Published in final edited form as:

Gene Expr Patterns. 2007 August ; 7(7): 738–745. doi:10.1016/j.modgep.2007.06.003.

***Mrf4* (*myf6*) is dynamically expressed in differentiated zebrafish skeletal muscle**

Yaniv Hinitz, Daniel P. S. Osborn, Jaime J. Carvajal[§], Peter W. J. Rigby[§], and Simon M. Hughes¹

MRC Centre for Developmental Neurobiology and Randall Division of Cell and Molecular Biophysics, New Hunt's House, Guy's Campus, King's College London, London SE1 1UL, UK

[§]Section for Gene Function and Regulation, Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB

Abstract

Mrf4 (*Myf6*) is a basic helix-loop-helix (bHLH) myogenic regulatory transcription factor (MRF) family which also contains *Myod*, *Myf5* and *myogenin*. *Mrf4* is implicated in commitment of amniote cells to skeletal myogenesis and is also abundantly expressed in many adult muscle fibres. The specific role of *Mrf4* is unclear both because *mrf4* null mice are viable, suggesting redundancy with other MRFs, and because of genetic interactions at the complex *mrf4/myf5* locus. We report the cloning and expression of an *mrf4* gene from zebrafish, *Danio rerio*, which shows conservation of linkage to *myf5*. *Mrf4* mRNA accumulates in a subset of terminally differentiated muscle fibres in parallel with myosin protein in the trunk and fin. Although most, possibly all, trunk muscle expresses *mrf4*, the level of mRNA is dynamically regulated. No expression is detected in muscle precursor cell populations prior to myosin accumulation. Moreover, *mrf4* expression is not detected in head muscles, at least at early stages. As fish mature, *mrf4* expression is pronounced in slow muscle fibres.

Keywords

mrf4; muscle; zebrafish; muscle pioneers; muscle fibre; fin; *myod*; *myogenin*; *myl2*; gene expression, craniofacial

1.0 Results

Mrf4 shows biphasic expression in rodent muscle development, being primarily expressed in terminally differentiated muscle fibres (Bober et al., 1991; Hinterberger et al., 1991). However, early expression is in the mouse ventrolateral dermomyotome, prior to muscle terminal differentiation (Summerbell et al., 2002). This early expression has been suggested to permit myogenesis of a subset of myotomal muscle fibres in *Myf5/Myod* double knockouts (Kassar-Duchossoy et al., 2004). Such a myogenic function has been associated with the presence of a serine-rich chromatin-remodelling region shared with *Myod* and *Myf5* (Bergstrom and Tapscott, 2001). *Mrf4* null mice are viable, although genomic manipulations cause suppression of the linked *Myf5* gene, making interpretation of phenotypes difficult (Braun and Arnold, 1995; Olson et al., 1996; Patapoutian et al., 1995; Zhang et al., 1995). *Mrf4* null mice have defects in neural control of adult gene expression (Thompson et al., 2005). Regulation at early and late stages is controlled by distinct genetic

¹Corresponding author: Simon M. Hughes, MRC Centre for Developmental Neurobiology, 4th floor south, New Hunt's House, Guy's Campus, King's College London, London SE1 1UL, UK. Tel.: +44 20 7848 6445; fax: +44 20 7848 6550; simon.hughes@kcl.ac.uk.

elements (Carvajal et al., 2001; Fomin et al., 2004; Pin et al., 1997). In adult muscle, *Mrf4* expression persists as the most abundant MRF and appears to be differentially regulated between different fibre types (Miner and Wold, 1990; Pin and Konieczny, 2002; Walters et al., 2000). To gain insight into the ancestral role of *Mrf4*, we analysed the sequence and expression of *mrf4* in zebrafish.

1.1 Sequence and phylogeny analysis of *mrf4*

We cloned zebrafish *mrf4*, sequenced both a BAC and cDNA clones and assembled an open reading frame (Fig. 1A). The sequence agreed with the latest release of the zebrafish genome project (Zv6), which shows *mrf4* linked to *myf5*. Zebrafish *mrf4*, like other MRF genes, has three exons separated by two introns. An open reading frame encoding 239 amino acids was identified (as in *Fugu* *Mrf4* and compared with 240 amino acids in *Xenopus* *Mrf4* and 242 amino acids in chicken, mouse, rat and human *MRF4*). Zebrafish *Mrf4* is highly homologous to *MRF4* from other vertebrates (Fig. 1B; ~56% similarity, comparable to the 55% and ~73% similarity between zebrafish *myf5* and *myoD*, respectively, with these genes in amniotes (Coutelle et al., 2001; Weinberg et al., 1996).

Homology is especially high in the basic helix-loop-helix domain that functions in the heterodimerisation and DNA binding of MRFs and in the serine-rich area which may be associated with ability to remodel chromatin (Fig. 1B; Gerber et al., 1997). The divergence between proteins generally matches known evolutionary relationships (Fig. 1C). No duplicate of the *mrf4* or *myf5* genes is apparent in the current genome release. Moreover, despite the proposed genome duplication in teleosts, *myoD* and *myogenin* also do not show duplication.

During 3' RACE a second *mrf4* transcript was also obtained, which showed an alternate third exon (Fig. 1D). Analysis of the genomic sequence revealed the insertion of a fish-specific DNA retrotransposon element (Izsvak et al., 1996) into the second intron of *mrf4*, presumably providing an alternative splice acceptor site. This transcript predicts a truncated *Mrf4* protein lacking the third exon and with five novel amino acids VPLLI at the C terminus. In situ mRNA hybridisation with a probe against the canonical third exon gives muscle-specific signal. A probe to the alternative third exon was not made due to predicted cross-reaction to widespread DNA transcripts.

1.2 Expression of *mrf4* in slow muscle

Two muscle fibre types form in the first few hours after somite formation: slow fibres arise first from adaxial cells adjacent to the notochord and fast fibres form later from more lateral somitic cells (Devoto et al., 1996). *Mrf4* mRNA is not detected by in situ hybridisation at the 2 cell, 64 cell, 1k cell, dome or tailbud stages, consistent with the lack of expression at 1-4 somite stage (www.zfin.org). *Mrf4* mRNA is first detected in cells adjacent to the notochord in nascent somites at the 5 somite stage, after *actin* (5s; Fig. 2A). As these rostral somites mature, *mrf4* expression is initially strong for around 3 hours, and then declines (Fig. 2B-F). From about the 6s stage, one somite is formed every 30 minutes, so six somites continue to show strong adaxial *mrf4* expression (Fig. 2E-H). Within the presomitic mesoderm (PSM), *mrf4* mRNA is absent from adaxial cells, which express *myoD* and the slow muscle determination factor *prdm1* (Fig. 2B,F,H). *Mrf4* mRNA accumulates much later than early differentiation markers such as actin (*actc*; Fig. 1C), but also after later ones, such as myosin mRNA (data not shown). Co-localization of myosin heavy chain protein (MyHC) and *mrf4* mRNA at 13s reveals that *mrf4* is detected exclusively in terminally differentiated slow fibres at this stage (Devoto et al., 1996; Fig. 2G). Similarly, in frogs, *Xmrf4* is co-localised with MyHC and absent from the PSM (Della Gaspera et al., 2006).

Early muscle differentiation is ablated by antisense morpholino knockdown of *myf5* and *myod* (Hammond et al., 2007). Such treatment prevents both *mrf4* mRNA and MyHC accumulation, consistent with expression of *mrf4* only in differentiated muscle (Fig. 2S). This contrasts with the ability of murine *Mrf4* to express in *Myf5:Myod* double mutants (Kassar-Duchossoy et al., 2004).

That early *mrf4* mRNA in differentiated slow muscle cells is supported by its dependence on Hedgehog (Hh) signalling, which drives slow myogenesis (Blagden et al., 1997; Du et al., 1997). In the Hh signalling mutant *gli2a/yot* (Karlstrom et al., 1999), *mrf4* mRNA is missing at 12s (Fig. 3A). Loss of slow muscle in the *prdm1* mutant (Baxendale et al., 2004) is also accompanied by loss of *mrf4* expression. Thus, *mrf4* appears to be transiently expressed for around three hours in slow muscle cells of each somite as it forms.

The transient expression of *mrf4* led us to ask whether all slow muscle fibres express *mrf4* similarly. Flatmounts of wild type embryos show that *mrf4* expression is only in some nascent fibres, those that are largest and located nearest to the midline (Fig. 2G inset). This suggests that muscle pioneer (MP) fibres express *mrf4*, because MPs were originally described as the first fibres to differentiate in the somite and remain close to notochord (Felsenfeld et al., 1991). In contrast, the slow fibres that translocate to the lateral myotome surface, precursors of the superficial slow fibres (SSF; Devoto et al., 1996), do not appear to express *mrf4* as highly as MPs, at least at these early developmental stages.

To test the hypothesis that MPs alone express *mrf4* at early stages, we examined *sonic hedgehog a* (*shha*; *syu*) mutants that lack MPs but have residual SSF differentiation. At 15s, *shha* mutant embryos contain medial slow muscle but lack *eng2a* expression, a marker of MPs (Fig. 3B; Coutelle et al., 2001; Schauerte et al., 1998). Despite the residual SSF precursors in *shha* mutants, no *mrf4* expression was detected prior to 18s (Fig. 3C; 43/159 embryos from a *shha* heterozygote cross). Similar results were observed in *spadetail* (*tbx16* mutants, 23/77 embryos) and *floating head* (*flh* mutants, 11/32 embryos) that also lack MPs but retain SSF cells (data not shown). In the hypomorphic *prdm1* mutant, *prdm1^{tp39}(ubo)*, which lacks MPs but has residual slow fibres at 10s (Baxendale et al., 2004; Roy et al., 2001), the high level *mrf4* in nascent MPs is also missing (Fig. 3D). During late somitogenesis, when MPs are forming in the caudal most somites, strong *mrf4* signal is absent from *shha*, *prdm1* and *gli2a* mutants, but visible in siblings (Fig. 3E,G,H arrowheads). More anteriorly in 24hpf wild type embryos, *mrf4* mRNA is absent from the location of the MPs at the dorsoventral midline, and this gap in expression is missing in *shha*, *prdm1* and *gli2a* mutants (Fig. 3E and data not shown). In contrast *mrf4* expression in slow fibres is not affected in *fgf8* morphants (Fig. 3J; 27/27 embryos) or mutants (17/17 embryos; Fig. 3K). Taken together, these data suggest that *mrf4* is transiently strongly expressed in MP fibres, in contrast to SSF precursors, as they begin to accumulate muscle MyHC protein adjacent to the midline.

Mrf4 is expressed in SSF at later stages. SSFs differentiate adaxially and then transverse the somite to form a monolayer of mononucleate fibres at the lateral myotome surface (Devoto et al., 1996). SSF and fast precursors express little, if any, *mrf4* prior to 18s when *mrf4* has declined in MPs of rostral somites (Fig. 2A-H). However, in *prdm1* hypomorphic mutants, which have some residual slow muscle (Roy et al., 2001), very low levels of *mrf4* mRNA are detected (Fig. 3D). Interestingly, heterozygous *gli2a* mutants also show reduced *mrf4* mRNA despite the presence of slow muscle, raising the possibility that Hh signalling can drive *mrf4* expression in SSFs (Fig. 3A). By 21s, *mrf4* mRNA is up-regulated in differentiated fibres of anterior somites (Fig. 2I). At this stage, differentiated SSF precursors have spread dorsoventrally to flank the neural tube and hypochord and commence lateral migration. *Mrf4* is detected co-localized with MyHC in migrating SSF

precursors of *shha* embryos, which lack MPs (Fig. 3F). Such mutants have no fast myosin protein at this stage. When the SSFs reach the lateral surface of the somite, *mrf4* is readily detected in the SSF monolayer at 24 hpf and beyond (Fig. 2J,M,R). High level *mrf4* mRNA is not observed in MPs at 24 hpf (Fig. 2J). Thus, the SSFs express *mrf4* later than MPs, starting as they begin migration.

1.3 *Mrf4* expression in later muscle development

Mrf4 is also expressed in fast fibres. *Mrf4* mRNA is not detected in fast fibre precursors marked by *myod* or *myog* mRNA in the lateral somite at early stages (Fig. 2B,D,H). However, at 21s, *mrf4* mRNA is detected in cells of the lateral somite (Fig. 2I). *Mrf4* mRNA in these cells accumulates shortly after differentiation markers such as myosin light chain *mylz2* mRNA (data not shown). By the end of somitogenesis, *mrf4* mRNA is clearly detected in dorsal and ventral somitic cells that are not MPs (Figs 2K and 3E). Both wild type, and also *gli2a* mutant embryos which entirely lack slow muscle fibres, show *mrf4* expression in fast muscle (Figs 2J and 3H,I). Thus, *mrf4* is expressed in fast fibres. *Fgf8* signalling is required for differentiation of fast but not slow fibres (Groves et al., 2005; Hammond et al., 2007). In *fgf8/ace* mutants, the amount of *mrf4* mRNA and differentiated fast fibre is substantially reduced compared to their siblings (17/56 embryos; Fig. 3K). After somitogenesis is complete around 24hpf, levels of detectable *mrf4* mRNA decline, although low levels of *mrf4* mRNA remain in muscle fibres at 48hpf and 5d of development, more prominently in slow fibres (Fig. 2L,M,Q,R).

1.4 *Mrf4* expression in fin and head muscle development

Mrf4 mRNA is first detected in pectoral fin muscles at 48hpf (long pec stage) in two muscle masses (dorsal and ventral; Fig. 2N). *Myogenin* (*myog*) mRNA and MyHC protein are also expressed at this stage in a similar pattern (Fig. 2O; Neyt et al., 2000), whereas *myod* mRNA is detected in the fin buds earlier, from around 30hpf (Neyt et al., 2000; Weinberg et al., 1996). *Mrf4* expression in fin muscles remains strong at 3d of development (Fig. 2P). In rodents, *Mrf4* is expressed after other MRFs, during secondary fibre myogenesis (Bober et al., 1991; Hinterberger et al., 1991). In contrast to fin expression, we found no *mrf4* mRNA in head muscles at least until 5d of development (Fig. 2N). On the contrary, *myog* (Fig. 2O) and muscle structural genes (Schilling and Kimmel, 1997; Xu et al., 2000 and data not shown) express in head muscle from around 48hpf. Our inability to detect *mrf4* mRNA in head muscle, contrasts with a recent report of *mrf4* mRNA in a similar pattern to *myogenin* mRNA in head muscles at 3d (Lin et al., 2006). Lack of early *Mrf4* expression in cranial muscle has also been described in embryonic mice (Bober et al., 1991). At no stage did we observe expression of *mrf4* in heart, smooth muscle or any other tissue except for skeletal muscle.

1.5 Conclusion

We have recently shown that muscle differentiation involves two steps: initial expression of early muscle genes followed by a second step regulating myofibril assembly (Hinitz & Hughes, 2007). Zebrafish *mrf4* expression parallels assembly of myosin protein into myofibrils. Expression is not detected in presomitic mesoderm, even though adaxial cells are terminally differentiating in this location as indicated by *actin* expression. *Mrf4* is dynamically expressed in subsets of terminally differentiated muscle fibres in both trunk somites and fins, but is not detected in head or fin muscle precursors, or in somitic cells lacking myosin. Transient expression in a subset of nascent slow fibres is followed by expression in both fast fibres and, more strongly, superficial slow. All manipulations examined that reduce myosin expression also diminish *mrf4* mRNA accumulation.

2.0 Experimental procedures

2.1 Zebrafish lines and maintenance

Mutant lines *shha*^{bx392} (Schauerte et al., 1998), *gli2a*^{ty119} (van Eeden et al., 1996), *flh*ⁿ¹ (Blagden et al., 1997), *spt*^{b104} (Kimmel et al., 1989), *prdm1*^{tp39} (van Eeden et al., 1996), *fgf8*^{ti282a} (Whitfield et al., 1996) were maintained on King's wild type background and staging and husbandry were as described (Westerfield, 1995).

2.2 Cloning zebrafish *mrf4*

A zebrafish BAC library was screened with a zebrafish *myf5* cDNA probe (Coutelle et al., 2001) and a single clone was isolated. Sequence analysis revealed no *mrf4* sequence in the approximately 10kb upstream of *myf5* (O. Coutelle and P.W.J.R., unpublished data). A probe generated from the end of this BAC was used to rescreen the library a single new clone isolated. Multiple restriction digests of this BAC were analysed by Southern blotting using mouse and *Fugu rubripes mrf4* probes in order to locate *mrf4*; an appropriate fragment was subcloned and sequenced. More sequence reads were added by sequencing a PCR product containing exon 1 and traces from the ongoing zebrafish genome project. DNA from 15s and 25s zebrafish embryos was extracted as described (Westerfield, 1995). Total RNA was extracted by using TRIZOL (Invitrogen) according to the manufacturer's protocol. Genomic PCR products were amplified using primers made according to the primary genomic reads obtained from the BAC library (FW: 5'TGAATCTGAAGCCCCGCAAC3' and REV: 5'ATTGCTCTGCTCCTGCTCATCC3'). A full-length cDNA was obtained by performing 3' and 5' RACE using Gene Racer kit (Invitrogen). Other genomic sequences were obtained from the zebrafish genome project and a sequence assembly mRNA made resolving ambiguity by reference to the original traces (GenBank: AY335193). Upon the release of Zv2 genome assembly all bases matched the genome sequence.

2.3 In situ mRNA hybridisation and immunohistochemistry

In situ mRNA hybridisation was conducted as previously described (Groves et al., 2005). A *mrf4* riboprobe was made by cloning the first exon PCR product into pGEM-T easy (Promega). For antisense probe plasmid was linearised with SpeI and T7 RNA polymerase used. Sense probe gave no signal above background. A second *mrf4* probe was made by amplifying the full sequence encoding Mrf4 with the reverse primer harbouring a T3 RNA polymerase binding site. This probe and a mixture of the two probes gave similar results. Other probes used were *myod*, *myogenin* (Weinberg et al., 1996), *eng2a* (Egger et al., 1992), *mylz2* (Xu et al., 2000), *prdm1* (Baxendale et al., 2004), *actc* (I.M.A.G.E clone 7284336). Probes were fluorescein or digoxigenin tagged by using Roche labelling mix. Antibody labelling of wholemount or sectioned embryos was performed as described (Groves et al., 2005), using primary antibodies: A4.1025 (IgG2a, recognises all MyHC, used 1:5; Dan-Goor et al., 1990), F59 (IgG1, recognises slow MyHC, used 1:5; Devoto et al., 1996). Antisense morpholinos to *myf5*, *myod* and *fgf8* were used as described (Groves et al., 2005; Hammond et al., 2007).

Acknowledgments

Thanks to Oliver Coutelle and Carlos Moreno de Barreda, who contributed to the cloning of zebrafish *mrf4*. We thank M. Westerfield, Z. Gong, F. Stockdale and E. Bandman for reagents. This work was supported by the MRC and the Institute of Cancer Research. YH received additional support from B'nai Brith, London, UK.

References

- Baxendale S, Davison C, Muxworthy C, Wolff C, Ingham PW, Roy S. The B-cell maturation factor Blimp-1 specifies vertebrate slow-twitch muscle fiber identity in response to Hedgehog signaling. *Nat Genet.* 2004; 36:88–93. [PubMed: 14702044]
- Bergstrom DA, Tapscott SJ. Molecular distinction between specification and differentiation in the myogenic basic helix-loop-helix transcription factor family. *Mol Cell Biol.* 2001; 21:2404–12. [PubMed: 11259589]
- Blagden CS, Currie PD, Ingham PW, Hughes SM. Notochord induction of zebrafish slow muscle mediated by Sonic Hedgehog. *Genes Dev.* 1997; 11:2163–2175. [PubMed: 9303533]
- Bober E, Lyons GE, Braun T, Cossu G, Buckingham M, Arnold HH. The muscle regulatory gene, Myf-6, has a biphasic pattern of expression during early mouse development. *J Cell Biol.* 1991; 113:1255–1265. [PubMed: 2045411]
- Braun T, Arnold HH. Inactivation of Myf-6 and Myf-5 genes in mice leads to alterations in skeletal muscle development. *EMBO J.* 1995; 14:1176–1186. [PubMed: 7720708]
- Carvajal JJ, Cox D, Summerbell D, Rigby PW. A BAC transgenic analysis of the Mrf4/Myf5 locus reveals interdigitated elements that control activation and maintenance of gene expression during muscle development. *Development.* 2001; 128:1857–68. [PubMed: 11311165]
- Coutelle O, Blagden CS, Hampson R, Halai C, Rigby PW, Hughes SM. Hedgehog signalling is required for maintenance of myf5 and myoD expression and timely terminal differentiation in zebrafish adaxial myogenesis. *Dev Biol.* 2001; 236:136–50. [PubMed: 11456450]
- Dan-Goor M, Silberstein L, Kessel M, Muhlrud A. Localization of epitopes and functional effects of two novel monoclonal antibodies against skeletal muscle myosin. *J. Muscle Res. Cell Motil.* 1990; 11:216–226. [PubMed: 1698201]
- Della Gaspera B, Sequeira I, Charbonnier F, Becker C, Shi DL, Chanoine C. Spatio-temporal expression of MRF4 transcripts and protein during *Xenopus laevis* embryogenesis. *Dev Dyn.* 2006; 235:524–9. [PubMed: 16258964]
- Devoto SH, Melancon E, Eisen JS, Westerfield M. Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. *Development.* 1996; 122:3371–3380. [PubMed: 8951054]
- Du SJ, Devoto SH, Westerfield M, Moon RT. Positive and negative regulation of muscle cell identity by members of the *hedgehog* and *TGF-β* gene families. *J Cell Biol.* 1997; 139:145–156. [PubMed: 9314535]
- Ekker M, Wegner J, Akimenko MA, Westerfield M. Coordinate embryonic expression of three zebrafish *engrailed* genes. *Development.* 1992; 116:1001–1010. [PubMed: 1363539]
- Felsenfeld AL, Curry M, Kimmel CB. The *fub-1* mutation blocks initial myofibril formation in zebrafish muscle pioneer cells. *Dev Biol.* 1991; 148:23–30. [PubMed: 1936560]
- Fomin M, Nomokonova N, Arnold HH. Identification of a critical control element directing expression of the muscle-specific transcription factor MRF4 in the mouse embryo. *Dev Biol.* 2004; 272:498–509. [PubMed: 15282164]
- Gerber AN, Klesert TR, Bergstrom DA, Tapscott SJ. Two domains of MyoD mediate transcriptional activation of genes in repressive chromatin: a mechanism for lineage determination in myogenesis. *Genes Dev.* 1997; 11:436–50. [PubMed: 9042858]
- Groves JA, Hammond CL, Hughes SM. Fgf8 drives myogenic progression of a novel lateral fast muscle fibre population in zebrafish. *Development.* 2005; 132:4211–22. [PubMed: 16120642]
- Hammond CL, Hinits Y, Osborn DP, Minchin JE, Tettamanti G, Hughes SM. Signals and myogenic regulatory factors restrict pax3 and pax7 expression to dermomyotome-like tissue in zebrafish. *Dev Biol.* 2007; 302:504–521. [PubMed: 17094960]
- Hinits Y, Hughes SM. Mef2s are required for thick filament formation in nascent muscle fibres. *Development.* 2007 In Eprint doi:10.1242/dev.007088.
- Hinterberger TJ, Sassoon DA, Rhodes SJ, Konieczny SF. Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Dev Biol.* 1991; 147:144–156. [PubMed: 1715299]

- Izsvak Z, Ivics Z, Garcia-Estefania D, Fahrenkrug SC, Hackett PB. DANA elements: a family of composite, tRNA-derived short interspersed DNA elements associated with mutational activities in zebrafish (*Danio rerio*). *Proc Natl Acad Sci U S A*. 1996; 93:1077–81. [PubMed: 8577717]
- Karlstrom RO, Talbot WS, Schier AF. Comparative synteny cloning of zebrafish you-too: mutations in the Hedgehog target *gli2* affect ventral forebrain patterning. *Genes Dev*. 1999; 13:388–93. [PubMed: 10049354]
- Kassar-Duchossoy L, Gayraud-Morel B, Gomes D, Rocancourt D, Buckingham M, Shinin V, Tajbakhsh S. *Mrf4* determines skeletal muscle identity in *Myf5:Myod* double-mutant mice. *Nature*. 2004; 431:466–71. [PubMed: 15386014]
- Kimmel CB, Kane DA, Walker C, Warga RM, Rothman MB. A mutation that changes cell movement and cell fate in the zebrafish embryo. *Nature*. 1989; 337:358–362. [PubMed: 2911386]
- Lin CY, Yung RF, Lee HC, Chen WT, Chen YH, Tsai HJ. Myogenic regulatory factors *Myf5* and *Myod* function distinctly during craniofacial myogenesis of zebrafish. *Dev Biol*. 2006; 299:594–608. [PubMed: 17007832]
- Miner JH, Wold B. Herculin, a fourth member of the MyoD family of myogenic regulatory genes. *Proc Natl Acad Sci USA*. 1990; 87:1089–1093. [PubMed: 2300571]
- Neyt C, Jagla K, Thisse C, Thisse B, Haines L, Currie PD. Evolutionary origins of vertebrate appendicular muscle. *Nature*. 2000; 408:82–6. [PubMed: 11081511]
- Olson EN, Arnold H-H, Rigby PWJ, Wold BJ. Know your neighbours: three phenotypes in null mutants of the myogenic bHLH gene *MRF4*. *Cell*. 1996; 85:1–4. [PubMed: 8620528]
- Patapoutian A, Yoon JK, Miner JH, Wang S, Stark K, Wold B. Disruption of the mouse *MRF4* gene identifies multiple waves of myogenesis in the myotome. *Development*. 1995; 121:3347–3358. [PubMed: 7588068]
- Pin CL, Konieczny SF. A fast fiber enhancer exists in the muscle regulatory factor 4 gene promoter. *Biochem Biophys Res Commun*. 2002; 299:7–13. [PubMed: 12435381]
- Pin CL, Ludolph DC, Cooper ST, Klocke BJ, Merlie JP, Konieczny SF. Distal regulatory elements control *MRF4* gene expression in early and late myogenic cell populations. *Dev Dynamics*. 1997; 208:299–312.
- Roy S, Wolff C, Ingham PW. The *u-boot* mutation identifies a Hedgehog-regulated myogenic switch for fiber-type diversification in the zebrafish embryo. *Genes Dev*. 2001; 15:1563–76. [PubMed: 11410536]
- Schauerte HE, van Eeden FJ, Fricke C, Odenthal J, Strahle U, Haffter P. Sonic hedgehog is not required for the induction of medial floor plate cells in the zebrafish. *Development*. 1998; 125:2983–2993. [PubMed: 9655820]
- Schilling TF, Kimmel CB. Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo. *Development*. 1997; 124:2945–60. [PubMed: 9247337]
- Summerbell D, Halai C, Rigby PW. Expression of the myogenic regulatory factor *Mrf4* precedes or is contemporaneous with that of *Myf5* in the somitic bud. *Mech Dev*. 2002; 117:331–5. [PubMed: 12204280]
- Thompson AL, Filatov G, Chen C, Porter I, Li Y, Rich MM, Kraner SD. A selective role for *MRF4* in innervated adult skeletal muscle: *Na(V) 1.4 Na⁺ channel* expression is reduced in *MRF4*-null mice. *Gene Expr*. 2005; 12:289–303. [PubMed: 16358417]
- van Eeden FJ, Granato M, Schach U, Brand M, Furutani-Seiki M, Haffter P, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Warga RM, Allende ML, Weinberg ES, Nüsslein-Volhard C. Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*. *Development*. 1996; 123:153–64. [PubMed: 9007237]
- Walters EH, Stickland NC, Loughna PT. *MRF-4* exhibits fiber type- and muscle-specific pattern of expression in postnatal rat muscle. *Am J Physiol Regul Integr Comp Physiol*. 2000; 278:R1381–4. [PubMed: 10801310]
- Weinberg ES, Allende ML, Kelly CS, Abdelhamid A, Murakami T, Andermann P, Doerre OG, Grunwald DJ, Riggleman B. Developmental regulation of zebrafish *MyoD* in wild-type, no tail and spadetail embryos. *Development*. 1996; 122:271–80. [PubMed: 8565839]
- Westerfield, M. *The Zebrafish Book - a guide for the laboratory use of zebrafish (Danio rerio)*. University of Oregon Press; 1995.

- Whitfield TT, Granato M, Van Eeden FJM, Schach U, Brand R, Furutani-Seiki M, Haffter P, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Nüsslein-Volhard C. Mutations affecting development of the zebrafish inner ear and lateral line. *Development*. 1996; 123:241–254. [PubMed: 9007244]
- Xu Y, He J, Wang X, Lim TM, Gong Z. Asynchronous activation of 10 muscle-specific protein (MSP) genes during zebrafish somitogenesis. *Dev Dyn*. 2000; 219:201–15. [PubMed: 11002340]
- Zhang W, Behringer RR, Olson EN. Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. *Genes Dev*. 1995; 9:1388–1399. [PubMed: 7797078]

Figure 1. Family relationship, sequence and structural comparison of Mrf4

A. Clustal alignment of the conceptual translation product of the zebrafish *mrf4* gene (Genebank accession No. AY335193) with representatives of other major vertebrate groups (human, mouse, chicken, *Xenopus laevis* and *Fugu rubripes*). The blue line above the sequence encoded by the third exon indicates the region missing in the alternatively spliced mRNA, which encodes the residues indicated.

B. Sequence pair distances of representative Mrf4 proteins (top) and bHLH domains (bottom) using the Clustal method with PAM 250 residue weight table.

C. An evolutionary tree showing the phylogenetic distance among predicted *mrf4* proteins.

D. Exon structure of *mrf4*, showing the DANA retrotransposon insertion (blue) and accompanying alternative splice. Canonical exons are shaded. The termination of the 3' untranslated region in exons 3 and alt. 3 is unknown. Distances are approximately to scale.

Figure 2. *Mrf4* mRNA is expressed in terminally differentiated skeletal muscle

In situ mRNA hybridisation for *mrf4* (purple, A-N,P,Q,R,S), *myoD* (red, B,H), *myogenin* (*myog* red, D; purple, O), *actin* (*actc* red, A,C), *prdm1* (red, F) and wholemount immunohistochemistry for MyHC (brown, G,J,S) in zebrafish embryos, viewed in dorsal flatmount (A-H), wholemount (K,L,N centre,O,Q,S lateral view; I,N right,P left dorsal view; N left ventral view) or transverse cryosection (J,M,R). Anterior is to the top (A-I,K,S), or left (L, N-Q) and dorsal to top (M,R). **A.** *Mrf4* is first detected adaxially in each somite at 5-6s, after *actin* (right panel). The horizontal line indicates the newest somite border (A-H). **B-D.** At 10-12s, *mrf4* mRNA is restricted to somitic adaxial cells, whereas *myoD* (B) and *actin* (C) expression are strong in adaxial cells of presomitic mesoderm (arrows) and *myod* (B) and *myog* (D) in the lateral fast muscle precursors (arrowheads). *Myogenin* mRNA (D) shows that *mrf4* is not expressed in fast precursors at this stage. Dual immunohistochemical detection of two mRNAs in a single embryo reveals expression of *mrf4* exclusively in the adaxial location of slow muscle obscuring the red *myod* or *actin* label. Note that *myogenin* expression is not detected in presomitic adaxial cells (Weinberg et al., 1996) due to short incubation with substrate. **E-H.** *Mrf4* is down regulated in rostral somites before 14s (E). *Mrf4* and *prdm1* mRNAs are co-expressed in differentiated adaxial cells whereas *prdm1* alone is expressed in presomitic adaxial cells (F). *Mrf4* transcripts co-localise with MyHC protein in adaxial cells of the newly-forming somite (G, asterisks). *Mrf4* expression is strongest in the six most recently formed somites, obscuring the MyHC signal (G, arrowheads; box shown magnified at right). In more mature somites, declining *mrf4* is detected in a similar location (G, bracketed region shown in inset, green arrowheads), whereas MyHC-expressing slow fibres are increasing (G inset, arrows). By 18s, *mrf4* is expressed most strongly in six nascent somites (H). **I.** At 22s, *mrf4* is up-regulated once

more in rostral somites. **J.** By 25s, *mrf4* mRNA is present throughout the somite and at high level in slow fibres after their migration (arrowheads). Re-staining of the same section shows that slow MyHC (F59) overlaps the high *mrf4* expressing cells (arrowheads, right panel). **K.** At 26hpf, *mrf4* is broadly expressed in muscle. **L,M.** *Mrf4* expression at 48hpf appears stronger in the superficial slow muscle cells. **N,O.** *Mrf4* and *myogenin* are expressed in dorsal and ventral muscle masses of pectoral fin at 48hpf (arrows). High magnifications of the fin show that *mrf4* and *myogenin* expression in dorsal and ventral muscle masses. *Myogenin* is detected in numerous ventral head muscle anlage (O left), but *mrf4* mRNA shows no such pattern (N left). **P.** *Mrf4* mRNA persists in pectoral fin at 72hpf. **Q,R.** *Mrf4* is weakly expressed at 5d and is more readily detected superficial slow cells. **S.** Embryos injected with *myf5* and *myoD* antisense morpholinos have no MyHC and lack *mrf4* expression at 12s.

Figure 3. *Mrf4* mRNA is expressed late in muscle terminal differentiation

In situ mRNA hybridisation for *mrf4* (blue, A,C-K), *eng2a* (B) and *myl2* (K) and wholemount immunohistochemistry for MyHC (brown, B,F) in zebrafish embryos viewed in wholemount (E,G,H,K lateral view, dorsal to right) or flatmount (A-D,J, dorsal view), anterior to top, or transverse section (F,I; dorsal to top). **A.** *Mrf4* expression is missing in *gli2a^{ty119/ty119}* embryos and reduced in heterozygotes *gli2a^{ty119/+}* compared to wild type siblings. **B.** At 15s, *shha* mutant embryos lack *eng2a*-expressing MPs, but have residual MyHC-expressing SSF precursors (black arrows), whereas sibling embryos show *eng2a* expression in MPs anterior to the three youngest somites (between red arrows) and MyHC in SSF precursors (black arrows). **C.** *shha* mutants show no *mrf4* MP expression prior to 16s, in contrast to their siblings. **D.** Similarly, *prdm1* mutants show faint *mrf4* expression in adaxial cells but lack transient strong expression. **E.** *Mrf4* is widely expressed at 24 hpf in wild type. Anteriorly, *mrf4* expression fills the bulk of the somite chevron. Note transient dorsoventral midline expression in the most caudal newly-formed somites (arrowhead). *shha* mutant lacks MP *mrf4* staining in caudal somites (arrowhead), but retains strong expression in more anterior U-shaped somites (arrow). **F.** Rostral section of a 21s *shha* embryo, identified by U-shape somites and lack of caudal MP *mrf4* expression, showing that MyHC closely co-localises with *mrf4* mRNA in differentiated SSF precursor fibres migrating towards the lateral surface of the somite. Eosin counterstain. **G,H.** At 24-25s, both *prdm1* (G) and *gli2a* (H) mutants and sibling show *mrf4* expression in fast muscle cells in more rostral somites, whereas only the sibling shows caudal slow expression (arrowheads). **I.** *Gli2a* mutant with no slow muscle (MPs or SSFs) expresses *mrf4* in fast muscle cells. **J.** *Fgf8* morphants show no change in *mrf4* expression at 15s. **K.** *Myl2* (*fast myosin light chain 2*) co-localizes with *mrf4* in fast fibres but not in slow fibres (arrowhead) in *fgf8*

mutants and siblings. In *fgf8* mutants, *mrf4* mRNA is reduced in parallel with differentiated fast muscle (insets), but is unchanged in slow.