Induced early expression of *mrf4* but not *myog* rescues myogenesis in the *myod/myf5* double-morphant zebrafish embryo

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Summary

Muscle regulatory factors activate myogenesis in all vertebrates, but their role has been studied in great detail only in the mouse embryo, where all but myogenin – Myod, Myf5 and Mrf4 – are sufficient to activate (albeit not completely) skeletal myogenesis. In the zebrafish embryo, *myod* and *myf5* are required for induction of myogenesis because their simultaneous ablation prevents muscle development. Here we show that *mrf4* but not *myog* can fully rescue myogenesis in the *myod/myf5* double morphant via a selective and robust activation of *myod*, in keeping with its chromatin-remodelling function in vitro. Rescue does not happen spontaneously, because the gene, unlike that in the mouse embryo, is expressed only at the onset of muscle differentiation, Moreover, because of the transient nature of morpholino inhibition, we were able to investigate how myogenesis occurs in the absence of a myotome. We report that in the complete absence of a myotome, subsequent myogenesis is abolished, whereas myogenesis does proceed, albeit abnormally, when the morpholino inhibition was not complete. Therefore our data also show that the early myotome is essential for subsequent skeletal muscle differentiation and patterning in the zebrafish.

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Key words: Skeletal myogenesis, MRF, Zebrafish, *myf5*, Myod, *mrf4*, Myogenin, Myotome

Introduction

Determination of skeletal muscle in vertebrates depends upon three members of a family of four b-HLH transcription factors, known as muscle regulatory factors (MRFs): Myf5, Mrf4, Myod and myogenin (Myog). Skeletal muscle histogenesis is a multi-step process, from precursor determination to patterning, fusion and activation of muscle-specific genes, and MRFs act at multiple steps in this process where they exert both overlapping and distinct functions. In the mouse, *Myf5* and *Myod* (official gene symbol *Myod1*) function in a large part redundantly in myoblast determination, so that deletion of one gene or the other does not significantly affect muscle development (Braun et al., 1992; Rudnicki et al., 1992), but deletion of both genes eliminates the skeletal muscle lineage (Rudnicki et al., 1993). Recently, it was demonstrated that *Mrf4* is also involved in mouse muscle determination. Kassar-Duchossoy et al. (Kassar-Duchossoy et al., 2004) have shown that the *Myf5/Myod* double mutant mice are in fact partial triple mutants, because the deletion of the *Myf5* locus also compromised the genetically linked *Mrf4* gene expression (Kassar-Duchossoy et al., 2004). Indeed, in mutant embryos where *Mrf4* expression is preserved, embryonic myogenesis takes place in the absence of *Myf5* and *Myod*, even though muscle rapidly degenerates in the foetal stage of development. These findings indicate that both *Myf5* and *Mrf4* act upstream of *Myod* to direct cells into the myogenic lineage (Kassar-Duchossoy et al., 2004). This is in agreement with previous expression data, which shows that *Mrf4* is transiently expressed during somitogenesis and later during fiber maturation (Bober et al., 1991; Hinterberger et al., 1991). The mouse myogenin gene (*Myog*) instead acts genetically downstream of *Myf5* and *Myod* to switch on muscle differentiation genes: in its absence, myoblasts are properly specified and positioned but there is a severe deficiency of muscle fibers (Hasty et al., 1993; Nabeshima et al., 1993). Mrf4 is not essential for later muscle development (Braun and Arnold, 1995; Patapoutian et al., 1995; Zhang et al., 1995), however mice *Mrf4* and *Myod* double mutants are phenotypically similar to *Myog* mutants, indicating that *Mrf4* and *Myod* play redundant roles in the activation of the differentiation program (Rawls et al., 1998).

In organisms other than mouse, the diverse roles of MRFs have been less extensively studied. In contrast to that observed in the mouse, initiation of *myf5* and *myod* (official symbol *myod1*) expression is presomitic in zebrafish, *Xenopus* and chick embryos (Della Gaspera et al., 2006; Hopwood et al., 1991; Jennings, 1992). Zebrafish *myf5* and *myod* are temporally and spatially expressed in largely overlapping patterns in adaxial cells and posteriorly in newly formed somites; however, *myf5* alone is expressed in the posterior presomitic mesoderm whereas *myod* expression appears in older somites (Coutelle et al., 2001; Weinberg et al., 1996). It has been reported that either *myf5* or *myod* is sufficient to promote slow muscle formation from adaxial cells, and that *myod* is required for fast muscle differentiation (Groves et al., 2005; Hammond et al., 2007). Downregulation of both Myf5 and Myod proteins abolishes slow muscle in early embryos (Hammond et al., 2007), whereas Myod but not Myf5 cooperates with Pbx homeodomain proteins to promote fast muscle differentiation (Maves et al., 2007). A possible role for zebrafish *mrf4* in muscle development has not yet been

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addressed, even though its pattern of expression has been described recently (Hinits et al., 2007).

Here, we report that, at variance with Myf5 and Myod, Mrf4 does not control early myogenesis in zebrafish; however, if heterochronically expressed, it is able to drive normal muscle differentiation in their absence via the selective activation of *myod*; *mrf4* does not naturally compensate for the absence of *myf5* and *myod*, as observed in the mouse, because its expression is late. By contrast, myogenin (gene: *myog*), the fourth MRF, is unable to rescue complete myogenesis in *myf5*/*myod* double morphants. Moreover, we observe that in embryos in which morpholino-mediated inhibition is incomplete, some muscle forms with a highly disorganised pattern, whereas in the complete absence of the early myotome, later myogenesis is abolished, underlining a crucial role of the myotome in zebrafish.

Results

myf5 and *myod* control determination of all skeletal muscles in zebrafish

To characterise the MRFs in zebrafish, we injected morpholinos against each gene alone, and in combination, into one- to two-cell-stage embryos. Whereas all the single morphants appeared normal at 24 hours post fertilisation (h.p.f.) (supplementary material Fig. S1), 90% (69/77) of the *myf5*/*myod* double morphants were immobile (Fig. 1A,B). In these embryos, skeletal myosin was either strongly reduced (25/69) or completely abolished (44/69) as revealed by antibody staining (Fig. 1C-E'). The morpholinos were designed to bind an exact sequence around the start codon of the mRNA of each gene and are therefore highly unlikely to cross react with one of the other MRF genes because their sequence similarity is negligible in this region. This result thus indicates that Myf5 and Myod are the only MRFs required for the induction of skeletal muscle in zebrafish and it also suggests a different function for Mrf4 in the fish compared with its role in the mouse.

mrf4 is expressed with *myog* only at the onset of muscle differentiation

We performed in situ hybridisation and real-time PCR to investigate whether *mrf4* is expressed in zebrafish muscle precursors. We could not detect zebrafish *mrf4* as early as *myod* or *myf5* expression, which can be visualised by in situ hybridisation from 70-80% epiboly onwards; *mrf4* expression was detected during early somitogenesis (from the 5-somite stage), similar to *myog* expression (Fig. 2, and data not shown). This pattern of expression of *mrf4* in the zebrafish is in agreement with a recent report (Hinits et al., 2007) and is difficult to reconcile with a possible role in muscle cell determination. Conversely, *mrf4* probably participates in muscle differentiation because its expression coincides with the earliest time point when we can detect myosin protein in differentiating muscle cells.

mrf4 and *myog* are expressed normally in single morphants but are absent in double morphants

The expression pattern of *mrf4* and *myog* suggests that they might be targets of Myf5 and/or Myod. We thus investigated the expression of the two genes in *myf5* and *myod* single morphants as well as in *myf5*/*myod* double morphants and found their expression to be normal in single morphants but strongly reduced or abolished in more than 85% of *myf5*/*myod* double morphants (52/60) (Fig. 3), as shown for skeletal myosin. These data are in agreement with recently published results of Maves et al. (Maves et al., 2007), who found that *myog*, *desmin*, *smyhc1* and *mylz2* are not expressed in *myf5*/*myod* double morphants. Thus Myod and Myf5 act redundantly for activation of *mrf4* and *myog*.

Premature expression of *mrf4* induces skeletal myogenesis in double-morphant embryos

Although *mrf4* does not appear to regulate muscle determination in zebrafish, we wondered whether this depends upon the different time of expression or rather upon some structural difference to mouse *Mrf4*. Thus we injected *myf5* and *myod* morpholinos together with different amounts of *mrf4* mRNA (20-100 pg) in the early fish embryo. Results showed that the majority of these fish were moving at 24 h.p.f. (supplementary material Movies 1-3; Table 1), and *myog* and myosin expression was rescued (Fig. 4). *myog* expression was normal in more than 50% of the rescued embryos (Fig. 4A) and only slightly decreased in the remaining embryos. Myosin staining revealed predominantly U-shaped somites, but was strongly positive in all rescued embryos (Fig. 4B,B'). By contrast, co-injection of either 100 pg or 200 pg, of *myog* mRNA did not activate neither mobility nor strong myosin expression (Table 1; Fig. 4C,C'). Semi-thin transverse sections revealed apoptotic and highly disorganised muscle in double morphants and quasi intact muscle in rescued embryos (Fig. 5). Electron microscopy of these sections showed organised sarcomeric structures in control and rescued embryos, which were either not present or highly disorganised in double morphants. For example, Fig. 5E shows longitudinally and transversally oriented sarcomeres in the same section.

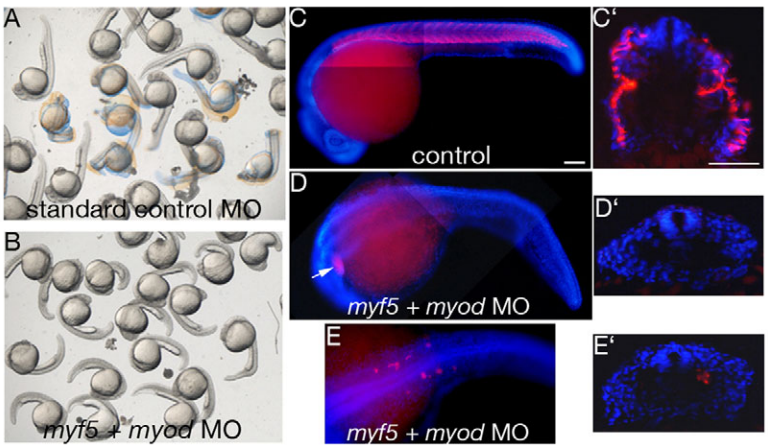

Fig. 1. Myf5 and Myod knockdown results in immobile embryos and absence of skeletal myosin. (A) 24 h.p.f. embryos injected with standard control morpholino look and move similarly to uninjected embryos. (B) Embryos co-injected with *myf5* and *myod* morpholinos (MO) look phenotypically normal but do not move. (C,D,E) Whole-mount myosin staining of control embryos (C), and *myf5*/*myod* morphants at 24 h.p.f. The embryo in D has no skeletal myosin and that in E very little skeletal myosin. The arrow in D indicates the heart, which expresses cardiac myosin independently of *myf5* and *myod*. (C',D',E') Cross sections of control embryos (C') and *myf5*/*myod* morphants (D',E') at 20-somite stage, stained for myosin. Myosin is in red and nuclei are blue. Scale bars: 100 μm in C and 50 μm in C'.

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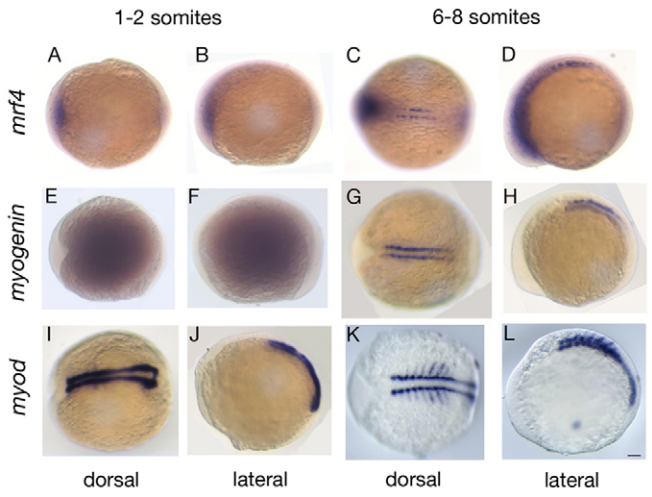

Fig. 2. *mrf4* and *myog* are expressed later than *myod*, after the onset of somitogenesis. (A-L) Whole-mount in situ hybridisation of *mrf4* (A-D), *myog* (E-H) and *myod* (I-L). (A,B,E,F,I,J) 1- to 2-somite stage. (C,D,G,H,K,L) 6- to 8-somite stage. (A,C,E,G,I,K) dorsal view anterior to the left. (B,D,F,H,J,L) lateral view anterior to the left. No signal for *mrf4* and *myog* can be detected at the 1- to 2-somite stage (A,B,E,F) when *myod* is already strongly expressed in adaxial cells and weakly expressed in the first somites (I,J). After the 5-somite stage, *mrf4* and *myog* start to be expressed in the adaxial cells (C,D,G,H), whereas *myod* is expressed both in adaxial cells and somites (K,L). Scale bar: 100 µm.

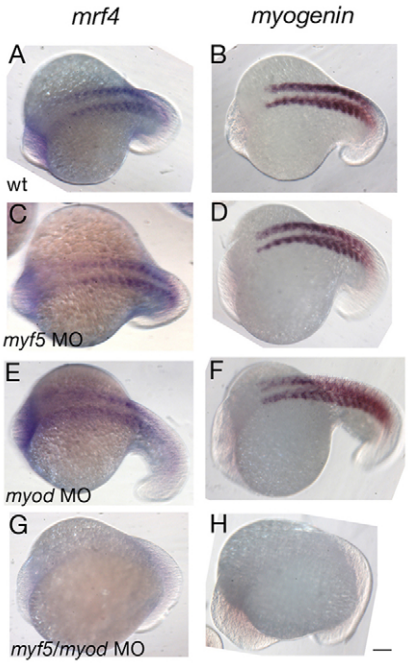

Fig. 3. *mrf4* and *myog* expression is unperturbed in single morphants but absent in double morphants. (A-H) Whole-mount in situ hybridisation for *mrf4* (A,C,E,G) and *myog* (B,D,F,H) in 20-somite embryos, anterior to the left. In situ signal in uninjected embryos (A,B) is undistinguishable from that in *myf5* (C,D) and *myod* (E,F) single morphants. No signal is detected in the majority of *myf5*/*myod* double morphants (G,H). Scale bar is 100 µm.

To understand whether rescued myogenesis is comparable to the normal process, we examined the onset of gene expression of *myog*, *pax3*, *pax7* and slow and fast myosin at their respective developmental stages. Except for *myog,* whose expression increased, and in some cases was more broadly expressed, in *mrf4*-rescued embryos, all other genes appeared to be expressed normally (Fig. 6). This result demonstrates that zebrafish *mrf4* is able to act as a muscle determination gene in the early embryo, just like mouse *Mrf4*, but cannot do so during zebrafish development probably because of its late onset of expression.

mrf4 rescues skeletal muscle via the activation of *myod*

To understand the molecular mechanisms underlying muscle rescue by *mrf4*, we examined the expression of Myod protein. The Myf5 antibody (Santa Cruz) recognises Myod in zebrafish, as reported by Hammond (Hammond et al., 2007) and confirmed by us here. *Myod* single morphants do not express Myod protein as expected, but *myf5* single morphants do (Fig. 7). Myod protein in double morphants is absent, as in *myod* single morphants, but is normal in rescued embryos compared with uninjected embryos (Fig. 7). We further performed real-time quantitative PCR experiments to compare the expression levels of *mrf4*, *myod* and *myf5* (which cannot be detected by antibody in the zebrafish) mRNAs in embryos injected with both *myf5* and *myod* morpholinos, in rescued embryos, and in embryos injected with two different concentrations of *mrf4* mRNA alone. In all cases gene expression levels were normalised to their levels in uninjected embryos. Interestingly, both *myf5* and *myod* mRNAs were more than tenfold upregulated in double-morphant embryos, whereas *mrf4* was reduced to one fifth of its normal expression level (Fig. 8), as previously demonstrated by the strong reduction of signal for *mrf4* revealed by in situ hybridisation (Fig. 3). Injection of 80 pg of *mrf4* mRNA together with *myf5* and *myod* morpholinos resulted in a more than 100-fold upregulation of *mrf4* mRNA at midsomitogenesis, confirming the good quality of the injected transcript, and a nearly 50-fold upregulation of *myod* mRNA, whereas the level of *myf5* was unchanged compared with that in the double morphants (Fig. 8). Injection of 50 pg or 20 pg *mrf4* mRNA alone increased *myf5* mRNA only marginally, but upregulated *myod* mRNA levels over 30-fold (Fig. 8). Injection of 20 pg of *mrf4* mRNA was sufficient to maintain mobility (Table 1), but no elevated levels of this mRNA could be detected by real-time PCR at midsomitogenesis compared with non-injected embryos (Fig. 8), indicating that physiological levels of *mrf4* mRNA are sufficient to induce at least functional fast muscle development in *myf5*/*myod* double morphants.

Taken together, these data indicate that *mrf4* rescues myogenesis in double-morphant fish via the activation of *myod*. This is the first evidence that *mrf4* is able to activate *myod* in vivo. Moreover, increased levels of *myod* morpholino (1 pmole instead of 0.5 pmole) compromised the rescue (data not shown), further supporting the finding that *mrf4* rescue is via activation of *myod*, and that *myod* is necessary for the rescue.

Table 1. *mrf4* can rescue mobility in double-morphant fish whereas *myog* cannot

	Motile*	Not motile*
Control fish	100% (60)	0% (0)
myf5/*myod* MO	10% (8)	90% (69)
myf5/*myod* MO + 100 pg *mrf4* RNA	61% (45)	39% (29)
myf5/*myod* MO + 50 pg *mrf4* RNA	69% (80)	31% (36)
myf5/*myod* MO + 20 pg *mrf4* RNA	66% (31)	34% (16)
myf5/*myod* MO + 100 pg *myog* RNA	20% (19)	80% (78)

*Total number of embryos in parentheses.

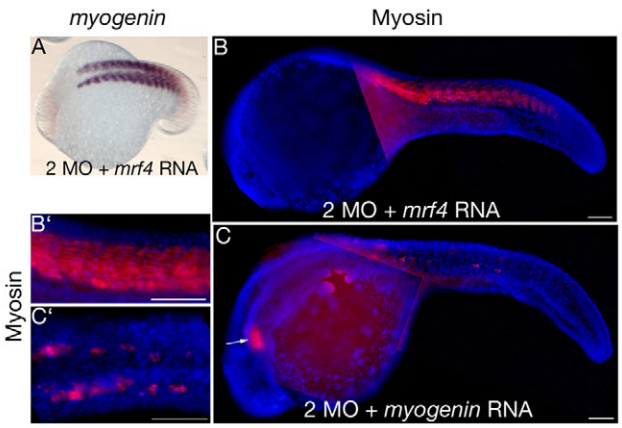

Fig. 4. Ectopic *mrf4* can rescue the muscle phenotype in *myf5*/*myod* double morphants. (A) In situ hybridisation for *myog* in *mrf4*-rescued embryos at 20-somite stage. (B-C') Myosin staining in *mrf4*-rescued (B,B') and *myog*-coinjected (C,C') double morphants at 24 h.p.f. B' and C' show part of the tail at higher magnification. Myosin is in red and nuclei are in blue. The arrow in C indicates the heart. Scale bars: 100 μm.

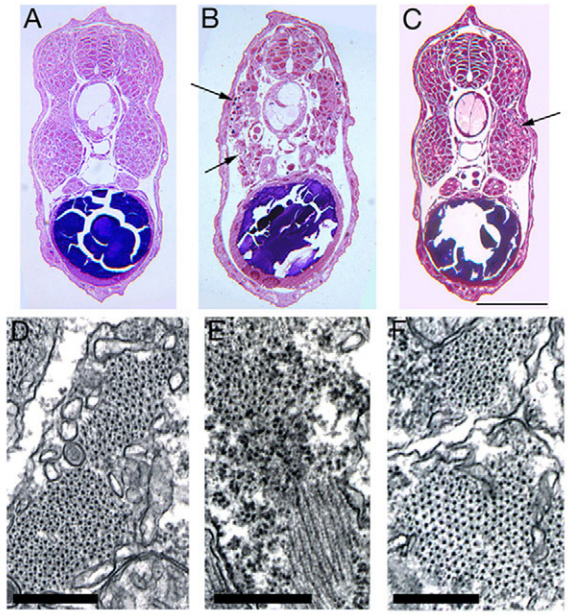

Fig. 5. Semi-thin sections and electron microscopy reveal disturbed muscle in double morphants and rescued muscle in *mrf4*-injected double morphants. (A-C) Transverse sections of control (A), double morphant (B), and rescued double morphant (C) embryos at 24 h.p.f., stained with gentian violet. Arrows in B and C indicate apoptotic and/or disturbed muscle. (D-F) Electron microscopy of control (D), double morphant (E), and rescued double morphant (F) embryos at 24 h.p.f. Transverse sections monitor highly organised sarcomeric structures in control and rescued embryos, whereas the sarcomeres are disoriented and unorganised in double morphants. Scale bars: 50 μm in C and 500 nm in D-F.

A crucial role for the myotome in muscle patterning and later myogenesis in zebrafish

Morpholinos have been reported to successfully block protein expression for at least 3 days in the developing zebrafish (Nasevicius and Ekker, 2000), during which they are progressively diluted and cleared from the tissue. This observation offers the unique opportunity to investigate how muscle development proceeds in a vertebrate embryo, after the transient repression of *myf5* and *myod*. Conditional mutants have not yet been studied in the mouse. We thus investigated whether the double-morphant embryos, with either strongly reduced or absent muscle, would recover over time, after the inhibition of protein synthesis is released. We followed double-morphant larvae for 8 days and found that after 5 d.p.f. they were still unable to move. However, at 7 d.p.f., the situation changed when some larvae started to tremble, and later to swim, whereas the majority of the double morphants was still immobile. The immobile larvae remained largely devoid of skeletal myosin (Fig. 9B,F) and they died the following day, probably due to the inability to ingest food. In the larvae that did regain motility, skeletal myosin was occasionally present but skeletal muscle was highly disorganised at 3 d.p.f. compared with control or *mrf4*-rescued larvae (Fig. 9, compare C to A,D). Muscle in 8-day-old recovered larvae was better organised but still was not comparable with uninjected or *mrf4*-rescued larvae (Fig. 9G,E,H), indicating that swimming does not require a perfect muscle organisation. These results were confirmed by coinjecting *myf5/myod* morpholinos into embryos of the α-actin-GFP transgenic zebrafish line (Higashijima et al., 1997). By following each embryo separately over a time course of 6 days, we confirmed that embryos devoid of GFP signal at 24 h.p.f. did not express GFP later on (8/8) (Fig. 10D-F), whereas those that faintly expressed GFP at 24 h.p.f. did increase the signal over time (2/2) (Fig. 10G-I), and even if the actin-GFP revealed disorganised somites, the embryos regained some mobility.

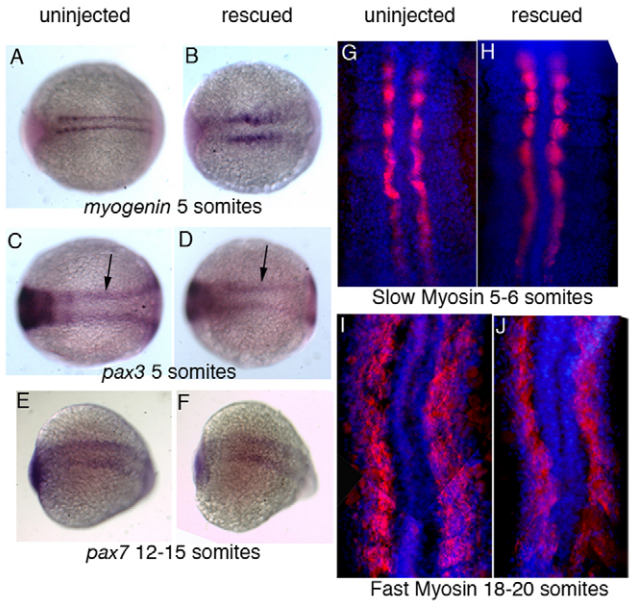

Fig. 6. Myogenesis in the *mrf4* rescue appears to proceed normally. The onset of expression of *myog* (A,B), *pax3* (C,D), *pax7* (E,F), slow Myosin (G,H) and fast Myosin (I,J) was examined in *mrf4*-rescued embryos (B,D,F,H,J) and uninjected embryos (A,C,E,G,I) at indicated developmental stages by in situ hybridisation (A-F, anterior to the left) or antibody staining (G-J, flat-mount views of tail region, anterior up). Except for *myog*, which is upregulated in the rescued embryos (compare B to A), all other genes seem to be expressed normally. The arrows in C and D indicate *pax3* expression in muscle precursors, the strong anterior staining is in the central nervous system. Slow and fast Myosin in G-J is stained in red, nuclei are in blue.

Discussion

Zebrafish *mrf4* can act as myogenic determination gene

Here we show that zebrafish myogenesis is entirely dependent on Myf5 and Myod because no muscle is formed in the absence of these proteins. Even though previous work had shown a block of

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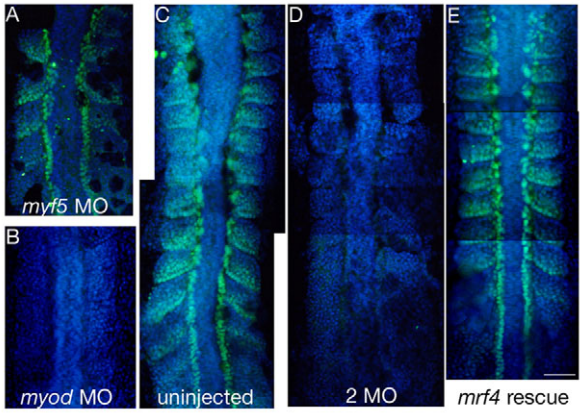

Fig. 7. *mrf4* rescues skeletal muscle via the activation of *myod*. (A-E) Flat mounts of whole-mount Myod antibody staining in *myf5* morphant (A), *myod* morphant (B), uninjected (C), double morphant (D), and *mrf4*-rescued (E) embryos at the 12-somite stage. Myod is not expressed in *myod* single and double morphants (B and D), but it is expressed normally in *mrf4*-rescued embryos (E). Myod is in green, nuclei are in blue; anterior is to the top. Scale bar: 50 µm.

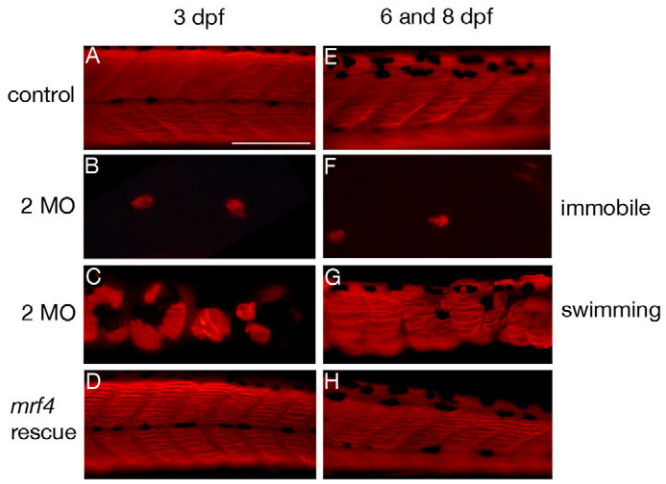

Fig. 9. The minority of double morphants recovers skeletal muscle 1 week after morpholino injection. (A-H) Whole-mount myosin staining of 3 d.p.f. (A-D), 6 d.p.f. (E,F) and 8 d.p.f. (G,H) in control (A,E), double morphant (B,C,F,G) and *mrf4*-rescued larvae (D,H). Control and rescued larvae show normal myosin expression (A,E,D,H), whereas most double morphants are still largely devoid of myosin at 3 d.p.f. and 6 d.p.f. (B,F) and remain immobile. Some double morphants recover and start to express myosin in an unorganised pattern 3 d.p.f. (C) and later regain mobility and improve myotome organisation (G), although skeletal muscle is still less organised compared with control (E) or rescued larvae (H). Scale bar: 100 µm.

adaxial myogenesis (Hammond et al., 2007), complete inhibition of subsequent fast myogenesis had not been documented before. At variance with the mouse, *mrf4* is expressed as late as *myog*, at the onset of muscle differentiation, and therefore has no role in zebrafish muscle specification. However, when prematurely expressed, zebrafish *mrf4* is able to drive myogenesis via the activation of Myod, whereas *myog* cannot do so. The ability of *mrf4* to rescue myogenesis is probably due to the activity of the third α-helix in the C-terminus of the protein, as described by Bergstrom and Tapscot (Bergstrom and Tapscott, 2001). These authors demonstrated that the third α-helix, conserved in all four MRFs, has evolved distinct functions in Myod and myogenin. Whereas in Myod it appears to be a domain critical for the efficient initiation of skeletal muscle gene expression, in myogenin it rather acts as a general transcription activation domain. They further showed that the C-terminal domain of Mrf4 can substitute for the domain in Myod, but the same domain of myogenin cannot. Also, either Mrf4 or Myod is required together with myogenin to mediate terminal

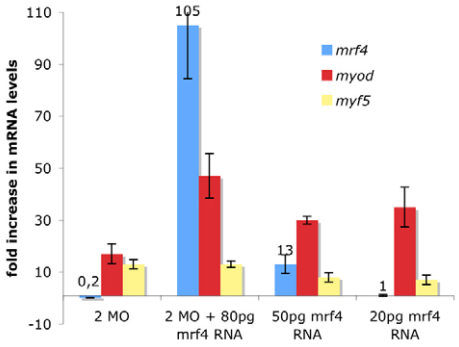

Fig. 8. *mrf4* is a strong activator of *myod* but not of *myf5*. Real-time PCR results show fold changes in *mrf4*, *myod* and *myf5* mRNA expression in double morphants, rescued and *mrf4* mRNA injected embryos normalised to a housekeeping gene and to their expression in uninjected embryos, which is set to 1. Error bars indicate s.d. RNA of all embryos was extracted at the 15-somite stage.

muscle cell differentiation, because mutation of *mrf4* and *myod* result in a severe skeletal muscle deficiency, despite normal expression of *myog* (Rawls et al., 1998). Myogenin is indeed the only MRF that failed to induce muscle-specific RNAs when ectopically expressed in non-muscle cells (Roy et al., 2002). Taken together, these data strongly indicate that Myf5, Mrf4 and Myod, which are lineage specification factors in the mouse, possess a greater intrinsic ability to initiate the expression of silent genes than myogenin, which rather acts as a differentiation factor. The third α-helices in zebrafish Mrf4 and myogenin protein are strongly conserved, containing only one and two amino acid changes, respectively, compared with the same domain in the mouse. It is therefore probably due to the different function of this domain that *mrf4* is able to rescue myogenesis in double-morphant zebrafish but *myog* is not.

It remains unknown why zebrafish and mouse *mrf4* have a similar molecular function and yet are expressed at different periods in the two organisms. It is also not clear when, in the course of vertebrate evolution, the expression of *mrf4* changed, i.e. when the gene acquired regulatory sequences able to respond to myogenic inducing factors in muscle progenitors. Also, in *Xenopus* embryos, *mrf4* is expressed late, but interestingly here it clearly precedes myogenin expression (Della Gaspera et al., 2006; Hopwood et al., 1989; Hopwood et al., 1991; Jennings, 1992; Nicolas et al., 1998), providing yet another relative expression pattern of the MRFs. However, no functional assays have been performed to elucidate the role of *mrf4* or *myog* in this context.

Zebrafish *mrf4* is a potent activator of *myod* but not of *myf5*

We show here that *mrf4* is able to activate Myod expression, despite the presence of the *myod* morpholino. Our real-time PCR results demonstrate that *mrf4* rescue leads to a 30- to 50-fold increase in

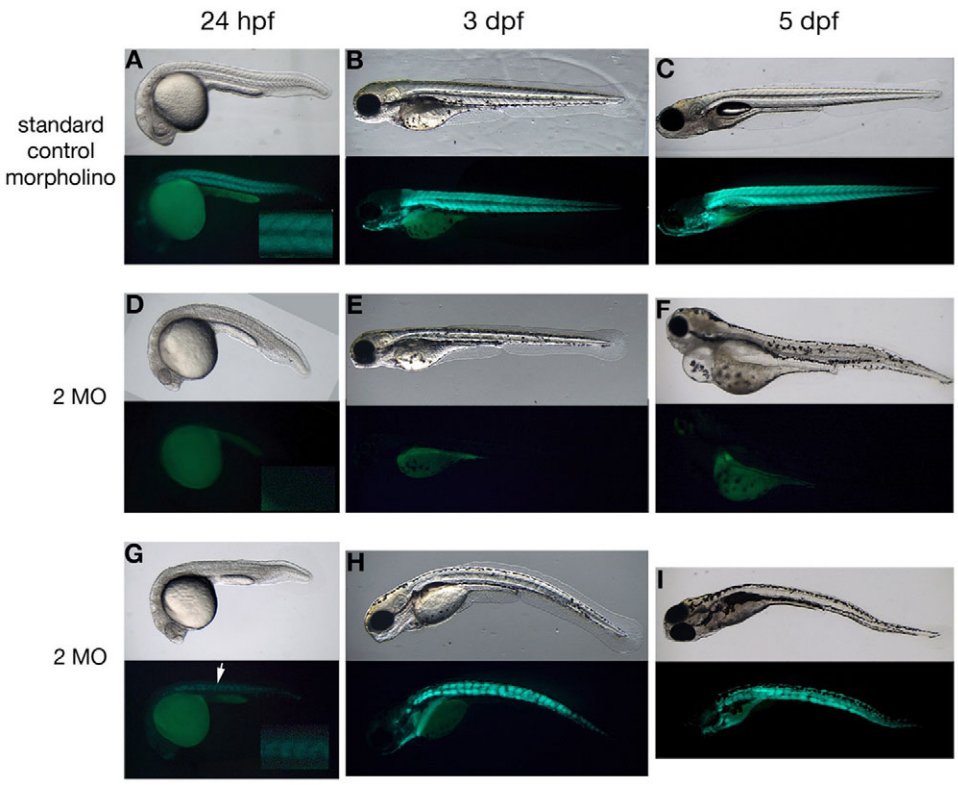

Fig. 10. GFP expression in α-actin-GFP transgenic embryos. (A-C) Embryo injected with the standard control morpholino and (D-I) two *myf5/myod* double-morphant embryos at 24 h.p.f. (A,D,G), 3 d.p.f. (B,E,H) and 5 d.p.f. (C,F,I). The majority of the double morphants never express GFP (D-F), however the double-morphant embryos that do express GFP at 24 h.p.f. (G, arrow points to weak GFP expression) increase GFP expression over time (see H,I). Each panel is composed of the normal light and the fluorescence image of the same embryo. A and G show a part of the tail at higher magnification.

myod mRNA, an amount that is probably sufficient to titrate out the amount of morpholino in the picomolar range and to explain the histochemical detection of the Myod protein in the nuclei of the rescued embryos. Following coinjection of *myod/myf5* morpholinos, both *myod* and *myf5* mRNAs are upregulated and this could be attributed to a compensation effect due to morpholino-mediated downregulation of Myf5 and Myod. Importantly, only *myod* mRNA levels are further increased by additional injection of *mrf4* mRNA, whereas *myf5* levels remain unchanged. Remarkably, even injection amounts of *mrf4* mRNA that cannot be detected by quantitative real-time PCR at levels higher than those detected in uninjected control embryos result in an >30-fold activation of *myod* mRNA, indicating that *mrf4* is a potent activator of *myod*. Increasing the amount of *myod* morpholino prevented rescue by *mrf4*, indicating that *myod* activation is necessary for *mrf4* rescue.

In addition, we also show by in situ hybridisation studies that *myog* mRNA is increased in early *mrf4*-rescued embryos, which could be either a direct or an indirect activation via *myod*. Most likely, both the direct activity of *mrf4* together with that of induced *myod* drive myogenesis in the rescued embryos.

Also, in the mouse, Mrf4 might have the ability to activate *Myod* because the *Myf5* single mutant does express *Myod* but the *Myf5/Mrf4* double mutant does not (Kassar-Duchossoy et al., 2004). Additionally, we demonstrate here that zebrafish *myod* can activate *mrf4* in *myf5* morphant zebrafish, indicating a positive-feedback loop between these two genes in zebrafish, which has not been reported in any species so far. For a schematic overview of the muscle gene interactions see Fig. 11.

The early myotome is required for muscle patterning in zebrafish

Thanks to the transient nature of morpholino inhibition, we have investigated the development of skeletal musculature in the absence of an anatomically defined myotome. In the mouse, ablation of *Myf5* and *Mrf4* delays the appearance of a myotome for over 2 days, whereas in *Myod* mutant embryos the myotome appears normal, probably due to the early expression of *Myf5* compared with *Myod*. However, even in the absence of an early myotome, skeletal myogenesis proceeds normally in the mouse, with only minor defects in the epaxial musculature (Kablar et al., 1997). By contrast, myotome absence precludes muscle patterning in the zebrafish. The minority of embryos that assemble fewer and disorganised muscle fibres compared with control embryos are probably those in which inhibition by morpholinos was not complete, and where some muscle had initially formed. Surprisingly, these embryos recover the ability to swim. In both immobile and motile surviving embryos, we found bundles of sarcomeres perpendicularly oriented to other bundles in the same cytoplasm of the few residual muscle cells. This observation suggests that an ordered pattern of MRF expression is also required to drive correct sarcomerogenesis in the embryonic muscle. Thus it appears that the myotome is crucial for further muscle development in zebrafish, consistent with the notion that further muscle development uses the myotome as a template and the adult muscle anatomy remains morphologically unchanged. By contrast, the large remodelling of muscle patterning that occurs in tetrapods probably developed upon later morphogenetic signals, so

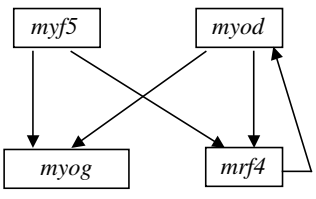

Fig. 11. Model of muscle gene interactions during zebrafish development.

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that the patterning role of the myotome was progressively diminished. Conditional *Myf5* and *Myod* ablation in the mouse, and morpholino approaches in other classes of vertebrates, might further address these issues in the future.

Materials and Methods

Zebrafish lines and maintenance

Breeding wild-type fish of the AB strain were maintained at 28°C on a 14 hours light/10 hours dark cycle. Embryos were collected by natural spawning, staged according to Kimmel (Kimmel et al., 1995), and raised at 28°C in fish water (Instant Ocean, 0.1% methylene blue) in Petri dishes (Haffter and Nüsslein-Volhard, 1996).

In situ hybridisation and immunohistochemistry

Whole-mount in situ hybridisation, WISH, was carried out as described (Thisse et al., 1993) on embryos fixed for 2 hours in 4% paraformaldehyde in PBS, then rinsed with PBS-Tween (PBT), dehydrated in 100% methanol and stored at –20°C until processed (Jowett and Lettice, 1994). Probes were transcribed with T7 polymerase for antisense and SP6 polymerase for sense probes, and in vitro labeled with digoxigenin (Roche). Primers for PCR probe templates for *mrf4* and *myog* are: *mrf4*_forward, 5′-ATTTAGGTGACACTATAGTTTTCAATGATTTGCGTTATCTT-3′; *mrf4*_reverse, 5′-TAATACGACTCACTATAGGGGAAGACTGCTGGACTCT-GAAGAC-3′; *myogenin*_forward, 5′-ATTTAGGTGACACTATAGGATAATTTC-TTCCAGTCCAGAATCA-3′; *myog*_reverse, 5′-TAATACGACTCACTATAGGG-CTGTCCACTATAGACGTCAGAGACC-3′. *Myod* probe was transcribed from a plasmid (kindly provided by Steve Wilson, University College London, UK) after linearisation with *Bam*HI.

For immunohistochemistry, embryos were fixed for 2 hours in fish fix (4% paraformaldehyde, 0.15 mM CaCl2, 4% sucrose, 0.1 M phosphate buffer pH 7.3) or for 10 minutes with a mix of 50% methanol and 50% acetone, washed several times in PBT and blocked in 10% donkey serum in PBT for 1 hour at room temperature. Primary antibody incubation was overnight at 4°C, followed by several washes in PBT and incubation of secondary antibody for 1 hour at room temperature. Nuclei were stained with Hoechst 33342. Primary antibodies are A4.1025 (anti-human all myosin) and EB165 (anti-chicken fast myosin heavy chain) purchased from Developmental Studies Hybridoma Bank (mouse hybridoma cells were grown in our lab and medium was collected and diluted 1:30 for antibody staining). Myod antibody is rabbit anti-Myf5 C-20 from Santa Cruz, and was diluted 1:100. Secondary antibodies are TRITC- or FITC-conjugated donkey anti-rabbit or anti-mouse from Molecular Probes, diluted 1:500. Images of embryos and sections were acquired using a fluorescence microscope equipped with a digital camera. Images were processed using the Adobe Photoshop software.

Injections and plasmids

myod mRNA was transcribed from a plasmid kindly provided by Steve Wilson. *mrf4* (AY335193), *myog* (AF202639) and *myf5* (AF270789) cDNAs were cloned by us in the pCS2^{+} expression plasmid after amplification of the genes from embryonic cDNA. *mrf4* and *myf5* were amplified with primers containing the *Eco*RI (in forward primer) and *Xho*I (in reverse primer) restriction sites, whereas *myog* primers have a *Bam*HI site in the forward primer and a *Xho*I site in the reverse primer. All cloned plasmids were verified by DNA sequencing. Synthetic capped *mgn* and *mrf4* mRNA was injected repeatedly (n>3) at 20, 50, 80, 100 and 200 pg per embryo. Injections were carried out on 1- to 2-cell-stage embryos (with Eppendorf FemtoJet Micromanipulator 5171); the dye tracer rhodamine dextran was co-injected as a control. To repress *mrf4* mRNA translation we designed an ATG-targeting morpholino (Gene Tools, LLC): *mrf4*-MO 5′-CGTTGGTCTCAAACAGGTCCATCAT-3′. To repress *myf5* we designed two *myf5* morpholinos against the ATG region and got similar results with both: *myf5* MO 5′-TACGTCCATGATTGGTTTGGTGTTG-3′; *myf5*B-MO 5′-GATCTGGGATGTGGAGAATACGTCC-3′. We could further rescue the *myf5/myod* double morphants by coinjection of *myf5* or *myod* mRNA. To repress *myod* mRNA translation we designed an ATG-targeting morpholino: *myod*-MO 5′-ATATC-CGACAACTCCATCTTTTTTG-3′; and as negative controls we injected 0.5 pmole of a 5 bp mismatch morpholino against *myod* 5′-ATtTCCcACAAgTCCATg-TTTTaTG-3′ that did result in an abnormal phenotype, or a standard control morpholino oligonucleotide (stdr-MO), a human β-thalassemia-specific morpholino that has not been reported to have other targets or generate any phenotypes in any known test system except human β-thalessemic hematopoietic cells. 0.5 pmole of *myod* and *mrf4* morpholinos and 0.25 pmole of *myf5* morpholino were injected in 1× Danieau buffer (pH 7.6) as suggested by Nasevicius and Ekker (Nasevicius and Ekker, 2000). (0.5 pmole morpholino correspond to approximately 4 ng.) All the morpholinos we injected have already been used by others and have been tested for their specificity (Chen and Tsai, 2002; Hammond et al., 2007; Lin et al., 2006; Wang et al., 2008).

Quantitative real-time RT-PCR

Total RNA was isolated from embryos at indicated developmental stages (1-2, 6-8 and 15 somites). Reverse transcriptions (RTs) were performed using 2 μg DNase-treated (DNA-*free*^{TM}, Ambion) total RNA in presence of random hexamers (Invitrogen^{TM}) and SuperScript II reverse transcriptase (Invitrogen^{TM}). Real-time PCRs were carried out in a total volume of 10 μl containing 1× iQ SYBR Green Super Mix (Bio-Rad) using 0.5 μl of the RT reaction. PCRs were performed using the Mx3000P Real Time Detection System (Stratagene). For normalisation purposes, 18S ribosomal RNA or elongation factor 1 alpha (*ef1alpha*) mRNA was amplified in parallel with the gene of interest. The following primers were used: *myf5*_sense, 5′-GAATAGCTACAACTTTGACG-3′; *myf5*_antisense, 5′-GTAAACTGGTCTG-TTGTTTG-3′; *mrf4*_sense, 5′-ACAACCTGAAGGAAAACCAT-3′; *mrf4*_antisense, 5′-TCTTCAGTGGAAATGCTGTC-3′; *myog*_sense, 5′-TCTGAAGAGGAG-CACATTGA-3′; *myog*_antisense, 5′-AGCCCTGATCACTAGAGGA-3′; 18S_sense: 5′-ACCTCACTAAACCATCCAATC-3′ and 18S_antisense, 5′-AGGAATTCC-CAGTAAGCGCA-3′; *ef1alpha*_sense, 5′-CAAGGAAGTCAGCGCATACA-3′; *ef1alpha*_antisense, 5′-TCTTCCATCCCTTGAACCAG-3′. All primer pairs are located in different exons. To calculate the fold increase in mRNA level of the gene of interest, normalised to the mRNA level of the housekeeping gene, the following equation was used: $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T=(C_{T,\text{Target}}-C_{T,\text{ef1alpha}})_{\text{Condition x}}-(C_{T,\text{Target}}-C_{T,\text{ ef1alpha}})_{\text{Condition 0}}$ (Livak and Schmittgen, 2001; Pfaffl, 2001). Condition x corresponds to the morpholino and mRNA injections and condition 0 to untreated embryos. Targets are the *mrf4*, *myf5* and *myod* genes. All samples were run in triplicate and s.d. was calculated.

Histological sections and electron microscopy

24 h.p.f. whole zebrafish embryos were manually dechorionated and fixed overnight at 4°C with 1.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.3. They were rinsed in the same buffer and postfixed for 1 hour in sodium-cacodylate-buffered 1% osmium tetroxide. The samples were then dehydrated in a graded ethanol series, transitioned to propylene oxide and embedded in Epon 812- Araldite. Sections were obtained using a Reichert Ultracut E. 0.5 μm sections were stained with gentian violet and photographed with a digital camera. Thin sections were cut at 70 nm and placed onto copper grids, stained with 2% aqueous uranyl acetate and lead citrate and analysed under a Jeol 100 SX electron microscope. Cryosections were performed on embryos following antibody staining. Embryos were embedded in 5% sucrose and 1.5% agarose, frozen in OCT and cut into 12- m-thin transverse sections on a Leica cryostat.

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References

Bergstrom, D. A. and Tapscott, S. J. (2001). Molecular distinction between specification and differentiation in the myogenic basic helix-loop-helix transcription factor family. *Mol. Cell. Biol.* **21**, 2404-2412.

Bober, E., Lyons, G., Braun, T., Cossu, G., Buckingham, M. and Arnold, H. H. (1991). The muscle regulatory gene, Myf-6, has a biphasic pattern of expression during early mouse development. *J. Cell Biol.* **113**, 1255-1265.

Braun, T. and Arnold, H. H. (1995). Inactivation of Myf-6 and Myf-5 genes in mice leads to alterations in skeletal muscle development. *EMBO J.* **14**, 1176-1186.

Braun, T., Rudnicki, M. A., Arnold, H. H. and Jaenisch, R. (1992). Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell* **71**, 369-382.

Chen, Y. H. and Tsai, H. J. (2002). Treatment with Myf5-morpholino results in somite patterning and brain formation defects in zebrafish. *Differentiation* **70**, 447-456.

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

Della Gaspera, B., Sequeira, I., Charbonnier, F., Becker, C., Shi, D. L. and Chanoine, C. (2006). Spatio-temporal expression of MRF4 transcripts and protein during Xenopus laevis embryogenesis. *Dev. Dyn.* **235**, 524-529.

Groves, J. A., Hammond, C. L. and Hughes, S. M. (2005). Fgf8 drives myogenic progression of a novel lateral fast muscle fibre population in zebrafish. *Development* **132**, 4211-4222.

Haffter, P. and Nüsslein-Volhard, C. (1996). Large scale genetics in a small vertebrate, the zebrafish.. *Int. J. Dev. Biol.* **40**, 221-227.

Hammond, C. L., Hinits, Y., Osborn, D. P., Minchin, J. E., Tettamanti, G. and Hughes, S. M. (2007). Signals and myogenic regulatory factors restrict pax3 and pax7 expression to dermomyotome-like tissue in zebrafish. *Dev. Biol.* **302**, 504-521.

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N. and Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**, 501-506.

Higashijima, S., Okamoto, H., Ueno, N., Hotta, Y. and Eguchi, G. (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. *Dev. Biol.* **192**, 289-299.

Hinits, Y., Osborn, D. P., Carvajal, J. J., Rigby, P. W. and Hughes, S. M. (2007). Mrf4 (myf6) is dynamically expressed in differentiated zebrafish skeletal muscle. *Gene Expr. Patterns* **7**, 738-745.

Hinterberger, T. J., Sassoon, D. A., Rhodes, S. J. and Konieczny, S. F. (1991). Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Dev. Biol.* **147**, 144-156.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1989). MyoD expression in the forming somites is an early response to mesoderm induction in Xenopus embryos. *EMBO J.* **8**, 3409-3417.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1991). Xenopus Myf-5 marks early muscle cells and can activate muscle genes ectopically in early embryos. *Development* **111**, 551-560.

Jennings, C. G. (1992). Expression of the myogenic gene MRF4 during Xenopus development. *Dev. Biol.* **151**, 319-332.

Jowett, T. and Lettice, L. (1994). Whole-mount in situ hybridizations on zebrafish embryos using a mixture of digoxigenin- and fluorescein-labelled probes. *Trends Genet.* **10**, 73-74.

Kablar, B., Krastel, K., Ying, C., Asakura, A., Tapscott, S. J. and Rudnicki, M. A. (1997). MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle. *Development* **124**, 4729-4738.

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V. and Tajbakhsh, S. (2004). Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature* **431**, 466-471.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

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

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402-408.

Maves, L., Waskiewicz, A. J., Paul, B., Cao, Y., Tyler, A., Moens, C. B. and Tapscott, S. J. (2007). Pbx homeodomain proteins direct Myod activity to promote fast-muscle differentiation. *Development* **134**, 3371-3382.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I. and Nabeshima, Y. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364**, 532-535.

Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216-220.

Nicolas, N., Gallien, C. L. and Chanoine, C. (1998). Expression of myogenic regulatory factors during muscle development of Xenopus: myogenin mRNA accumulation is limited strictly to secondary myogenesis. *Dev. Dyn.* **213**, 309-321.

Patapoutian, A., Yoon, J. K., Miner, J. H., Wang, S., Stark, K. and Wold, B. (1995). Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. *Development* **121**, 3347-3358.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.

Rawls, A., Valdez, M. R., Zhang, W., Richardson, J., Klein, W. H. and Olson, E. N. (1998). Overlapping functions of the myogenic bHLH genes MRF4 and MyoD revealed in double mutant mice. *Development* **125**, 2349-2358.

Roy, K., de la Serna, I. L. and Imbalzano, A. N. (2002). The myogenic basic helix-loop-helix family of transcription factors shows similar requirements for SWI/SNF chromatin remodeling enzymes during muscle differentiation in culture. *J. Biol. Chem.* **277**, 33818-33824.

Rudnicki, M. A., Braun, T., Hinuma, S. and Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell* **71**, 383-390.

Rudnicki, M. A., Schnegelsberg, P. N., Stead, R. H., Braun, T., Arnold, H. H. and Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351-1359.

Thisse, C., Thisse, B., Schilling, T. F. and Postlethwait, J. H. (1993). Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. *Development* **119**, 1203-1215.

Wang, Y. H., Li, C. K., Lee, G. H., Tsay, H. J., Tsai, H. J. and Chen, Y. H. (2008). Inactivation of zebrafish mrf4 leads to myofibril misalignment and motor axon growth disorganization. *Dev. Dyn.* **237**, 1043-1050.

Weinberg, E. S., Allende, M. L., Kelly, C. S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O. G., Grunwald, D. J. and Riggleman, B. (1996). Developmental regulation of zebrafish Myod in wild-type, no tail and spadetail embryos. *Development* **122**, 271-280.

Zhang, W., Behringer, R. R. and Olson, E. N. (1995). Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. *Genes Dev.* **9**, 1388-1399.

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