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DYRK2 displays muscle fiber type specific function during zebrafish early somitogenesis

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ABSTRACT Dual specificity tyrosine-phosphorylation regulated kinase 2 (DYRK2) is a serine/threonine kinase. In zebrafish, *DYRK2* is expressed in the lateral somites and adaxial cells at the early stage of embryo development. However, its role in early myogenesis had not been elucidated yet. Here, we report that *DYRK2* mRNA and *MyoD* mRNA were colocalized in the muscle progenitor cells in somites, including both the posterior compartment of the lateral somites and adaxial cells. Knockdown of *DYRK2* reduced the levels of *MyoD* transcripts in the muscle progenitor cells in somites. In contrast, overexpression of *DYRK2* increased the levels of *MyoD* transcripts in the muscle progenitor cells in somites. The effects of knockdown and overexpression of *DYRK2* on the expression of *MyoD* in the posterior compartment of the lateral somites were much greater than in the adaxial cells. Further studies indicated that forced expression of *DYRK2* increased the levels of fast-twitch skeletal myosin RNA. Moreover, knockdown or forced expression of *DYRK2* affected the levels of fast-twitch skeletal myosin protein. Together, these data indicate that *DYRK2* is expressed in the developing muscle progenitor cells in somites and that it positively regulates fast-twitch muscle differentiation, at least at the early stages.

KEY WORDS: *zebrafish, DYRK2, in situ hybridization, muscle development*

Skeletal muscle is a major component of vertebrate anatomy. Vertebrate skeletal muscle is composed of two major types of muscle fibers that are functionally adapted through differences in their physiological and metabolic properties, namely fast-twitch muscle fibers and slow-twitch muscle fibers. Most mammalian skeletal muscles are heterogeneous in composition, containing both fast- and slow-twitch fibers and are capable of performing various functions (Schiaffino and Reggiani, 2011). By contrast, zebrafish embryonic myotome shows a discrete temporal and spatial separation of fiber type ontogeny, providing an excellent model for studying the molecular mechanisms involved in fiber type specification (Jackson *et al.*, 2015). Zebrafish myogenesis begins prior to somite formation with the activation of the myogenic regulatory factors (MRFs), *MyoD* and *Myf5* (Coutelle *et al.*, 2001; Weinberg *et al.*, 1996). During segmentation period, *MyoD* transcripts are specifically expressed both in the adaxial cells and the posterior compartment of the lateral somites, which will differentiate into slow- and fast-twitch muscle fibers, respectively (Ochi and Westerfield, 2007; Weinberg *et al.*, 1996).

Dual specificity tyrosine(Y)-phosphorylation regulated kinase 2 (DYRK2) is a member of the DYRK family that belongs to the CMGC group of protein kinases (Nihira and Yoshida, 2015). DYRK2 consists of a conserved kinase domain and an adjacent N-terminal DYRK homology box. DYRK2 autophosphorylates a critical tyrosine residue in its own activation loop of the kinase domain and phosphorylates its substrates on serine and/or threonine residues. Once DYRK2 is fully translated and released from the ribosome, the transitional tyrosine kinase activity is lost and it then functions only as a serine/threonine kinase (Lochhead *et al.*, 2005). DYRK2 is involved in regulating key developmental and cellular processes, such as neurogenesis, cell proliferation, cytokinesis, and cellular differentiation (Taira *et al.*, 2007; Woods *et al.*, 2001; Yoshida, 2008).

Our previous study have showed that zebrafish *DYRK2* was specifically expressed in the lateral somites and adaxial cells that will develop into muscles (Sun *et al.*, 2010), indicating that DYRK2 may

Abbreviations used in this paper: DYRK2, dual specificity tyrosine-phosphorylation regulated kinase 2.

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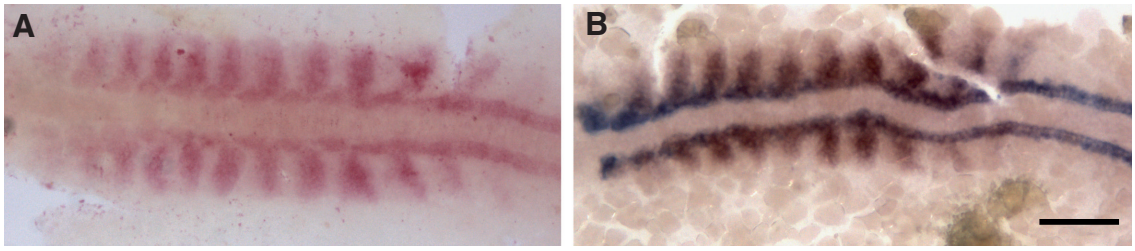

Fig. 1. One and two-color *in situ* hybridization in zebrafish embryos showing *DYRK2* (blue) and *MyoD* (red) expression. *Zebrafish embryos at the 11-somite stage. Head to the left, dorsal view.* **(A)**, *One-color* in situ *hybridization showing* MyoD *expression in the posterior compartment of the lateral somites and adaxial cells.* **(B)**, *Two-color* in situ *hybridization of* DYRK2 *and* MyoD *showing* DYRK2 *also expression in the posterior compartment of the lateral somites and adaxial cells. Scale bar, 10μm.*

be involved in the early development of muscle fiber specialization. However, the role of *DYRK2* in this process remains unclear. In this study, we investigated the role of DYRK2 in regulating *MyoD* expression, and its effects on fast-twitch muscle differentiation, in zebrafish embryos. We showed that *DYRK2* mRNA and *MyoD* mRNA were colocalized in the muscle progenitor cells in somites, and DYRK2 was a positive regulator of *MyoD* transcripts. Our further analyses revealed that DYRK2 also contributed to fast-twitch muscle fibers differentiation both at the mRNA and protein level. Our findings implied that DYRK2 was a key factor regulating muscle development at the early stage of embryo development.

Results

Zebrafish DYRK2 *mRNA and* MyoD *mRNA are colocalized in the muscle progenitor cells in somites during early embryogenesis*

Our previous study has shown that zebrafish *DYRK2* mRNA was expressed in somite cells that will develop into muscles (Sun *et al.*, 2010). Based on the anatomical location and distribution of the hybridization signal and published literature (Maves *et al.*, 2007), we speculated that the *DYRK2*-expressing cells were located in the lateral somites and adaxial cells. To test this idea, we performed one-color (Fig. 1A) or two-color (Fig. 1B) *in situ* hybridization analysis using digoxigenin-labelled *DYRK2* probe and fluorescein-labelled *MyoD* probe. As shown in Fig. 1B, *DYRK2* mRNA signals colocalized perfectly with *MyoD* mRNA signals, suggesting that the *DYRK2* gene is specifically expressed in the muscle progenitor cells in somites including both the posterior compartment of the lateral somite and adaxial cells.

Knockdown of DYRK2 *reduces levels of* MyoD *transcripts in the muscle progenitor cells in somites during early embryogenesis*

To investigate the possible role of endogenous *DYRK2* in embryonic myogenesis, morpholino-mediated knockdown of *DYRK2* was

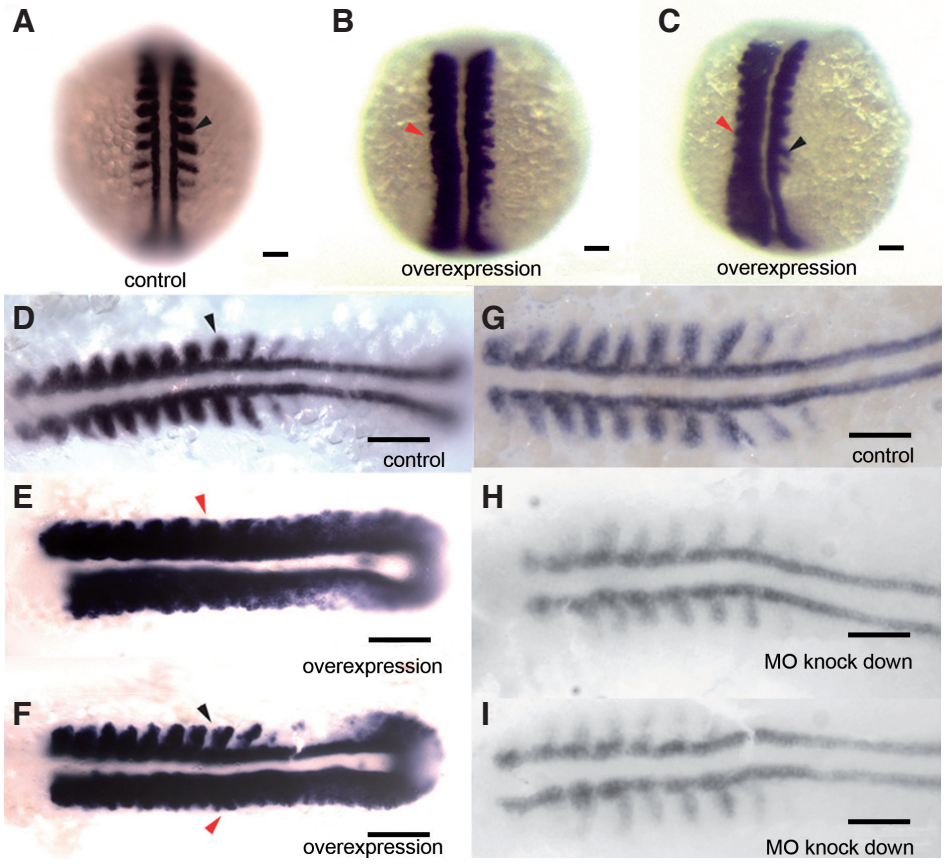

Fig. 2. Effect of *DYRK2* forced expression and knockdown on the expression of *MyoD* mRNA in zebrafish embryos. *Zebrafish embryos at the 10-13-somite stage.* **(A-C)** *Dorsal views with anterior to the top.* **(D-I)** *Dorsal views with anterior to the left.* **(A,D)** *Control* GFP *mRNA-injected embryos.* **(G)** *A control MO-injected embryo.* **(A,D,G)**, MyoD *expression in the posterior compartment of the lateral somites and adaxial cells.* **(B,E)** MyoD *mRNA showed increased expression in the posterior compartment of the lateral somites and adaxial cells from the front to back on both sides when* DYRK2 *mRNA was injected into one-cell stage embryos.* **(C,F)** MyoD *mRNA showed high-level expression at the side injection side when* DYRK2 *mRNA was injected into one of the two cells at the two-cell stage embryos.* **(H,I)**, *Konckdown of* DYRK2 *by ATG-MO attenuated* MyoD *expression in the posterior compartment of the lateral somites and adaxial cells. The red and black arrowheads indicate the injected and uninjected side of embryos respectively. Scale bar, 10 μm.*

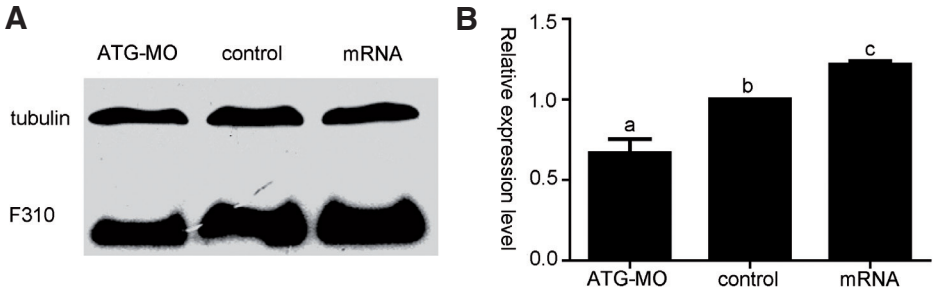

Fig. 3. Effect of *DYRK2* knockdown and forced expression on the expression of fast-twitch skeletal myosin in zebrafish embryos analyzed by Western immunoblot. *Embryos injected with ATG-MO or mRNA of* DYRK2 *displayed a decreased and an increased expression level of fast-twitch skeletal myosin.* **(A)** *Western immunoblot analysis of F310 and γ-tubulin indicated, Images were representative images from three independent experiments.* **(B)** *The relative expression levels of F310 were determined by densitometry and normalized by the γ-tubulin protein levels. Control group was set as 1. Data shown are means ± SEM, n = 3. Different letters represent statistically significant differences (p<0.05).*

carried out. The specificity and efficacy of MOs were verified (data not shown). At 10-13-somite stage, MOs injected embryos were collected for *in situ* hybridization analysis of *MyoD*. As shown in Fig. 2 G-I, knockdown of *DYRK2* decreased *MyoD* expression levels in both the lateral somites and adaxial cells, and the effect in former one was more dominant.

Overexpression of DYRK2 *increases levels of* MyoD *transcripts in the muscle progenitor cells in somites during early embryogenesis*

We next analyzed the effect of *DYRK2* ectopic expression on embryonic myogenesis by *in situ* hybridization using *MyoD*. Ectopic expression of *DYRK2* caused marked changes in *MyoD* expression patterns between the *GFP* mRNA-injected control and *DYRK2* mRNA injected embryos. As shown in Fig. 2 A,B,D,E, injection of *DYRK2* mRNA at the one-cell stage resulted in robust expression of *MyoD* in the developing somite cells including both the lateral somites and adaxial cells. Further, injection of *DYRK2* mRNA in one cell at the two-cell stage only resulted in robust expression of *MyoD* in the injected side of the developing somites (Fig. 2 A,C,D,F). Moreover, the effects of overexpression of *DYRK2* on the expression of *MyoD* in the lateral somites were much greater than that in the adaxial cells.

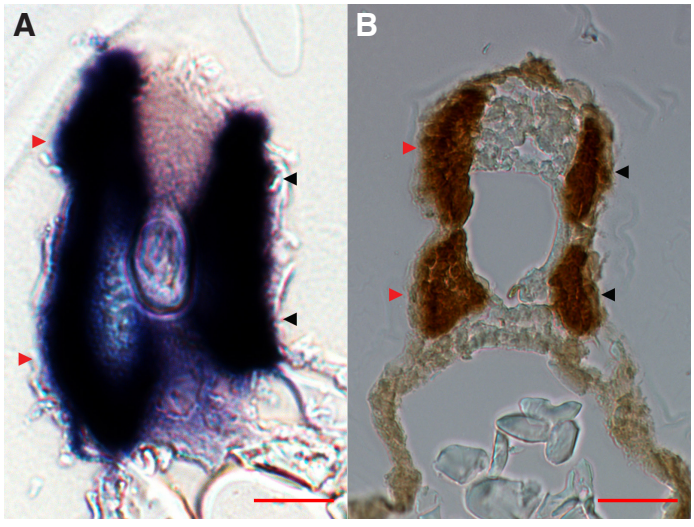

Fig. 4. Effect of *DYRK2* forced expression on the expression of fast-twitch skeletal myosin both at the mRNA and protein level. DYRK2 *mRNA was injected into one of the two cells at the two-cell stage embryos, and at 24hpf, some embryos were fixed and analyzed by* in situ *hybridizaition using the antisense probes against zebrafish fast muscle myosin heavy chain2 (myhz2) (Du* et al., *2008)* **(A)***; and at 30hpf, some embryos were fixed and analyzed by immunostaining using the F310 anti-fast-twitch skeletal myosin light chain antibody* **(B)**. *Transverse cryosections have dorsal towards the top. The red and black arrowheads indicate the injected and uninjected side of embryos respectively. Scale bar, 20 μm.*

DYRK2 *regulates the expression of myosin in fast-twitch muscles during early embryogenesis*

We next examined the effects of *DYRK2* knockdown and forced expression on the fast-twitch skeletal muscle differentiation. Compared with the control group, embryos injected with *DYRK2* MO had significantly lower fast-twitch skeletal myosin levels. Furthermore, forced expression of *DYRK2* significantly increased fast-twitch skeletal myosin levels, indicating that *DYRK2* played a role in fast-twitch skeletal muscle fiber differentiation (Fig. 3 A,B). Further, injection of *DYRK2* mRNA in one cell at the two-cell stage obviously increased fast-twitch skeletal myosin mRNA (Fig. 4A) and protein (Fig. 4B) expression in the injected side of the developing somites.

Discussion

In this study, we have showed that zebrafish *DYRK2* mRNA was specifically co-expressed with *MyoD* mRNA in the muscle progenitor cells in somites including both the posterior compartment of the lateral somites and adaxial cells during early embryogenesis, which were the fast- and slow-twitch muscle precursor cells, respectively. Moreover, we have proved that zebrafish *DYRK2* positively regulated *MyoD* gene expression and fast-twitch muscle fiber differentiation during embryonic myogenesis. These results provided the first *in vivo* evidence that the *DYRK2* gene may play a critical role in the process of fast-twitch muscle differentiation during embryonic myogenesis.

MyoD is essential for initiating the skeletal muscle program in the embryos (Weintraub, 1993). In this paper, we found *DYRK2* and *MyoD* mRNA co-localized in the posterior compartment of the lateral somites and adaxial cells in zebrafish embryonic myogenesis. Moreover, both of their expression occurs during zebrafish embryogenesis far in advance of any overt myogenic differentiation (Sun *et al.*, 2010; Weinberg *et al.*, 1996). Therefore, we speculated that *DYRK2* was an important factor in regulating embryonic myogenesis.

Our loss- and gain-of-function analysis results provide strong evidence supporting the view that DYRK2 plays an important role in regulating embryonic myogenesis. Overexpression of a func-

tional DYRK2 protein increased expression of *MyoD* both in the posterior compartment of the lateral somites and the adaxial cells. It is of interest to note that the increased levels of *MyoD* transcripts in the lateral somites was much higher than that in the adaxial cells, which implied that DYRK2 might play a much stronger role in embryological development of fast-twitch muscle than in the slow-twitch muscle. Furthermore, targeted knockdown of DYRK2 significantly reduced expression of *MyoD* both in the lateral somites and the adaxial cells, and also the effect in the former one was much higher than that in the latter one. The knockdown data also support the point of view that DYRK2 had an important role in embryonic myogenesis, especially in embryological development of fast-twitch muscle. Further research showed that DYRK2 positively regulated fast-twitch skeletal myosin mRNA and protein expression levels. To our knowledge, this study is the first to provide *in vivo* evidence showing DYRK2 is a positive regulator of fast-twitch muscle development in a vertebrate embryo.

Protein kinase DYRK2 phosphorylates its substrates on serine/threonine residues (Lochhead *et al.*, 2005). Therefore, we speculate that DYRK2 might play a role in mediating embryonic myogenesis by phosphorylating one or some key factors of the differentiation of muscle and transformation between the fast- and slow-twitch muscle, such as Gli2, Pbx2, and Pbx4 (Katoh and Katoh, 2008; Maves *et al.*, 2007; Varjosalo *et al.*, 2008; Yao *et al.*, 2013). Further studies on the searching for the phosphorylated substrates of DYRK2 would be more interesting.

In summary, we provide evidence that zebrafish DYRK2 is a key factor in muscle development during embryogenesis, especially in the fast-twitch muscle development. Zebrafish embryonic myotome shows a discrete temporal and spatial separation of fiber type ontogeny, providing an excellent model for studying the molecular mechanisms involved in fiber type specification. Our study has also presented the opportunity to further elucidate the mechanism and the signalling pathways by which DYRK2 regulates muscle development using this unique vertebrate model organism.

Materials and Methods

Experimental animals

Zebrafish (*Danio rerio*) were maintained on a 14-h light, 10-h dark cycle at 28 °C and fed twice a day. Embryos were obtained by natural crossing. Fertilized eggs were raised in embryo medium at 28.5 °C and staged according to the standard method (Kimmel *et al.*, 1995). 2-Phenylthiourea (Sigma-Aldrich, USA) 0.003% (wt/vol) was added to the water in some experiments to prevent pigmentation. All experiments were conducted according to the regulations of local and central governments, and approved by the Institutional Animal Care and Use Committee of Institute of Oceanology, Chinese Academy of Sciences.

Morpholino knockdown

Morpholino antisense oligos (Gene Tools, USA) were synthesized. The translation blocker (ATG-MO) was based on a sequence near the ATG (in bold) start site. The splicing blocker (E2I2-MO) was based on the sequence at the exon 2 and intron 2 junctions: ATG-MO: 5'- GGTTTTCTGCTTAATATCAT**CAT**GG -3'; and E2I2-MO: 5'- GAACAGGAAGTCGGACTTACAGTGT -3'. Besides, a standard control MO (CMO): 5'-CCTCTTACCTCAGTTACAATTTATA-3' were used as control. Morpholino antisense oligos were dissolved in 1X Danieau buffer to a final concentration of 1 mM. Next, 1–2 nl (5–10 ng) was injected into each embryo. The specificity and efficacy of each of these MO sequences have been confirmed as reported previously (Tan *et al.,* 2006).

Overexpression of DYRK2

A previous pBlueScript SK (Stratagene, USA) vector (Sun *et al.*, 2010) containing the entire ORF of zebrafish *DYRK2* was digested with *Sal* I and *BamH* I and subcloned into *Sal*I and *BamH* I sites of pSP64 vector. Capped mRNA was synthesized with a mMESSAGE mMACHINE SP6 Kit (Ambion, USA) and linearized plasmid DNA as template. 2nl mRNA (25 ng/μl) was microinjected into one cell of zebrafish embryos at the one-cell or two-cell stage, respectively. *GFP* mRNA (25 ng/μl) injected embryos were used as controls. After injection, embryos were placed in embryo rearing medium and kept at 28.5°C.

Whole-mount in situ *hybridization and transverse cryosection*

One-color or two-color *in situ* hybridization using digoxigenin-labeled or fluorescein-labeled antisense riboprobes was performed as reported previously (Tan and Du, 2002). For the *DYRK2* knockdown/ overexpression and *Myod* expression experiments, all of the treatments, including microinjection, fixation, *in situ* hybridization and revelation, were in parallel. For the *DYRK2* overexpression and *fMHC* expression experiments, after whole-mount *in situ* hybridization, some embryos were proceed for transverse cryosection at 10 μm. pGEM-myhz2 plasmid for synthesizing *fMHC* antisense probe was kindly provided by Dr. Shao jun Du. Images were captured with a QImaging 5.0RTV CCD Camera mounted on an Olympus microscope (Olympus, Japan).

Analysis of protein expression by Western blot in injected zebrafish embryos

Wild-type embryos were injected with *GFP* or *DYRK2* capped mRNA or *DYRK2* ATG-MO at the one-cell stage. Injected embryos were analyzed by immunostaining by using the F310 anti-fast-twitch skeletal myosin light chain antibody (DSHB, USA) and anti-γ-tubulin antibody (Sigma, USA) at 30 hpf. Briefly, zebrafish embryos were dechorionated, deyolked, homogenized and boiled in 2× sodium dodecyl sulfate (SDS) lysis buffer (125mM Tris-HCl, pH6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.02% Bromophenol Blue) (2.5μl for each embryo) containing 10μg/ml aprotinin, 10μg/ml leupeptin, 10μg/ml pepstatin, and 100mM PMSF. The homogenates were collected by low speed centrifugation at 3000 rpm for 1min. 10μl were loaded each lane on a 10% SDS-PAGE and then analyzed by Western immunoblot.

Whole-mount immunostaining and transverse cryosection

The whole mount immunostaining was carried out with the injected embryos at 30 hpf using the F59 anti-slow-twitch skeletal myosin heavy chain antibody (DSHB, USA), and the F310 anti-fast-twitch skeletal myosin light chain antibody (DSHB, USA) as previously described(Tan *et al.*, 2006). Anti-mouse IgG secondary antibody was labeled with horseradish peroxidase (Invitrogen, USA). Following whole-mount immunostaining, some embryos were proceed for transverse cryosection at 10 μm.

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