, enabled rescue of embryos from the defects induced by *tbx1*-MO (Figs. 6F, H). This evidence strongly suggests that *tbx1* is an upstream modulator of *myf5*, regulating the specification of cranial muscle development through *myf5*; however, the findings also indicate that *tbx1* is not a direct upstream regulator of *six1a*.

Next, we studied whether the EYA-DACH-SIX-PAX pathway, which plays a critical function in the trunk muscle development of mouse, is also involved in the head muscle development of zebrafish. When we detected the *myf5* and *myod* expressions in the *eya1*-knockdown morphants, we found that both *myf5* (Figs. 7A vs. C) and *myod* (Figs. 7D vs. F) were normally expressed in the *eya1*-MO-injected

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029

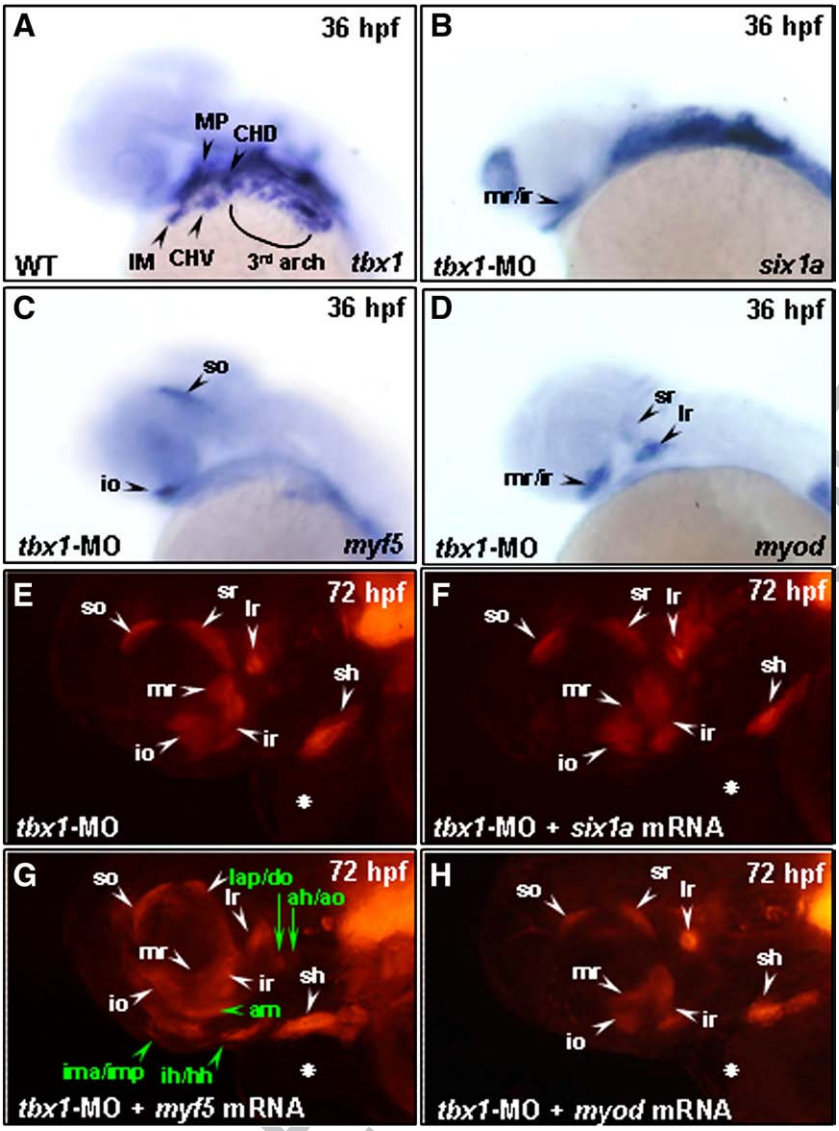

Fig. 6. The function of Six1a in branchial muscle development requires Tbx1 and Myf5 to play a specification role on arch muscle cell fate. Embryos derived from the wild-type strain (A–D) and from the transgenic line Tg(α-*actin*:RFP) (E–H) were examined at lateral view. Whole-mount *in situ* hybridization was used to detect the *tbx1* expression in arch muscle and cranial mesoderm in wild-type embryos at 36 hpf (A). Compared to the above control embryos, the expressions of *six1a* (B), *myf5* (C) and *myod* (D) in the 10 ng group of *tbx1*-MO-injected embryos were lost in arch muscles, but retained in extraocular muscles. We also observed that all the pharyngeal arch muscles were lost, but that 6 extraocular muscles developed normally in the 10 ng group of *tbx1*-MO-injected embryos derived from Tg(α-*actin*:RFP) at 72 hpf (E). With co-injection of 10 ng of *tbx1*-MO and either 150 pg of *six1a* mRNA (F), 100 pg of *myf5* mRNA (G) or 50 pg of *myod* mRNA (H) in embryos, we found that only *myf5* mRNA enabled embryos to rescue the RFP expression in lap, do, ah, ao, am, ima/imp and ih/hh (marked in green typeface of G). The heart defect induced by injection of *tbx1*-MO is labeled with a white star. ah, adductor hyoideus; am, adductor mandibulae; ao, adductor operculi; do, dilator operculi; hh, hyohyoideus; ih, interhyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; io, inferior oblique; ir, inferior rectus; lap, levator arcus palatini; lr, lateral rectus; mr, medial rectus; sh, sternohyoideus; so, superior oblique and sr, superior rectus. Embryos were all lateral views.

embryos. In addition, we injected *eya1*-MO into the embryos derived from transgenic line *Tg(α-actin:RFP)* and found that the head muscles were still developed normally in the *eya1* morphants (Figs. 7I, L), even though the *eya1* morphant suffered from reduced size of the inner ear (Supplemental Fig. S5), a phenotype similar to that of the *eya1* mutant described by Kozlowskia et al. (2005). In contrast, when we detected the *myf5* and *myod* expressions in the *pax3*-knockdown morphants, we found that *myf5* was detected in the head muscles that originated from the mesoderm (Figs. 7A vs. B), and *myod* was detected only in so, io, sr and lr muscle primordia in the *pax3*-MO-injected embryos (Figs. 7D vs. E), suggesting that *pax3* is necessary for the development of mr and ir, all arch muscles and sh. Moreover, when we injected *pax3*-MO into the *Tg(α-actin:RFP)* embryos, we found that only the so, io, sr and lr muscles remained unchanged, which was similar to that of *six1a*-MO morphants (Figs. 7G, H, J, K). When we co-injected *six1a* mRNA with *pax3*-MO, it was observed that the head muscle defects induced by *pax3*-MO could not be rescued (Figs. 7M, P). Similarly, neither *myf5* nor *myod* mRNA was able to rescue the *pax3*-morphant defects (Figs. 7N, O, Q, R). To better understand the role *pax3* plays in craniofacial muscle development, we analyzed the expression pattern of *pax3* by whole-mount *in situ* hybridization during late embryogenesis. Results showed that *pax3* was not detected in the cranial muscles, with the exception of sh (Supplemental Fig. S6). However, the absence of Pax3 function caused a severe defect in the head muscles. Based on this evidence, we suggest that the modulation of *pax3* on the cranial muscle development from head mesoderm is indirect in manner. It is also clear that *six1a* and *pax3* do not function in the same regulatory network during cranial muscle development.

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029

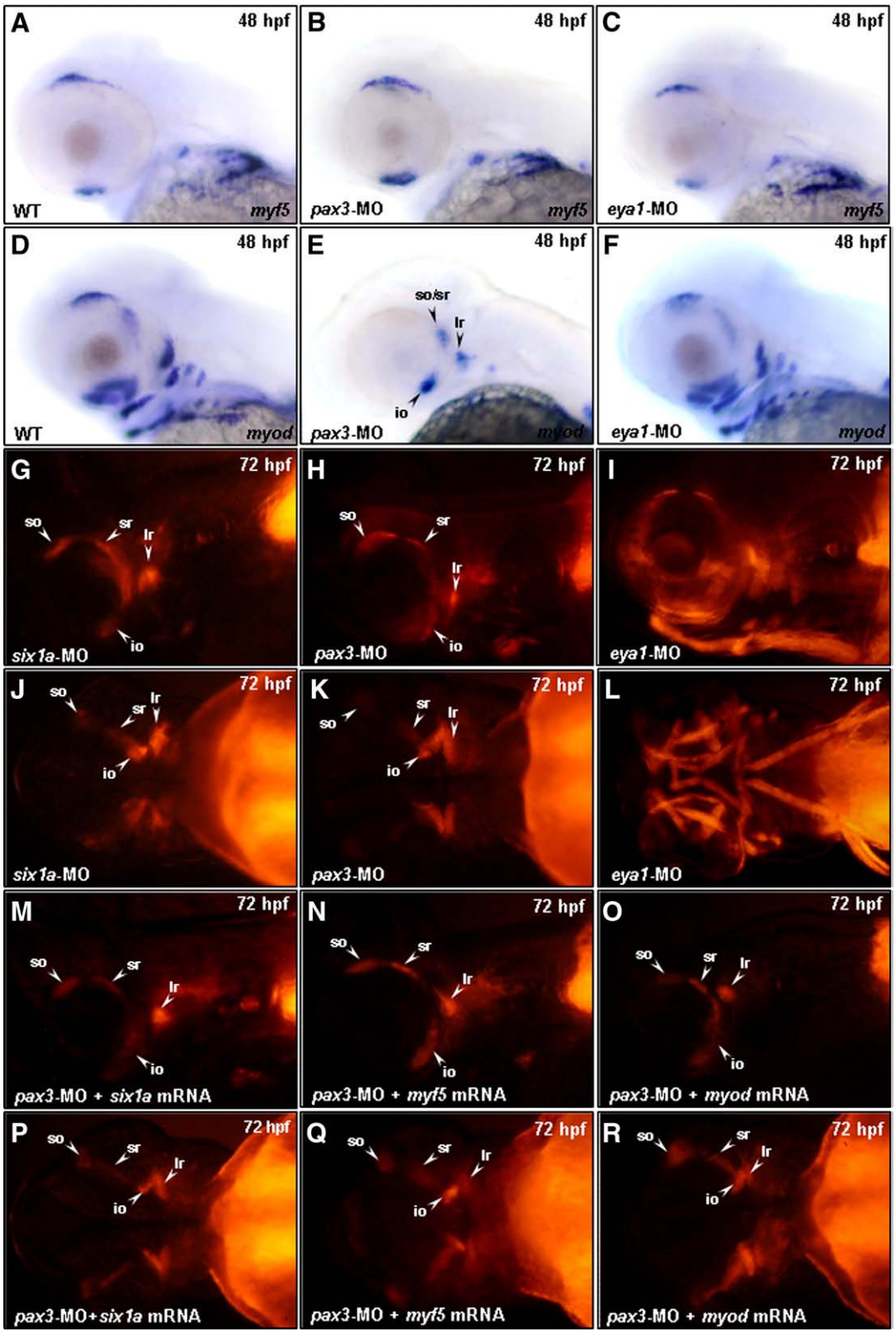

Fig. 7. Similar to Six1a, Pax3 is required for the development of mr, ir and all arch muscles. Embryos derived from the wild-type strain (A–F) and from the transgenic line Tg(α-*actin*: RFP) (G–R) were examined. Whole-mount *in situ* hybridization was used to detect the expressions of *myf5* and *myod* at 48 hpf in the wild-type embryos, which served as a control group (A, D). Compared to the control group, embryos injected with 3 ng of *pax3*-MO exhibited a normal expression of *myf5* in head muscle primordia at 48 hpf (B), but these embryos expressed *myod* only in so/sr, lr, io, and some remnant branchial muscles (E). Embryos injected with 10 ng of *eya1*-MO expressed *myf5* and *myod* normally in head muscle primordia at 48 hpf (C, F). We also noticed that the RFP expression of embryos injected with *six1a*-MO was similar to that of control group at 72 hpf (G vs. J). The RFP signal appeared in the so, io, sr, lr and some remnant branchial muscles in *pax3*-MO-injected embryos, which was similar to that of the *six1a* morphants at 72 hpf (G vs. H and J vs. K). The RFP signal appeared in all cranial muscles of the *eya1*-MO-injected embryos at 72 hpf (I and L). By co-injection of 10 ng of *pax3*-MO with 150 pg of *six1a* mRNA (M, P), 100 pg of *myf5* mRNA (N, Q) or 50 pg of *myod* mRNA (O, R), we found that the defective expressions of *six1a*, *myf5* and *myod* could not be rescued in the *pax3* morphants.

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029

Discussion

Myf5 and Myod play crucial functions in modulating the expression of genes encoding the muscle structural proteins and, finally, permitting the assembly of myofibers (Molkentin and Olson, 1996; Buckingham, 2001). In previous studies, we demonstrated that the role of myogenic regulatory factor *myf5* is markedly different from that of *myod* during craniofacial muscle development in zebrafish through three pathways (Lin et al., 2006). However, it remained unclear whether other factors might be involved in the regulation of *myf5* and *myod* expression in craniofacial muscle development. Here, we study the roles that transcriptional factors *six1a*, *tbx1* and *pax3* play in regulating *myf5* and *myod* expressions in craniofacial muscle development. Based on expression patterns and knockdown phenotypes, we, therefore, proposed four putative regulatory pathways along which these myogenic regulatory factors function with particular focus on Myf5 and Myod (Fig. 8).

Regulatory networks of Tbx1, Six1a, Pax3, Myf5 and Myod during cranial myogenesis

During zebrafish cranial muscle development, the arch I and II mesoderm cores are subdivided into dorsal and ventral mesoderm cores. The dorsal mesoderm cores are the precursors of lap, do, am, ah, and ao, whereas the ventral mesoderm cores are the precursors of ima, imp, ih, and hh. We previously found that both *myf5* and *myod* are expressed in the dorsal mesoderm cores, but only *myod* is expressed in the ventral mesoderm (Lin et al., 2006). Nathan et al. (2008) also showed that chick *myf5* expresses in the dorsal region of arch I mesoderm core at an early stage and functions synergistically with Isl1 to commit cell fates to be different from those in ventral arch I mesoderm core.

Pathway I is involved in dorsal arch muscle development. In this study, we demonstrate that the expressions of *myf5*, *six1a* and *myod* are not detected in the dorsal arch region of embryos treated with *tbx1*-MO. However, only injection of *myf5* mRNA can enable rescue of the embryos from the defects induced by *tbx1*-MO (Fig. 6). We therefore propose that *tbx1* determines cell fates to myogenic lineage through the modulation of *myf5*. Once *myf5* is expressed, *myod* expression starts to increase. On the other hand, we found that *myf5* was expressed normally in the embryos injected with either *six1a*- or *pax3*-MO (Figs. 2, 7), whereas *myod* (Figs. 2, 7) and *myogenin* (Fig. 2) transcripts were severely reduced in the dorsal arch of *six1a*-and *pax3*-morphants. In addition, injection of *myod* mRNA enables rescue of embryos from the defects induced only by *six1a*-MO (Figs. 3, 7). Taken together, we conclude that *six1a* and *pax3* are not involved in

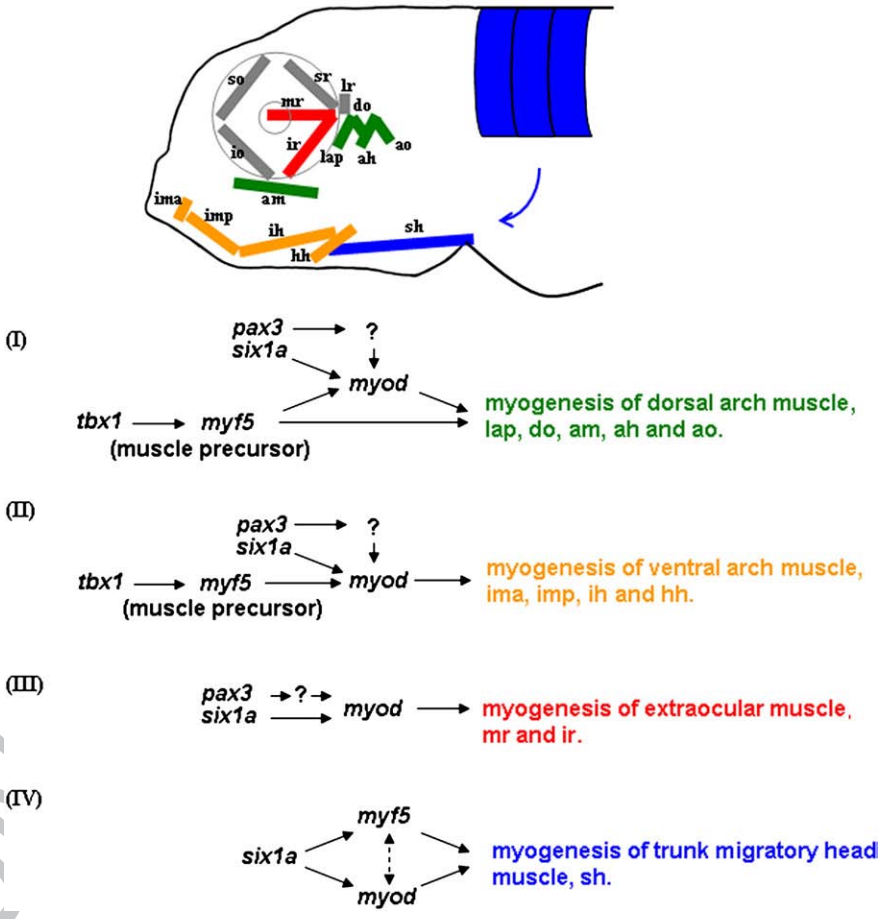

Fig. 8. Regulatory network model for *tbx1*, *six1a*, *pax3*, *myf5* and *myod*, which are involved in the craniofacial muscle development of zebrafish embryos. Based on the evidence presented in this study, we propose that the development of all cranial muscles of zebrafish is through four regulatory pathways, which is a modification of model presented by Lin et al. (2006). To summarize, Pathway I (marked in green): for dorsal arch muscles, lap, do, ah, ao and am. The myogenic regulatory factor *tbx1* activates *myf5* to initiate myogenesis. As a consequence, the basal level of *myf5* triggers *myod* expression to further myogenic processes. Subsequently, *myod*, which is directly controlled by *six1*, but indirectly controlled by *pax3*, maintains and enhances a high level of myogenesis. Pathway II (marked in yellow): for the precursors of ventral arch muscles, ima, imp, ih and hh. Before subdivision, *tbx1* activates *myf5* expression to determine the myogenic cell fate. After subdivision, the major role of *myod* is to trigger the continuation of myogenic processes. Subsequently, *myod*, which is directly controlled by *six1a*, but indirectly controlled by *pax3*, maintains and enhances a high level of myogenesis. Pathway III (marked in red): for extraocular muscles, mr and ir. The myogenic regulatory factor *myod* initiates myogenesis, and its expression is controlled by *six1a* directly, and by *pax3* indirectly, to maintain and enhance myogenesis. Pathway IV (marked in blue): for trunk migratory head muscle, sh. The MRF *six1a* directly controls both *myf5* and *myod* in the myogenesis process, but *myf5* and *myod* have redundant function.

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029

myogenesis initiation. Rather, they are required for maintaining a high level of *myod* transcripts so that myogenesis can be processed. We also notice that *myod* mRNA enables rescue of embryos from the defects induced by *six1a*-MO, but not by *pax3*-MO, suggesting that, while the expression of *myod* in the dorsal arch is directly controlled by *six1a*, it is only indirectly controlled by *pax3*, perhaps through the interaction of still unknown regulatory modules.

Pathway II is involved in ventral arch muscle development. Ventral mesodermal core primordia originate from the *myf5*-positive core and require Tbx1 and Myf5 to initiate myogenic lineage. In this study, we found that the expressions of *six1a* and *myod* are lost in the ventral arch region of *tbx1* morphants, but that this defect can be rescued by overexpression of *myf5* mRNA (Fig. 6). Therefore, we propose the involvement of two steps in the development of ventral arch muscle: *tbx1* initiates *myf5* expression in the beginning, and, subsequently, *six1a* enhances and maintains *myod* expression. Furthermore, similar to Pathway I in dorsal arch, we also found that the expressions of *myod* (Figs. 2, 7) and *myogenin* (Fig. 2) are severely reduced in ventral arch when embryos are injected with both *six1a*-and *pax3*-MO. Thus, we conclude that *six1a* and *pax*3 are required to maintain *myod* expression in the ventral mesodermal core cells. Again, as in Pathway I, we found that overexpression of *myod* mRNA enables rescue of embryos from the defects induced only by *six1a*-MO, but not *pax3*-MO, suggesting that the regulation of *myod* in the ventral arch is directly controlled by *six1a*, but indirectly controlled by *pax3*.

Pathway III is involved in the development of extraocular muscles, mr and ir. We found that *six1a* and *myod* transcripts are detected, but not *myf5* transcript, in mr and ir (Fig. 1). Meanwhile, the expressions of *myod* (Figs. 2, 7>) and *myogenin* (Fig. 2) were lost in the mr and ir of *six1a*- and *pax3*-morphants. Injection of *myod* mRNA enables rescue of embryos from the defects induced by *six1a*-MO, but not induced by *pax3*-MO (Figs. 3, 7). Therefore, *myod* is required for the development of mr and ir, and *myod* is directly regulated by *six1a*. Interestingly, although *six1a* and *myod* transcripts are expressed in all extraocular muscles, the so, io, sr and lr are still observed in the *six1a* morphants, suggesting that other factors may be involved in controlling the development of so, io, sr and lr.

Pathway IV is involved in the development of sh primordia, which originates from anterior trunk somites. We found that the transcript of sh was lost in the *six1a* morphants, but injection of either *myf5* mRNA or *myod* mRNA enables embryos to be rescued from this defect (Fig. 5). Since sh muscle is derived from anterior trunk somites, the regulatory mechanisms controlling the muscle development between head and trunk paraxial mesoderm are different. This evidence suggests that *six1a* is governed by a head-specific regulatory cascade in cranial myogenesis, which is fundamentally distinct from that which is governed by a trunk-specific regulatory cascade. Taken together, we conclude that *six1a* performs its function in head muscle development, but it does so separately and by two distinct pathways, one where muscle development originates from head mesoderm and one where muscle development originates from the trunk.

Networks supporting modulation of Sixla and MRFs are dependent on mesoderm origin

The branchiomeric muscles originate from cranial paraxial mesoderm. Knockdown of Six1a function results in greatly decreased expressions of *myod* and *myogenin*, while *myf5* is expressed normally. Because of the existence of *myf*5 in these muscles, the remaining arch muscles in Six1a knockdown embryos are observed. Nevertheless, *myod* transcripts are reduced, with the result that these muscles eventually lose their function (Fig. 3). Like branchiomeric muscles, the extraocular muscles originate from cranial paraxial mesoderm. Both *six1a* and *myod* transcripts are expressed in mr and sr muscles at 32 hpf, whereas *myf5* is not expressed (Fig. 2). Thus, when *six1a* is knocked down, the *myod* transcripts are lost, with the result that muscle primordia of mr and ir are not developed (Fig. 3). On the other hand, unlike branchiomeric muscles and extraocular muscles, the cranial muscle sh originates from trunk paraxial mesoderm, which is named dermomyotome. During sh muscle development, Six1a is required for both *myf5* and *myod* expressions (Fig. 5). This result is consistent with what we observe in fb and phm which originate from anterior trunk mesoderm in zebrafish. Therefore, both *myf5* mRNA and *myod* mRNA were injected in the attempt to rescue muscle defects of *six1a* morphants. While injection of *myf5* mRNA could rescue only the defective sh muscle in *six1a* morphants, injection of *myod* mRNA enabled rescue of embryos from all defective cranial muscles (Fig. 4). Based on these findings, it seems clear that the modulatory network between Six1a and these two MRFs in cranial paraxial mesoderm is quite different from that which is observed in trunk (sh) paraxial mesoderm. This conclusion is similar to, and supported by, the findings of Grifone et al. (2005) in mice. They described how *six1* and *six4* genes control *mrf4* expression and that $six1^{-/-}six4^{-/-}$ embryos display reduced and delayed expressions of *myod* and *myogenin*, whereas the early activation of *myf5* transcripts in the epaxial somite is unaffected. However, in the limb muscles, the Six1/4 are shown to be involved in m*yf5* transcription through binding the MEF3 site in the 145-bp regulatory sequence located at −57.5 kb of *myf5* gene (Giordani et al., 2007).

The *six* genes constitute a large family of genes that are highly conserved within the animal kingdom. In mammals, six members of the Six family have so far been identified, and these can be divided into three subclasses designated as Six1/2, Six3/6 and Six4/5 subfamilies (Laclef et al., 2003; Seo et al., 1999). Moreover, it has been subsequently demonstrated that Six1, Six2, Six4 and Six5 have a similar binding specificity to the ARE/MEF3 site possessing a consensus sequence TCAGGTTTC (Ohto et al., 1999; Spitz et al., 1998). In mice, the defects of muscle hypoplasia in $six1^{-/-}six4^{-/-}$ embryos are more severe than those seen in $six1^{-/-}$ embryos (Grifone et al., 2005; Laclef et al., 2003). Similarly, the reduced expression of *myf5* in the hind limb of $six1^{-/-}six4^{+/-}$ mice is more severe than that observed in $six1^{-/-}$ embryos. These lines of evidence suggest that Six4 in myogenic progenitor cells displays a redundant function with Six1. Meanwhile, in zebrafish, three isoforms, *six4.1–4.3*, and their expression patterns have been defined, and *six4.2* is expressed in the presomitic mesoderm, somites and pectoral fin bud (Kobayashi et al., 2000). In addition, Bessarab et al. (2008) reported that the fast muscles differentiate abnormally in the trunk muscles of *six1a* morphants in contrast to the slow muscles which develop normally. The expression of *myogenin* is reduced in all somites in the *six1a* morphants at the 9-somite stage. However, *myogenin* increases its expression at the 10-somite stage and finally reaches its normal expression level at the 13-somite stage. In our case, we also noticed that the cranial muscles in *six1a* morphants are partially developed. Thus, we speculate that zebrafish *six4.2* may have redundant function with *six1* during muscle development of zebrafish. The zebrafish Six4.2, like mouse Six4, might partially compensate for the absence of Six1 to activate MRFs in the trunk muscle cells. According to this hypothesis, the selective muscle hypoplasia described in *six1* morphants could result from either insufficient levels of Six4.2 to compensate for Six1 in the affected myogenic precursor cells or from the existence of specific Six1 target genes.

Comparison of Six1a, Pax3 and Eya1 functions in head muscle development between zebrafish and other model animals

Genetic studies in *Drosophila* have identified that the eyeless (*pax*) is synergistic with DNA binding homeodomain factors, such as sine oculis (*so*/*six*), and nuclear cofactors, such as eyes absent (*eya*) and dachshund (*dach*) (Cheyette et al., 1994; Bonini et al., 1993; Mardon et al., 1994). Mutation of any gene encoding for these proteins leads

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029

to the failure of eye formation, and even the ectopic expressions of these genes cause an additional eye formation (Bonini et al., 1997; Shen and Mardon, 1997). Dach2, Six1 and Eya2 were first described in the chick model system, and this report demonstrates that they synergistically regulate the expressions of Pax3 and MRFs during myogenesis (Heanue et al., 1999). Thereafter, mouse Six1 was found to have synergistic genetic interactions with Eya factor (Li et al., 2003), and Six1 knockout mice displayed defects in the kidney, muscle and inner ear (Ozaki et al., 2004). Li et al. (2003) concluded that binding Eya protein, which possesses phosphatase, can switch the Six1–Dach function from transcriptional repression to activation. Through this process, co-activators are recruited to regulate specific gene targets for controlling the proliferation and survival of precursor cells during mammalian organogenesis. Moreover, mouse *Six* and *Eya* genes are activated independently in the ventrolateral part of somitic dermomyotome, and the induction of *Pax3* in this region also relies on the interactions between Six and Eya proteins (Grifone et al., 2007). Band-shifted assay and chromatin immunoprecipitation studies reveal that *Pax3* expression is directly controlled by Six1 protein through binding to a conserved MEF3 site located in *Pax3* hypaxial enhancer sequence. In zebrafish, *eya1* and *six1* are proven to act synergistically in adenohypophyseal cell development (Nica et al., 2006). This evidence suggests that the interaction of Six1a, Eya1 and Pax3 may be conserved among species in trunk muscle development. However, in this study, we found that the functions of Six1a, Eya1 and Pax3 in the cranial muscle development of zebrafish are different from their functions in trunk muscle development: *eya1* does not affect head muscle development, whereas the influence of *pax3* on the gene expressions of MRFs in the head muscles is similar to *six1a*.

The function of *myod* in head muscle development is directly modulated by Six1a, but not *myf5*, with the exception of sh muscle which originates from trunk muscle. When the function of either Tbx1 or Myf5 is lost, Six1a is unable to initiate myogenesis in arch muscles. Knockdown of *six1a* results in the reduction of *myod* transcripts, suggesting that the role of Six1a in arch muscle does not involve lineage of specification. Instead, Six1a activates and maintains *myod* expression in order to help the proliferation and differentiation of muscle cells. Moreover, knockdown of tbx1 and *myf5* does not induce the defect occurring in extraocular muscles mr and ir, where *tbx1* and *myf5* are not expressed. Thus, *six1a* is capable of displaying the lineage of specification through the activation of *myod* expression.

Regarding Eya1, when we knocked down *eya1*, the expressions of *myf5* and *myod* in the head muscles of wild-type were normal, as well as the appearance of RFP in the head muscle of transgenic line (Fig. 7), suggesting that the loss of Eya1 function does not affect the cranial muscle development of zebrafish. In ZFIN, three Eya genes, Eya1, Eya3 and Eya4, are identified. The *eya1* (Sahly et al., 1999) and *eya4* (Schonberger et al., 2005) have been described as expressing in branchial arches (ba) and the developing somites (so) which are precursors of myogenic cells in zebrafish embryos. No major defects in head myogenesis were observed in the *eya1*-MO-injected embryos. Thus, we cannot exclude the possibility that either Eya4 or other Eya proteins could participate in a Six transcriptional complex which functions to activate downstream targets. Nevertheless, in zebrafish, adenohypophyseal cells in the *eya1* mutant maintain either an undifferentiated or de-differentiated state and therefore fail to initiate *gsud* and *pomc* expressions, suggesting that Eya1 is required for lineage-specific differentiation (Nica et al., 2006). However, knockdown of *six1* does not affect adenohypophyseal cells in lineage-specific differentiation steps in wild-type embryos. Nevertheless, when *six1* is knockdown in the embryos derived from *eya1* mutant, the proliferation of adenohypophyseal cells is affected and the specification defects are enhanced (Nica et al., 2006). Although *eya1* and *six1* are co-expressed in all adenohypophyseal cells, this evidence suggests that the stratum and extent of gene regulation are different. Therefore, we conclude that Six1a plays a more dominant function than Eya1 during head muscle development of zebrafish.

Regarding Pax3, when we knocked down *pax3*, the defective phenotypes of cranial muscles were found to be similar to those of *six1a* morphants (Fig. 7). However, co-injection of *six1a*-, *myf5*- or *myod* mRNA failed to rescue the defects induced by *pax3*-MO (Fig. 7). By comparing the expression patterns among *pax3*, *six1a*, *myf5* and *myod*, it can be seen that *pax3* is not expressed in head muscle primordia (Supplemental Fig. S6). Based on these results, we suggest that the modulation of *pax3* in head muscles may be through an indirect means. Moreover, we found that *myf5* is normally expressed in the cranial muscles in *pax3* morphants, which is consistent with the result obtained from mouse *myf5* studies, demonstrating that *myf5* has a function in cranial muscle development of *splotch* mutant (Tajbakhsh et al., 1997). Again, this evidence strongly supports our hypothesis that the regulatory networks involved in craniofacial muscle development and trunk muscle development are not identical. In addition, lost FoxD3 expression in the *splotch* mutant indicates that Pax3 plays important roles in neural crest development (Dottori et al., 2001). Zebrafish *pax3* mRNA is also expressed in the cranial neural crest precursors and is required for specification of two specific lineages of neural crest, xanthophores and enteric neurons (Minchin and Hughes, 2008). In chicken, ablation of the cranial neural crests influences paraxial mesoderm migration, resulting in the abnormal patterning of head muscles. Moreover, the muscle precursor cells fail to undergo terminal myogenic differentiation (Rinon et al., 2007). Therefore, we speculate that the lost function of Pax3, which leads to induction of defective cranial muscles, may be the result of abnormal neural crest cell development. Taken together, we conclude that the interaction among Six1a, Eya1 and Pax3 in the cranial muscle development of zebrafish is processed in a pathway very different from the Eya1–Six1–Pax3 pathway observed in the trunk muscle development of other model animals.

Upstream regulators of Six1a and possible pathways involved in head muscle development

In mice and chicks, transcription factors Pitx2, Tbx1, Tcf21 (Capsulin), and Msc (MyoR) are necessary for activation of MRFs during branchiomeric myogenesis. In contrast, Wnt and BMP signaling display negative modulators in head muscle development (Grifone and Kelly, 2007). Here, we are the first to demonstrate that zebrafish Tbx1 initiates myogenesis in arch muscles through *myf5*, while *six1a* functions in head muscle development through *myod* (Fig. 6). These data strongly support our previous conclusions reported in Lin et al. (2006), which indicated that the regulatory pathways involving *myf5* and *myod* are different during head muscle development.

By using P19 cells, Petropoulos and Skerjanc (2002) reported that canonical Wnt3a/β-catenin/Lef/TCF signaling activates the expressions of transcription factors, including Pax3, Mox1, Gli2, and Six1. In addition, Pax3 induces Six1 and Eya2 expressions in skeletal myogenesis (Ridgeway and Skerjanc, 2001). Thus, we speculate that Wnt signaling may also play an important role in head muscle development of zebrafish. Nevertheless, further study of the relationship between Wnt and Six1a during cranial myogenesis is more than justified.

Uncited reference

Tajbakhsh and Cossu, 1997

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.04.029.

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029

References

Akimenko, M.A., Ekker, M., Wegner, J., Lin, W., Westerfield, M., 1994. Combinatorial expression of 3 zebrafish genes related to distal-less-part of a homeobox gene code for the head. J. Neurosci. 14, 3475–3486.

Bessarab, D.A., Chong, S.W., Korzh, V., 2004. Expression of zebrafish six1 during sensory organ development and myogenesis. Dev. Dyn. 230, 781–786.

Bessarab, D.A., Chong, S.W., Srinivas, B.P., Korzh, V., 2008. Six1a is required for the onset of fast muscle differentiation in zebrafish. Dev. Biol. 323, 216–228.

Bonini, N.M., Leiserson, W.M., Benzer, S., 1993. The eyes absent gene-genetic-control of cell-survival and differentiation in the developing *Drosophila* eye. Cell 72, 379–395.

Bonini, N.M., Bui, Q.T., Gray-Board, G.L., Warrick, J.M., 1997. The *Drosophila* eyes absent gene directs ectopic eye formation in a pathway conserved between flies and vertebrates. Development 124, 4819–4826.

Bricaud, O., Collazo, A., 2006. The transcription factor six1 inhibits neuronal and promotes hair cell fate in the developing zebrafish (*Danio rerio*) inner ear. J. Neurosci. 26, 10438–10451.

Buckingham, M., 2001. Skeletal muscle formation in vertebrates. Curr. Opin. Genet. Dev. 11, 440–448.

Buckingham, M., 2006. Myogenic progenitor cells and skeletal myogenesis in vertebrates. Curr. Opin. Genet. Dev. 16, 525–532.

Chai, Y., Maxson, R.E., 2006. Recent advances in craniofacial morphogenesis. Dev. Dyn. 235, 2353–2375.

Chen, R., Amoui, M., Zhang, Z.H., Mardon, G., 1997. Dachshund and eyes absent proteins form a complex and function synergistically to induce ectopic eye development in *Drosophila*. Cell 91, 893–903.

Cheyette, B.N.R., Green, P.J., Martin, K., Garren, H., Hartenstein, V., Zipursky, S.L., 1994. The *Drosophila* sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual-system. Neuron 12, 977–996.

Dastjerdi, A., Robson, L., Walker, R., Hadley, J., Zhang, Z., Rodriguez-Niedenfuhr, M., Ataliotis, P., Baldini, A., Scambler, P., Francis-West, P., 2007. Tbx1 regulation of myogenic differentiation in the limb and cranial mesoderm. Dev. Dyn. 236, 353–363.

David, N.B., Saint-Etienne, L., Tsang, M., Schilling, T.F., Rosa, F.M., 2002. Requirement for endoderm and FGF3 in ventral head skeleton formation. Development 129, 4457–4468.

Dong, F.Y., Sun, X.X., Liu, W., Ai, D., Klysik, E., Lu, M.F., Hadley, J., Antoni, L., Chen, L., Baldini, A., Francis-West, P., Martin, J.F., 2006. Pitx2 promotes development of splanchnic mesoderm-derived branchiomeric muscle. Development 133, 4891–4899.

Dottori, M., Gross, M.K., Labosky, P., Goulding, M., 2001. The winged-helix transcription factor Foxd3 suppresses interneuron differentiation and promotes neural crest cell fate. Development 128, 4127–4138.

Drivenes, O., Seo, H.C., Fjose, A., 2000. Characterisation of the promoter region of the zebrafish six7 gene. Biochim. Biophys. Acta-Gene Struct. Expr. 1491, 240–247.

Giordani, J., Bajard, L., Demignon, J., Daubas, P., Buckingham, M., Maire, P., 2007. Six proteins regulate the activation of Myf5 expression in embryonic mouse limbs. Proc. Natl. Acad. Sci. U. S. A. 104, 11310–11315.

Grifone, R., Kelly, R.G., 2007. Heartening news for head muscle development. Trends Genet. 23, 365–369.

Grifone, R., Laclef, C., Spitz, F., Lopez, S., Demignon, J., Guidotti, J.E., Kawakami, K., Xu, P. X., Kelly, R., Petrof, B.J., Daegelen, D., Concordet, J.P., Maire, P., 2004. Six1 and Eya1 expression can reprogram adult muscle from the slow-twitch phenotype into the fast-twitch phenotype. Mol. Cell Biol. 24, 6253–6267.

Grifone, R., Demignon, J., Houbron, C., Souil, E., Niro, C., Seller, M.J., Hamard, G., Maire, P., 2005. Six1 and Six4 homeoproteins are required for Pax3 and Mrf expression during myogenesis in the mouse embryo. Development 132, 2235–2249.

Grifone, R., Demignon, J., Giordani, J., Niro, C., Souil, E., Bertin, F., Laclef, C., Xu, P.X., Maire, P., 2007. Eya1 and Eya2 proteins are required for hypaxial somitic myogenesis in the mouse embryo. Dev. Biol. 302, 602–616.

Heanue, T.A., Reshef, R., Davis, R.J., Mardon, G., Oliver, G., Tomarev, S., Lassar, A.B., Tabin, C.J., 1999. Synergistic regulation of vertebrate muscle development by Dach2, Eya2, and Six1, homologs of genes required for *Drosophila* eye formation. Genes Dev. 13, 3231–3243.

Kawakami, K., Ohto, H., Takizawa, T., Saito, T., 1996. Identification and expression of six family genes in mouse retina. FEBS Lett. 393, 259–263.

Kawakami, K., Sato, S., Ozaki, H., Ikeda, K., 2000. Six family genes—structure and function as transcription factors and their roles in development. BioEssays 22, 616–626.

Kelly, R.G., Jerome-Majewska, L.A., Papaioannou , V.E., 2004. The del22q11.2 candidate gene Tbx1 regulates branchiomeric myogenesis. Hum. Mol. Genet. 13, 2829–2840.

Kobayashi, M., Toyama, R., Takeda, H., Dawid, B., Kawakami, K., 1998. Overexpression of the forebrain-specific homeobox gene six3 induces rostral forebrain enlargement in zebrafish. Development 125, 2973–2982.

Kobayashi, M., Osanai, H., Kawakami, K., Yamamoto, M., 2000. Expression of three zebrafish Six4 genes in the cranial sensory placodes and the developing somites. Mech. Dev. 98, 151–155.

Kobayashi, M., Nishikawa, K., Suzuki, T., Yamamoto, M., 2001. The homeobox protein Six3 interacts with the Groucho corepressor and acts as a transcriptional repressor in eye and forebrain formation. Dev. Biol. 232, 315–326.

Kozlowskia, D.J., Whitfieldc, T.T., Hukrieded, N.A., Lamb, W.K., Weinberga, E.S., 2005. The zebrafish dog-eared mutation disrupts eya1, a gene required for cell survival and differentiation in the inner ear and lateral line. Dev. Biol. 277, 27–41.

Laclef, C., Hamard, G., Demignon, J., Souil, E., Houbron, C., Maire, P., 2003. Altered myogenesis in Six1-deficient mice. Development 130, 2239–2252.

Lee, H.C., Huang, H.Y., Lin, C.Y., Chen, Y.H., Tsai, H.J., 2006. Foxd3 mediates zebrafish myf5 expression during early somitogenesis. Dev. Biol. 290, 359–372.

Lee, H.C., Tsai, J.N., Liao, P.Y., Tsai, W.Y., Lin, K.Y., Chuang, C.C., Sun, C.K., Chang, W.C., Tsai, H.J., 2007. Glycogen synthase kinase 3α and 3β have distinct functions during cardiogenesis of zebrafish embryo. BMC Dev. Biol. 7, 93–107.

Li, X., Oghi, K.A., Zhang, J., Krones, A., Bush, K.T., Glass, C.K., Nigam, S.K., Aggarwal, A.K., Maas, R., Rose, D.W., Rosenfeld, M.G., 2003. Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. Nature 426, 247–254.

Lin, C.Y., Yung, R.F., Lee, H.C., Chen, W.T., Chen, Y.H., Tsai, H.J., 2006. Myogenic regulatory factors Myf5 and Myod function distinctly during craniofacial myogenesis of zebrafish. Dev. Biol. 299, 594–608.

L'Honore, A., Coulon, V., Marcil, A., Lebel, M., Lafrance-Vanasse, J., Gage, P., Camper, S., Drouin, J., 2007. Sequential expression and redundancy of Pitx2 and Pitx3 genes during muscle development. Dev. Biol. 307, 421–433.

Lu, J.R., Bassel-Duby, R., Hawkins, A., Chang, P., Valdez, R., Wu, H., Gan, L., Shelton, J.M., Richardson, J.A., Olson, E.N., 2002. Control of facial muscle development by MyoR and capsulin. Science 298, 2378–2381.

Mardon, G., Solomon, N.M., Rubin, G.M., 1994. Dachshund encodes a nuclear-protein required for normal eye and leg development in *Drosophila*. Development 120, 3473–3486.

Miller, C.T., Schilling, T.F., Lee, K.H., Parker, J., Kimmel, C.B., 2000. sucker encodes a zebrafish Endothelin-1 required for ventral pharyngeal arch development. Development 127, 3815–3828.

Minchin, J.E., Hughes, S.M., 2008. Sequential actions of Pax3 and Pax7 drive xanthophore development in zebrafish neural crest. Dev. Biol. 319, 530.

Molkentin, J.D., Olson, E.N., 1996. Defining the regulatory networks for muscle development. Curr. Opin. Genet. Dev. 6, 445–453.

Mootoosamy, R.C., Dietrich, S., 2002. Distinct regulatory cascades for head and trunk myogenesis. Development 129, 573–583.

Nica, G., Herzog, W., Sonntag, C., Nowak, M., Schwarz, H., Zapata, A.G., Hammerschmidt, M., 2006. Eya1 is required for lineage-specific in the zebrafish differentiation, but not for cell survival adenohypophysis. Dev. Biol. 292, 189–204.

Noden, D.M., Francis-West, P., 2006. The differentiation and morphogenesis of craniofacial muscles. Dev. Dyn. 235, 1194–1218.

Ohto, H., Kamada, S., Tago, K., Tominaga, S., Ozaki, H., Sato, S., Kawakami, K., 1999. Cooperation of Six and Eya in activation of their target genes through nuclear translocation of Eya. Mol. Cell Biol. 19, 6815–6824.

Ozaki, H., Watanabe, Y., Takahashi, K., Kitamura, K., Tanaka, A., Urase, K., Momoi, T., Sudo, K., Sakagami, J., Asano, M., Iwakura, Y., Kawakami, K., 2001. Six4, a putative myogenin gene regulator, is not essential for mouse embryonal development. Mol. Cell. Biol. 21, 3343–3350.

Ozaki, H., Nakamura, K., Funahashi, J., Ikeda, K., Yamada, G., Tokano, H., Okamura, H., Kitamura, K., Muto, S., Kotaki, H., Sudo, K., Horai, R., Iwakura, Y., Kawakami, K., 2004. Six1 controls patterning of the mouse otic vesicle. Development 131, 551–562.

Petropoulos, H., Skerjanc , I.S., 2002. beta-catenin is essential and sufficient for skeletal Myogenesis in P19 cells. J. Biol. Chem. 277, 15393–15399.

Pignoni, F., Hu, B.R., Zavitz, K.H., Xiao, J.A., Garrity, P.A., Zipursky, S.L., 1997. The eye-specification proteins so and eya form a complex and regulate multiple steps in *Drosophila* eye development. Cell 91, 881–891.

Piotrowski, T., Ahn, D.G., Schilling, T.F., Nair, S., Ruvinsky, I., Geisler, R., Rauch, G.J., Haffter, P., Zon, L.I., Zhou, Y., Foott, H., Dawid , I.B., Ho, R.K., 2003. The zebrafish van gogh mutation disrupts tbx1, which is involved in the DiGeorge deletion syndrome in humans. Development 130, 5043–5052.

Rawls, A., Olson, E.N., 1997. MyoD meets its maker. Cell 89, 5–8.

Ridgeway, A.G., Skerjanc , I.S., 2001. Pax3 is essential for skeletal myogenesis and the expression of Six1 and Eya2. J. Biol. Chem. 276, 19033–19039.

Rinon, A., Lazar, S., Marshall, H., Buchmann-Moller, S., Neufeld, A., Elhanany-Tamir, H., Taketo, M.M., Sommer, L., Krumlauf, R., Tzahor, E., 2007. Cranial neural crest cells regulate head muscle patterning and differentiation during vertebrate embryogenesis. Development 134, 3065–3075.

Ruf, R.G., Xu, P.X., Silvius, D., Otto, E.A., Beekmann, F., Muerb, U.T., Kumar, S., Neuhaus, T.J., Kemper, M.J., Raymond Jr., R.M., Brophy, P.D., Berkman, J., Gattas, M., Hyland, V., Ruf, E.M., Schwartz, C., Chang, E.H., Smith, R.J., Stratakis, C.A., Weil, D., Petit, C., Hildebrandt, F., 2004. SIX1 mutations cause branchio-oto-renal syndrome by disruption of EYA1–SIX1–DNA complexes. Proc. Natl. Acad. Sci. U. S. A. 21, 8090–8095.

Sahly, I., Andermann, P., Petit, C., 1999. The zebrafish eya1 gene and its expression pattern during embryogenesis. Dev. Genes Evol. 209, 399–410.

Schilling, T.F., Kimmel, C.B., 1994. Segment and cell-type lineage restrictions during pharyngeal arch development in the zebrafish embryo. Development 120, 483–494.

Schonberger, J., Wang, L., Shin, T.J., Kim, S.D., Depreux, F.F.S., Zhu, H., Zon, L., Pizard, A., Kim, J.B., MacRae, C.A., Mungall, A.J., Seidman, J.G., Seidman, C.E., 2005. Mutation in the transcriptional coactivator EYA4 causes dilated cardiomyopathy and sensorineural hearing loss. Nat. Genet. 37, 418–422.

Seo, H.C., Saetre, B.O., Havik, B., Ellingsen, S., Fjose, A., 1998. The zebrafish Pax3 and Pax7 homologues are highly conserved, encode multiple isoforms and show dynamic segment-like expression in the developing brain. Mech. Dev. 70, 49–63.

Seo, H.C., Curtiss, J., Mlodzik, M., Fjose, A., 1999. Six class homeobox genes in *Drosophila* belong to three distinct families and are involved in head development. Mech. Dev. 83, 127–139.

Shen, W.P., Mardon, G., 1997. Ectopic eye development in *Drosophila* induced by directed dachshund expression. Development 124, 45–52.

Shih, H.P., Gross, M.K., Kioussi, C., 2007. Cranial muscle defects of Pitx2 mutants result from specification defects in the first branchial arch. Proc. Natl. Acad. Sci. U. S. A. 104, 5907–5912.

Spitz, F., Demignon, J., Porteu, A., Kahn, A., Concordet, J.P., Daegelen, D., Maire, P., 1998.

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029

Expression of myogenin during embryogenesis is controlled by Six/sine oculis homeoproteins through a conserved MEF3 binding site. Proc. Natl. Acad. Sci. U. S. A. 95, 14220–14225.
Tajbakhsh, S., Cossu, G., 1997. Establishing myogenic identity during somitogenesis. Curr. Opin. Genet. Dev. 7, 634–641.
Tajbakhsh, S., Rocancourt, D., Cossu, G., Buckingham, M., 1997. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell 89, 127–138.
Tirosh-Finkel, L., Elhanany, H., Rinon, A., Tzahor, E., 2006. Mesoderm progenitor cells of common origin contribute to the head musculature and the cardiac outflow tract. Development 133, 1943–1953.
Tzahor, E., Kempf, H., Mootoosamy, R.C., Poon, A.C., Abzhanov, A., Tabin, C.J., Dietrich, S., Lassar, A.B., 2003. Antagonists of Wnt and BMP signaling promote the formation of vertebrate dead muscle. Genes Dev. 17, 3087–3099.
von Scheven, G., Alvares, L.E., Mootoosamy, R.C., Dietrich, S., 2006. Neural tube derived signals and Fgf8 act antagonistically to specify eye versus mandibular arch muscles. Development 133, 2731–2745.
Wargelius, A., Seo, H.C., Austbo, L., Fjose, A., 2003. Retinal expression of zebrafish six3.1 and its regulation by Pax6. Biochem. Biophys. Res. Commun. 309, 475–481.
Xu, P.X., Adams, J., Peters, H., Brown, M.C., Heaney, S., Maas, R., 1999. Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. Nat. Genet. 23, 113–117.
Xu, P.X., Zheng, W.M., Huang, L., Maire, P., Laclef, C., Silvius, D., 2003. Six1 is required for the early organogenesis of mammalian kidney. Development 130, 3085–3094.

Please cite this article as: Lin, C.-Y., et al., The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish, Dev. Biol. (2009), doi:10.1016/j.ydbio.2009.04.029