

Mef2s are required for thick filament formation in nascent muscle fibres

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During skeletal muscle differentiation, the actomyosin motor is assembled into myofibrils, multiprotein machines that generate and transmit force to cell ends. How expression of muscle proteins is coordinated to build the myofibril is unknown. Here we show that zebrafish Mef2d and Mef2c proteins are required redundantly for assembly of myosin-containing thick filaments in nascent muscle fibres, but not for the earlier steps of skeletal muscle fibre differentiation, elongation, fusion or thin filament gene expression. *mef2d* mRNA and protein is present in myoblasts, whereas *mef2c* expression commences in muscle fibres. Knockdown of both Mef2s with antisense morpholino oligonucleotides or in mutant fish blocks muscle function and prevents sarcomere assembly. Cell transplantation and heat-shock-driven rescue reveal a cell-autonomous requirement for Mef2 within fibres. In nascent fibres, Mef2 drives expression of genes encoding thick, but not thin, filament proteins. Among genes analysed, myosin heavy and light chains and myosin-binding protein C require Mef2 for normal expression, whereas actin, tropomyosin and troponin do not. Our findings show that Mef2 controls skeletal muscle formation after terminal differentiation and define a new maturation step in vertebrate skeletal muscle development at which thick filament gene expression is controlled.

KEY WORDS: Mef2c, Mef2d, Myosin, Muscle, Zebrafish, Myofibril, Somite, *tnnc*, Myogenin, Hoover, *prdm1*, *eng2a*, *acta1*, *actc1*, *smyhc1*, *myhz1*, *tpma*, *mybpc1*, *hsp90a*

INTRODUCTION

Skeletal myogenesis involves three steps: (1) commitment of proliferative mesodermal cells as myoblasts, (2) terminal differentiation, often accompanied by cell fusion, and (3) assembly of the contractile myofibril, which is composed of serial sarcomeres of thick myosin filaments alternating with thin actin filaments. This third step is poorly understood but clearly requires ordered synthesis and assembly of specific proteins. In mouse and zebrafish somites, transcripts encoding thin filament proteins are expressed before those for thick filament proteins (Lyons et al., 1990; Xu et al., 2000). Consistent with this, one model of myofibril assembly suggests that myofibrils are initiated on actin stress fibres by formation of z bodies, aggregates containing thin filament proteins found later in mature z lines, which align and anchor the thin filaments (van der Ven et al., 1999; Wang et al., 2005). Myosin and other thick filament proteins would then integrate into the thin filament structure, perhaps mediated by the giant molecular ruler titin (Lange et al., 2006). What triggers expression of myosin and other thick filament proteins is unknown.

High throughput studies are revealing the complex temporal succession of gene expression during and following myoblast terminal differentiation in culture (Penn et al., 2004; Tapscott, 2005). As terminal differentiation leads on to myofibrillogenesis, distinct combinations of transcription factors are expressed (Tapscott, 2005). Among such transcription factors, members of the myocyte enhancer factor 2 (Mef2) and serum response factor (SRF) families of MADS domain-containing proteins are expressed in muscle from jellyfish to humans and upregulated during muscle terminal

differentiation (Black and Olson, 1998; Spring et al., 2002). In culture, Mef2 can collaborate with MyoD-family proteins to enhance myogenic conversion of non-muscle cells (Molkentin et al., 1995). In vivo, both Mef2 and SRF proteins can regulate many heart and skeletal muscle genes (Balza and Misra, 2006; Black and Olson, 1998; Niu et al., 2005). SRF proteins can drive *C. elegans* myogenesis, are required for murine myocardiogenesis and also regulate cytoskeletal components in non-muscle cells (Fukushige et al., 2006; Niu et al., 2005; Posern and Treisman, 2006). Thus, these MADS proteins appear to regulate the specialised muscle cytoskeleton. Yet the precise functions of Mef2 proteins during myoblast differentiation in vivo remain unclear.

In invertebrates, the requirement for Mef2 genes is highly variable, suggesting evolutionary flexibility as animal phyla diverged (Dichoso et al., 2000; Lilly et al., 1995). In vertebrates, *Mef2c* is required for cardiac morphogenesis and right ventricle formation and *Mef2a* mutants suffer from structural defects in cardiac muscle (Lin et al., 1997; Naya et al., 2002). Mutations in *MEF2A* in humans are also associated with cardiovascular disease (Bhagavatula et al., 2004; Gonzalez et al., 2006). However, understanding of how vertebrate Mef2 proteins function to regulate skeletal muscle development in vivo is lacking. Mef2 function in vertebrate skeletal myogenesis is unclear because several Mef2 genes are expressed in the early myotome, and because mice lacking *Mef2c* die early in development from cardiovascular defects (Lin et al., 1997). As fish embryos can develop for several days without a functioning heart because oxygen is delivered by diffusion, we set out to study the in vivo function of the Mef2 gene family in zebrafish muscle.

In zebrafish, several populations of skeletal muscle precursors arise and undergo terminal differentiation to make contractile muscle within hours of formation of the mesoderm (Stickney et al., 2000). As in mice, Mef2 genes in zebrafish are expressed in both cardiac and skeletal muscle precursors and in neural tissue (Ticho et al., 1996) (www.zfin.org). Here we show that *mef2d* mRNA and protein are expressed in muscle precursors, whereas *mef2c* appears after muscle terminal differentiation. Mutant or antisense-mediated

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knockdown reveals that Mef2s are required to upregulate several genes encoding the major components of the thick filament, but not those of thin filaments. These findings reveal a molecular mechanism controlling myofibril assembly.

MATERIALS AND METHODS

Zebrafish lines and maintenance

Mutant *hoo*^{tn213} (Piotrowski et al., 1996), *smo*^{b641} (Barresi et al., 2000) and transgenic *Tg(acta1:GFP)* (Higashijima et al., 1997) *D. rerio* lines were maintained on King's wild-type background, and staging and husbandry were as described (Westerfield, 1995).

In situ mRNA hybridisation and immunohistochemistry

In situ mRNA hybridisation and immunohistochemistry were performed as described (Hammond et al., 2007). Fluorescein- or digoxigenin-tagged probes used were *mef2c* and *mef2d* (Ticho et al., 1996), *myod* and *myogenin* (Weinberg et al., 1996), *smyhcl* (Bryson-Richardson et al., 2005), *myhz1*, *mylz2*, *mybpc1*, *tnnc*, *tpma* (Xu et al., 2000), *acta1*, *actc1*, *hsp90a* (I.M.A.G.E. clones 6997034, 7284336 and 7259827, respectively). Anti-Mef2 was raised in rabbit against a C-terminal peptide of human MEF2 (c-21, Santa Cruz; used at 1:200). Anti-Mef2c is a rabbit polyclonal made against aa 140-238 of human MEF2C (McDermott et al., 1993) (81.8% identity to aa 139-237 of zebrafish Mef2c; 1:1000), A4.1025 [recognises all myosin heavy chain (MyHC) proteins (Dan-Goor et al., 1990); 1:5], F59 and S58 [anti-slow MyHC, DSHB (Devoto et al., 1996); 1:5], EB165 [anti-fast MyHC, DSHB; 1:1], anti- α -actinin (Sigma; 1:500), anti-tropomyosin (CH1, Sigma; 1:1000), anti-titin T12 (a gift from D. Furst, University of Bonn, Germany; 1:1), anti-cardiac actin (Ac1-20.4.2, Progen), anti-MyBP-C (rabbit polyclonal, gift from M. Gautel, King's College London, UK), anti-Pax3/7 (DP312) (Hammond et al., 2007). Slow MyHC was detected with S58 in dual staining with EB165 and with F59 elsewhere. Secondary reagents were Alexa (Invitrogen) or peroxidase (Vector) conjugates. Embryos were dissected, flatmounted in glycerol or Citifluor (Agar) and images recorded on a Zeiss Axiophot with Axiocam using Openlab software, or on a Zeiss LSM510. Except where stated otherwise, confocal images are short stacks of one side of the embryo at around somites 10-13.

Embryo manipulation

Antisense morpholino oligonucleotides (MOs) (Gene-Tools, 0.5-8 ng per embryo, see Fig. 1D for sequences) and plasmid DNA were injected into 1- to 2-cell stage embryos. *myogenin:GFP* plasmid DNA (Du et al., 2003) was kindly provided by S. J. Du (University of Maryland Biotechnology Center, Baltimore, MD). Cell transplantations from donor embryos injected at the 1- to 2-cell stage with ~1% FITC-dextran (Invitrogen) were made at sphere stage into age-matched hosts. Rescue experiments were conducted by co-injecting MOs with *hs-mef2d-IRES-GFP* into 1- to 2-cell stage embryos. *hs-mef2d-IRES-GFP* was made by cloning the full-length coding sequence of *mef2d*, generated by PCR using the 5' primer 5'-TCTAGATCTA-GAATGGGACGAAAGAAAATTCAGATTCAGC-3' with a 5 nt mismatch to the *mef2d/c* and *mef2c/d* MOs and the 3' primer 5'-GTCGACTTAT-GTGACCCAGGTGTCCA-3', shuttling through pGEM-Teasy (Promega) into the *XbaI* and *Sall* sites of *hsp70-4-MCS-IRES-mGFP6* plasmid (gift of S. Gerety and D. Wilkinson, NIMR, London, UK). DNA sequence was verified. Embryos were heat shocked at 39°C for 1 hour, recovered for 1 hour at 28.5°C and then fixed for immunofluorescence. For quantitative data supporting all experiments, see Table S1 in the supplementary material.

RESULTS

Mef2d and Mef2c are sequentially expressed in developing muscle

To understand the role of Mef2s, we first analysed when and where Mef2 mRNA and protein accumulates. In agreement with a previous study (Ticho et al., 1996), *mef2d* expression was seen to follow that of *myod* in skeletal muscle precursors and was maintained in differentiated fibres (Fig. 1A,D), but was undetectable in the developing heart (data not shown). In skeletal muscle, *mef2d* mRNA accumulated in parallel with *actin* mRNA and before expression of

genes encoding myosin heavy chain (MyHC) proteins (Fig. 1A). By contrast, *mef2c* mRNA is first detected in skeletal muscle fibres just as they undergo differentiation and appears early in heart primordial cells (Fig. 1A,D) (Ticho et al., 1996). Both *mef2d* and *mef2c* mRNAs persist until after 24 hpf (Fig. 1A). Using a general anti-Mef2 antibody, Mef2 proteins were detected in differentiated cardiomyocytes and their precursor cell nuclei in heart primordium and in skeletal myoblasts and differentiated fibres in the somites (Fig. 1B,D and see Fig. S1 in the supplementary material). A specific anti-Mef2c antibody detected nuclear Mef2c protein in heart and differentiated skeletal muscle, but not in skeletal myoblasts (Fig. 1B,D and see Fig. S2 in the supplementary material). Anti-Mef2c does not react with tissues expressing *mef2d* in the absence of *mef2c* mRNA, and is therefore Mef2c-specific (Fig. 1D). Thus, accumulation of Mef2 proteins matches their mRNAs.

Several morpholino/mutant combinations deplete Mef2 proteins

We designed four antisense morpholino oligonucleotides (MOs) to block translation of the mRNAs encoding Mef2d and Mef2c (Fig. 1C). We also searched for mutants affecting Mef2 and noticed a loss of Mef2c-specific immunoreactivity and mRNA in skeletal muscle of fish carrying the *hoover* (*hoo*) mutation (see Fig. S2C,D in the supplementary material). Like *hoo* mutants, mice lacking Mef2c in neural crest lineages show jaw defects (Verzi et al., 2007). Using the general anti-Mef2 and specific anti-Mef2c antibodies, we showed that each MO knocks down the predicted target(s) (summarised in Fig. 1D, for data see Figs S1, S2 in the supplementary material). Importantly: (1) the *mef2d* MO abolishes Mef2 immunoreactivity from regions where *mef2d* but not *mef2c* is expressed; (2) the *mef2c* MO ablates Mef2c protein from muscle; (3) injection of the *mef2d/c* MO or *mef2c/d* MO alone, which are each predicted to knockdown both Mef2c and Mef2d, or of *mef2c+mef2d* MOs, eliminates all Mef2 immunoreactivity from muscle, as does injection of the *mef2d* MO into the *hoo* mutant (see Fig. S1F in the supplementary material). Thus, we have three independent ways to eliminate Mef2 proteins from skeletal muscle that all gave similar results; we hereafter refer to these treated fish as 'mef2 morphants'.

Mef2 is required for myofibrillogenesis and muscle function

What is the effect of Mef2 loss? *mef2* morphants lacked somitic Mef2 protein and had a severe skeletal muscle defect (Fig. 2 and see Figs S1-S3 in the supplementary material). Embryos were ventrally curved with little or no motility at any stage (Fig. 2A) and a dramatic loss of MyHC accumulation (Fig. 2B-D). These manipulations did not have common effects on the heart, which in most cases appeared wild-type at 24 hpf (data not shown). Neither knockdown of Mef2c or Mef2d alone, nor *hoo* mutation, gave any obvious phenotype during the segmentation period (see Fig. S2C and Fig. S3 in the supplementary material). Thus, loss of Mef2c and Mef2d proteins in skeletal muscle causes a severe defect in muscle structure and function.

mef2 morphant muscle failed to mature after terminal differentiation. In fast muscle cells of *mef2* morphants, almost no MyHC was detected with anti-MyHC antibodies (Fig. 2B,C arrowheads). Nevertheless, injecting *mef2d/c* MO into embryos of the muscle actin reporter line *Tg(acta1:GFP)*, which marks all terminally differentiated muscle, confirmed that both slow and fast fibres differentiated, elongated and migrated normally in *mef2* morphants (Fig. 2D). Co-injecting *mef2d/c* MO and *myogenin:GFP* plasmid DNA, which expresses chimaerically but

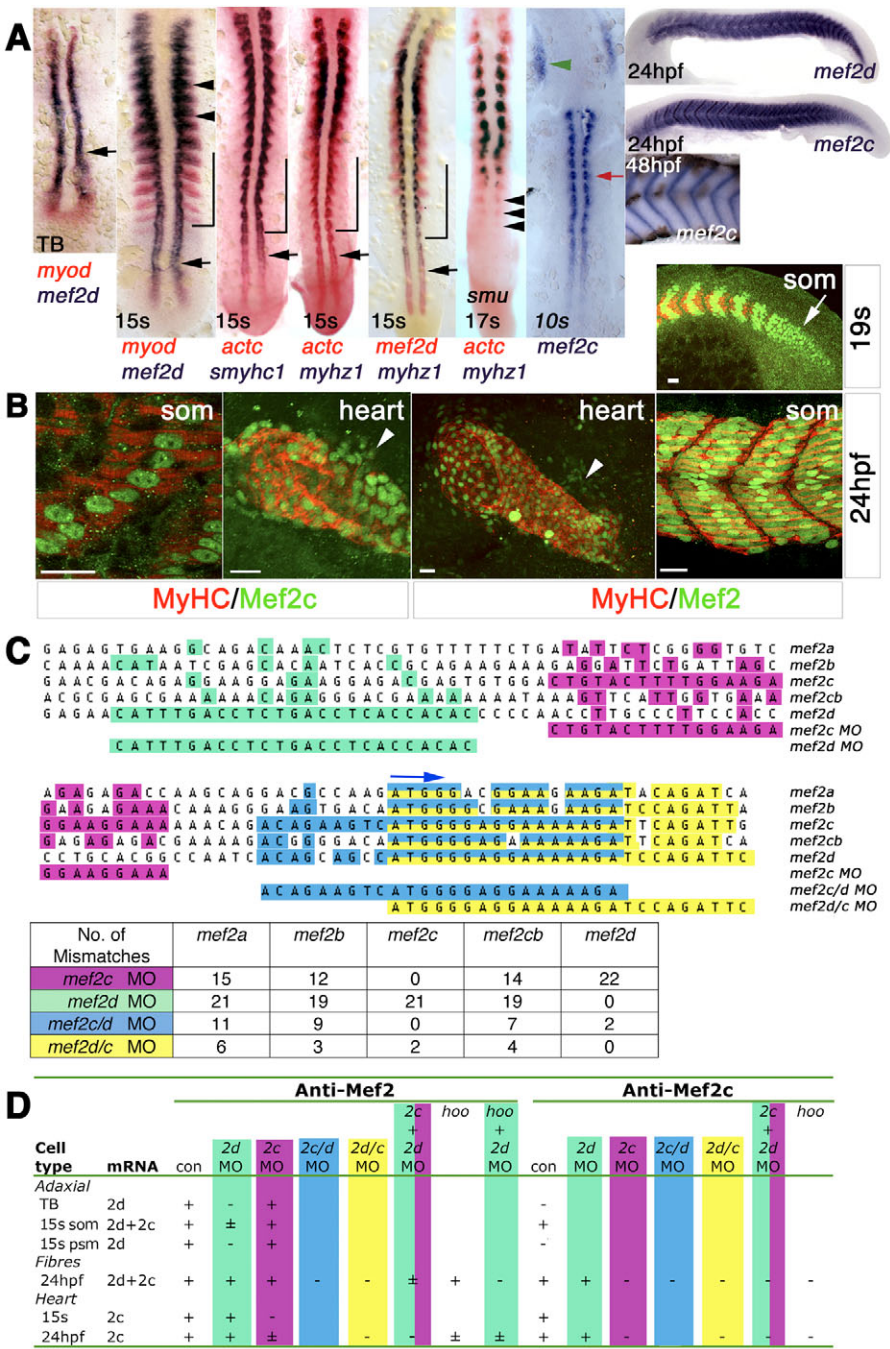


Fig. 1. Mef2d and Mef2c expression during zebrafish skeletal myogenesis and cardiogenesis. (A) In situ mRNA hybridisation of *mef2d*, *mef2c*, *myod*, *actc1* (*actc*), *smyhc1* and *myhz1* viewed in dorsal (anterior to top) or lateral (right-most panels; anterior to left, dorsal up) flatmount. *Mef2d*, *myod* and *actc1* mRNAs coincide in early adaxial slow (black arrow) and lateral fast (black arrowheads) myoblasts and precede *smyhc1* and *myhz1* in differentiated slow (bracket) and fast muscle fibres. Sequential expression of *actc1* and *myhz1* during fast muscle differentiation is confirmed in *smu* (*smo*) mutant, which lacks slow muscle. *Mef2c* expression commences as adaxial cells form slow fibres (red arrow) and in bilateral heart fields (green arrowhead). *Mef2c* expression in fast muscle parallels terminal differentiation. TB, tailbud; s, somite. (B) Immunodetection of Mef2 and MyHC in wholemount embryos viewed in dorsal (heart; anterior to top) or lateral [somite (som); anterior to left, dorsal up] flatmount. Confocal single scans of anti-Mef2c and z-stacks of anti-Mef2 antibodies detect muscle nuclei in skeletal myoblasts in the presomitic mesoderm (arrow), muscle fibres, heart tube and undifferentiated cardiomyoblasts (arrowheads). Scale bars: 20 μ m. (C) Sequence alignment of start codon region (blue arrow) of five zebrafish Mef2 genes and comparison with MOs employed. Coloured squares highlight identity with the MO. The table shows the number of mismatches. (D) Location of anti-Mef2 and anti-Mef2c immunoreactivity in relation to *mef2d* and *mef2c* mRNAs and the effect of the indicated MO combinations (see Figs S1-S3 in the supplementary material). +, \pm and - indicate normal, low and no nuclear reactivity, respectively; where there is no entry, nuclear reactivity was not determined.

specifically in fast muscle precursors, showed that these cells undergo fusion into multinucleate fibres with three to four nuclei (Fig. 2E). Despite the presence of terminally differentiated and fused fast fibres, fast MyHC was absent from fast fibres at both protein and mRNA levels (Fig. 2C,F, arrowheads). Thus, in the absence of Mef2 proteins, fast fibre development is halted after terminal differentiation but prior to myosin expression and myofibrillogenesis.

A defect in slow fibre development appeared in *mef2* morphant embryos after the terminal differentiation step. In wild-type fish, slow fibres initially expressed low levels of fast MyHC, encoded by the *myhz1* gene (Fig. 2F,G), and high levels of slow MyHC, encoded by *smyhc1* (Fig. 3A,B). Fast MyHC is lost from slow cells by 24 hpf (Fig. 2C) (Bryson-Richardson et al., 2005). *mef2* morphants initiated

normal terminal differentiation of slow muscle and expressed *smyhc1* and *myhz1* at the 15-somite stage (Fig. 2F and Fig. 3A,B). Moreover, *mef2* morphant slow fibres migrated to the lateral myotome surface as normal (Fig. 2B-D). Thereafter, however, fibre maturation and myofibrillogenesis failed. Fast MyHC persisted with slow MyHC in slow fibres (Fig. 2C,F) and the normal downregulation of *myogenin* mRNA failed to occur in *mef2* morphants (Fig. 2H). At 24 hpf, the mononucleate slow fibres in control embryos had thick, regularly striated myofibril bundles (Fig. 2D). By contrast, *mef2* morphant slow fibres had aggregates of MyHC at their ends, connected by a thin myofibril with striped MyHC bands, similar to younger fibres in unmanipulated embryos (compare 24 hpf morphants in Fig. 2C,D and Fig. 3D with control at 22 somites in Fig. 3C). Normal maturation of slow fibres involves downregulation of *prdm1*

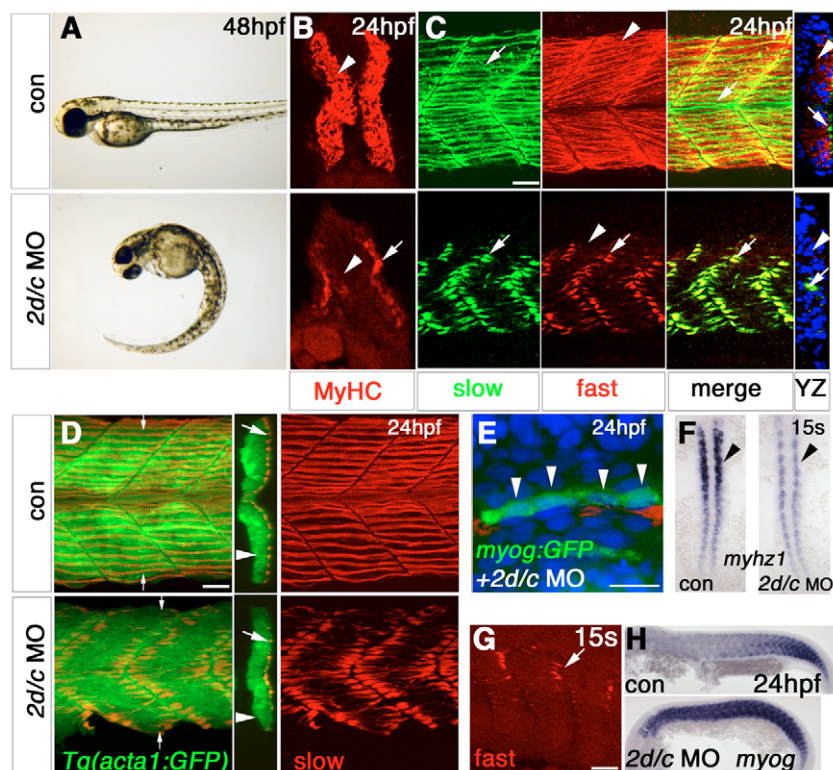


Fig. 2. Mef2 knockdown blocks fast muscle myofibril assembly. Zebrafish embryos in bright field (A,F,H), after immunodetection of all MyHC (B), slow MyHC (C-E) or fast MyHC (C,G) or in situ mRNA hybridisation (F, dorsal flatmount; H, lateral flatmount). (A,B) *mef2d/c* MO causes tail curvature (A) and ablates MyHC (B) in fast (arrowhead), but not slow (arrow) fibres. (C) *mef2d/c* morphants retain fast MyHC in slow fibres (arrows), but have essentially no fast MyHC in fast fibres (arrowheads), as shown in the yz optical transverse reconstruction. (D) Injection of *mef2d/c* MO into *Tg(acta1:GFP)* fish disrupts slow myofibrillogenesis (red) but not actin reporter expression (green). yz reconstruction at small arrows. Large arrows, slow fibres; arrowheads, fast fibres. (E) *myogenin:GFP* DNA co-injected with *mef2d/c* MO into wild-type embryos yields elongated GFP-filled fibres (green) with three to four nuclei (DAPI-stained, blue, arrowheads) at 24 hpf, despite disorganised slow MyHC (red). (F) Dorsal flatmounts showing decrease in *myh21* in fast precursors (arrowhead) but no change in adaxial slow precursors. (G) In wild-type embryos, fast MyHC is present in slow fibres at 15 somites (arrow) but is lost by 24 hpf (arrows, upper panels in C). (H) Persistence of *myogenin* mRNA in *mef2d/c* morphants. Scale bars: 20 μ m.

(Baxendale et al., 2004) and expression of *eng2a*; both events failed in *mef2* morphants (Fig. 3F,G). In summary, slow fibres in *mef2* morphants fail to mature beyond the initial terminal differentiation step and have a severe defect in myofibrillogenesis.

To examine the effect of differing amounts of Mef2 protein, we injected *mef2d* MO into embryos from a cross between two *hoo* heterozygotes. This treatment yielded embryos with strong, weak and no Mef2 immunoreactivity in the predicted 1:2:1 ratio (Fig. 3E; no

Mef2 10/37, weak Mef2 21/37). Embryos had wild-type, mild and severe muscle phenotypes correlating with the loss of Mef2 (Fig. 3E). By contrast, all uninjected embryos from such a cross appeared wild type and had Mef2-immunoreactive nuclei in muscle, as did wild-type embryos injected with *mef2d* MO (data not shown). Thus, loss of Mef2c and Mef2d function arrests slow muscle development at a nascent fibre stage, and the extent of myofibril assembly parallels the level of residual Mef2 protein, at least at low Mef2 levels.

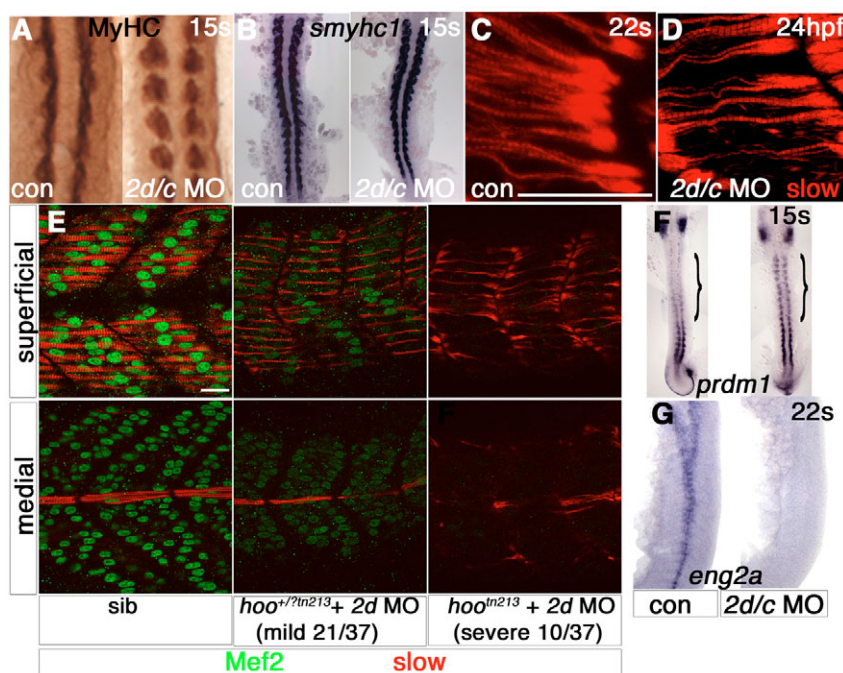


Fig. 3. Slow muscle myofibril defects correlate with the extent of Mef2 depletion. Zebrafish embryos in bright field (A,B,F,G), after immunodetection of all MyHC (A), slow MyHC (C-E) or Mef2 (E) or in situ mRNA hybridisation (B,F, dorsal flatmounts; G, lateral flatmount). (A,B) Slow muscle differentiation at 15 somites is unaffected by *mef2d/c* MO (A, somites 6-10). (C,D) Immature slow fibres in *mef2d/c* morphant at 24 hpf (D) are of comparable maturity to nascent fibres in a 20-hpf control embryo at the same rostrocaudal position (C). (E) Injection of *mef2d* MO into a *hoo*^{tn213} heterozygote cross yielded three phenotypes at the frequencies shown. Putative heterozygotes have diminished Mef2 in both slow (superficial slice) and fast (medial slice) fibres and thinner slow myofibril bundles. Putative mutants contained no Mef2 and had immature slow fibres. (F,G) *mef2d/c* morphants show persistence of *prdm1* in adaxial cells (F, bracket) and loss of *eng2a* expression (G). Scale bars: 20 μ m.

Mef2 acts cell-autonomously to rescue myofibrillogenesis

To prove that Mef2 acts cell-autonomously within muscle fibres to control myofibrillogenesis, we transplanted fluorescein-labelled cells from *mef2d/c* MO donor embryos into a wild-type host and found that the donor-derived slow cells did not contain Mef2 and failed to assemble mature myofibrils (Fig. 4A). Conversely, when wild-type donor cells were implanted into a *mef2d/c* morphant host, single donor slow fibres contained nuclear Mef2 and had a significantly more mature structure than the surrounding host fibres (Fig. 4B). We conclude that Mef2 proteins act cell-autonomously within the mononucleate slow fibres to promote myofibril assembly.

Normal Mef2 levels are not required for efficient terminal differentiation. Generation of both slow and fast donor-derived muscle fibres occurred at a similar frequency independent of whether the donor was MO-injected or wild type (Fig. 4D). However, when the cell autonomy of myofibril defects was tested in multinucleate fast fibres, a consistently non-cell-autonomous result was obtained, as would be expected if the more abundant cell type dominated within a syncytial fibre (Fig. 4D). We conclude that Mef2 is not essential for terminal differentiation of either slow or fast fibres, but is required for myofibril assembly.

To prove that morphant defects are due to loss of Mef2 function and to confirm the cell-autonomous requirement for Mef2d for myofibril assembly, *mef2* morphants were rescued by overexpression of a *mef2d* cDNA engineered to lack the MO target sequence. We injected *hs-mef2d-IRES-GFP* plasmid DNA together with the *mef2d/c* MO into 1- to 2-cell stage embryos and applied heat shock at 22 hpf. Both within somites and elsewhere, GFP-labelled cells contained immunoreactive Mef2d protein, whereas surrounding cells lacking GFP did not contain Mef2 (Fig. 4C). All GFP-expressing slow fibres had significantly better-assembled myofibrils as compared with adjacent slow fibres lacking GFP (Fig. 4C). Thus, Mef2d expression at a late stage is sufficient to rescue slow fibre maturation.

Mef2 is required for expression of major components of thick filaments

We next examined expression of genes encoding major components of the sarcomere. In *mef2* morphants, mRNAs encoding certain thick filament proteins were downregulated, whereas mRNAs encoding thin filament proteins were, if anything, upregulated (Fig. 5 and see Fig. S4A,C in the supplementary material). *hoo* mutants treated with the *mef2d* MO showed similar changes (see Fig. S4B in the supplementary material). Fast fibres failed to express myosin genes *myhz1* and *mylz2* (Fig. 2B,C, Fig. 5A-C). Interestingly, the fast myosin light chain (MyLC) gene *mylz2* has several Mef2 elements in its proximal promoter that are essential for activation in vitro (Xu et al., 1999). As described above, slow fibres commence normal expression of both myosin genes *smyhcl* and *myhz1* (Bryson-Richardson et al., 2005; Xu et al., 2000), but remain immature, failing to downregulate *myhz1*. Expression of the slow fibre-specific thick filament gene, *mybpc1*, was greatly reduced (Fig. 5D). Thus, with the exception of the MyHC genes expressed in nascent slow fibres, all genes examined encoding thick filament-associated proteins were downregulated in *mef2* morphants. By contrast, probes to thin filament genes encoding skeletal and cardiac actin, *acta1* or *actc1*, and *Tg(acta1:GFP)* and *troponin C (tnnc)*, showed no change in mRNA level in *mef2* morphant somites (Fig. 2D, Fig. 5E,G and data not shown), whereas α -tropomyosin (*tpma*) and *hsp90a*, a gene implicated in myosin folding (Barral et al., 2002), were upregulated

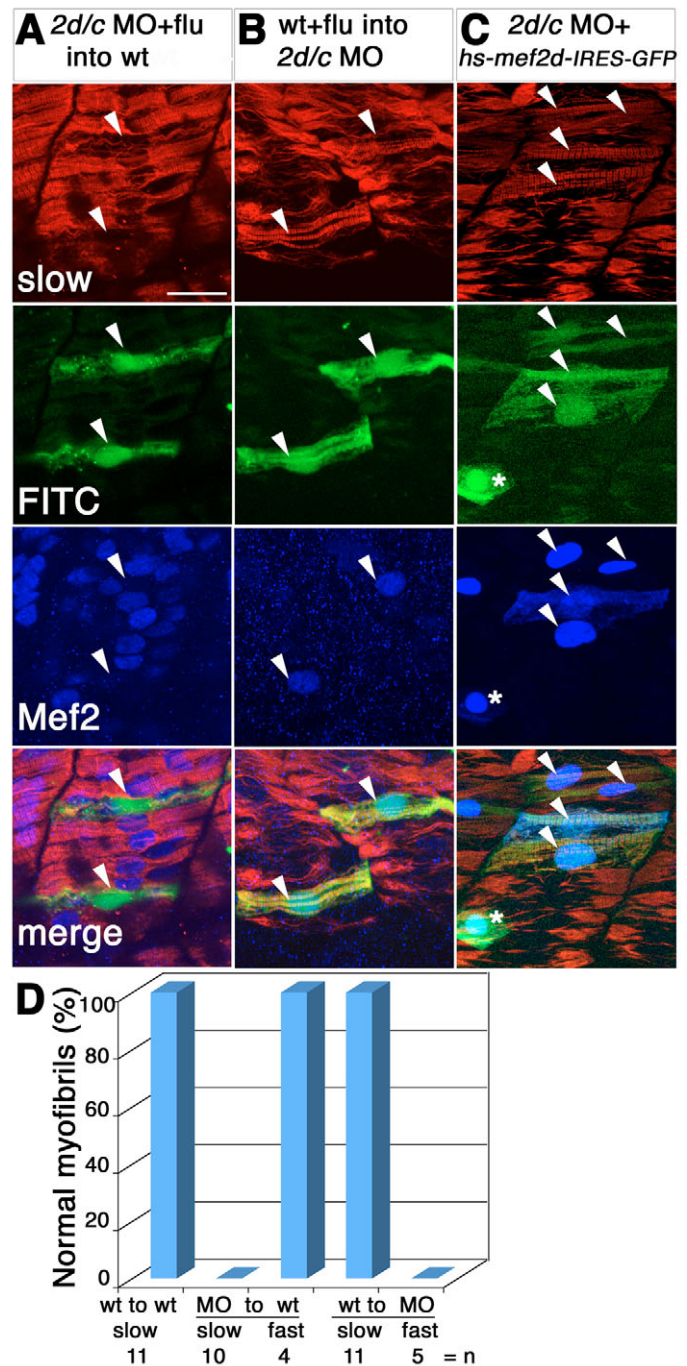


Fig. 4. Mef2 controls slow fibre myofibril assembly cell-autonomously and rescues the morphant. Detection of slow MyHC (red), Mef2 (blue) and FITC or GFP (green) in manipulated 24-hpf zebrafish embryos. (A,B) Transplantation of FITC-dextran-labelled cells (arrowheads, green) from *mef2d/c* morphants into wild-type host (A) or from wild-type donor into *mef2d/c* morphant host (B). Note the immature character of the FITC-labelled transplanted fibres in A and the greater maturity of transplanted cells in B, compared with their neighbours. (C) Co-injection of *hs-mef2d-IRES-GFP* and *mef2d/c* MO followed by heat shock at 22 hpf rescues myofibril structure in the GFP-expressing cells, which also contain nuclear Mef2 (arrowheads). Asterisk indicates a Mef2-positive skin cell. (D) Bar chart showing quantification of transplantation results.

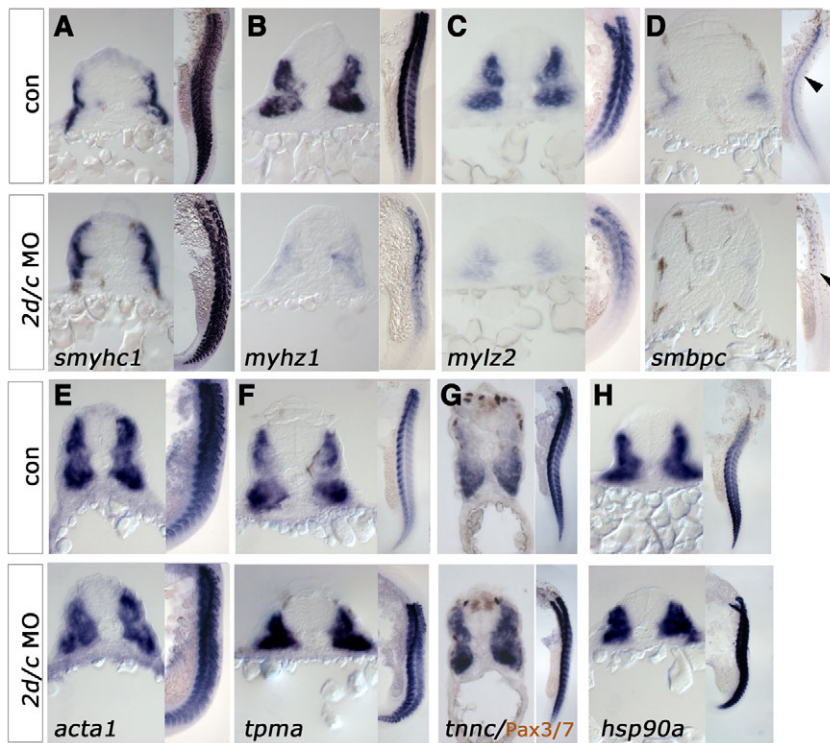


Fig. 5. Mef2 controls a transcriptional module involved in thick filament assembly. In situ hybridisation for mRNAs encoding sarcomeric components in 24-hpf control or *mef2d/c* MO-injected zebrafish embryos. In each panel, cryosections of wholemount embryos, dorsal up, are to the left and flatmounts, anterior up and dorsal to right, are to the right. (A–D) Embryonic fast *myhz1* (B), *mylz2* (C, 22s) and *smbpc* (D) mRNAs are grossly downregulated in morphants. Slow fibre-specific *smyhc1* mRNA (A) remains abundant. (E–G) mRNA for thin filament-related genes detected with probes to *acta1* (E, 21s) or *tnnc* (G) are unaltered in morphants, whereas *tpma* (F) is upregulated. Note the lack of change in nuclear Pax3/7 protein (F, brown). (H) mRNA for the myosin chaperone protein *hsp90a* is also upregulated.

(Fig. 5F,H). We conclude that Mef2c and Mef2d redundantly drive expression of genes involved in thick filament assembly in zebrafish skeletal muscle.

Thin filament assembly is halted in the absence of Mef2

The consequence of loss of Mef2 activity and thick filament proteins is disruption of sarcomere assembly, apparently at the z-body stage (Wang et al., 2005). Although MyHC accumulation in fast fibres failed completely, some MyHC clusters and single myofibrils did form in slow fibres (Fig. 6A,F). In *mef2* morphants, actin was present in the cytoplasm of both fast and slow fibres, but was only located in a regular sarcomeric array in the residual thin myofibrils of slow fibres (Fig. 6A). Actin, along with other thin filament components, α -tropomyosin and α -actinin, showed diffuse cytoplasmic staining, often in puncta, concentrated at fibre ends and near the plasma membrane. In slow fibres, actin extended well beyond regions of residual MyHC accumulation (Fig. 6A). In morphant embryos, titin arrays and ordered I-Z-I structure were only observed where MyHC-containing sarcomeres formed (Fig. 6B–D,F). Most of the rudimentary sarcomeric structure that formed in *mef2*-morphant slow fibres was concentrated near the somite border. Cytoplasmic puncta and the level of thick filament components, such as MyHC and slow myosin-binding protein C, were greatly reduced as compared with thin filament proteins (Figs 2, 3; Fig. 6A,E). Overall, there is a more severe disruption of thick filament components, and aggregates of thin filament proteins form, often at the cell periphery.

DISCUSSION

Although muscle fibres require both myosin and actin to generate force, thick and thin filament proteins are independently regulated during myogenesis. Actin and other z-line and thin filament proteins are expressed and assembled prior to myosin and thick filament proteins during myotube formation (Lyons et al., 1990; Wang et al.,

2005; Xu et al., 2000). Moreover, isoforms of thin and thick filament proteins are substituted at different times during fibre maturation (Schiaffino and Reggiani, 1996). How are the genes independently regulated? Actin expression rapidly succeeds *myod* expression in zebrafish, which we suggest constitutes a first phase of myofibrillogenesis. Our data show that Mef2s drive thick filament gene expression in nascent fast fibres, indicating that Mef2 activity directs a second phase of myofibrillogenesis. It is striking that the Mef2-related protein Srf regulates the actin cytoskeleton in both muscle and non-muscle cells (Niu et al., 2005; Posern and Treisman, 2006; Treisman, 1987). Srf is highly expressed as zebrafish muscle differentiates (Vogel and Gerster, 1999) (www.zfin.org), raising the possibility that it drives thin filament genes during the first phase. The widespread low-level expression of Srf and Mef2 proteins in non-muscle tissue at later stages of mammalian development might indicate roles for each, beyond muscle, in regulation of the actin and myosin cytoskeletons, respectively.

Our findings show that Mef2 proteins are required for the maturation and assembly of sarcomeric structure in nascent muscle fibres and provide *in vivo* evidence consistent with one suggested mechanism of myofibril assembly. Myofibrillogenesis is initiated by formation of pre-myofibrils, in which z-bodies decorate actin stress fibres near the cell periphery (Wang et al., 2005). The actin-, α -actinin-, titin z-line region- and α -tropomyosin-containing puncta observed in *mef2* morphants might be z-bodies. Thus, in the absence of the second phase of myofibrillogenesis, zebrafish muscle appears to be stuck in a pre-myofibril state. Mef2 is required to permit these z-body-like structures to assemble efficiently into large z-lines. In fast fibres, the absence of thick filament components might account for the failure of sarcomere assembly, as occurs when MyLC is lacking in heart (Rottbauer et al., 2006). The ability of slow fibres, which provide a mononucleate fibre scaffold for the myotome, to initiate myofibrillogenesis in *mef2* morphants is consistent with their early myosin expression and weak early motility and is reminiscent of the lack of muscle defects in the mononucleate muscles of C.

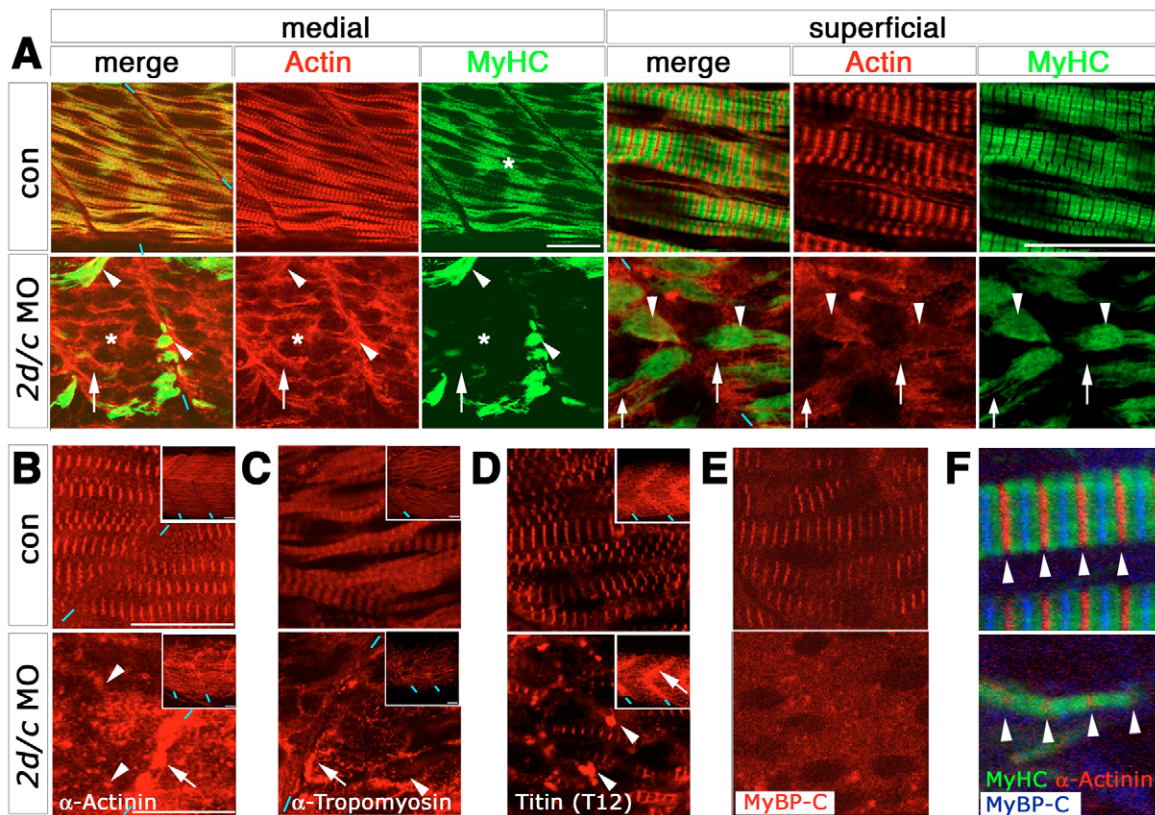


Fig. 6. Mef2 controls a transcriptional module involved in thick filament assembly. Confocal immunofluorescence of sarcomeric components in a 24-hpf midbody somite of control or *mef2d/c* MO-injected zebrafish embryos in fast (A, medial) or slow (A, superficial, B-F) fibres; lateral view slice and insets of stack, anterior to left, dorsal to top. (A) MyHC is absent from fast fibres (asterisk) and concentrated at fibre ends in slow fibres (arrowhead) in morphants, whereas actin is diffusely dispersed in cytoplasm of both fibre types (arrows). Blue dashes indicate somite borders. (B-D) z-line components α -actinin and titin and thin filament protein α -tropomyosin are concentrated in cytoplasmic puncta (arrowheads) and at fibre termini (arrows) after Mef2 knockdown. (E,F) M-line component MyBP-C is absent from residual myofibrils in morphants with regular sarcomere z-line spacing (arrowheads). Scale bars: 20 μ m.

elegans embryos lacking MEF-2 (Dichoso et al., 2000). However, *mef2* morphant slow myofibrils are immature: they retain fast MyHC but lack MyBP-C and fail to grow, similar to the phenotype of mice lacking titin's M-line region (Weinert et al., 2006). Strikingly, although fast fibres are devoid of myofibrils, all slow fibres appear to have a single thin myofibril spanning their length. This suggests that myofibril initiation and growth are two separate processes, at least in slow fibres. In slow fibres, the role of Mef2 appears to be to permit growth of these initial myofibrils. Therefore, the data implicate Mef2 in a second transcriptional phase occurring in both fast and slow nascent fibres.

How do the observations described here relate to data implicating Mef2 in terminal differentiation, adult fibre properties and expression of a wide range of muscle genes (Blais et al., 2005; Nakagawa et al., 2005; Sandmann et al., 2006; Tapscott, 2005; van Oort et al., 2006; Xu et al., 2006; Haberland et al., 2007)? In cultured cells, Mef2d seems to act together with MyoD-family transcription factors to promote a late step in terminal differentiation of myoblasts (Penn et al., 2004; Tapscott, 2005). In zebrafish, as in mice, the MyoD family drives differentiation of the earliest muscle fibres (Hammond et al., 2007) and these cells express Mef2d but not Mef2c. Nevertheless, the earliest fish muscle precursors undergo apparently normal terminal differentiation, actin expression, elongation and fusion into myotubes in the absence of detectable Mef2 protein. This is

surprising when one considers the potent ability of Mef2 to cooperate with MyoD to drive myogenic conversion of 10T1/2 cells (Molkentin et al., 1995). Our data indicate a need to re-examine myogenic conversion assays performed using expression of thick filament components as a readout: perhaps, in vivo, Mef2 is not normally required for cell-cycle exit and initiation of muscle gene expression indicative of terminal differentiation. Despite the lack of immunologically detectable Mef2 protein, morpholino knockdowns might not represent a null condition. However, we did not observe under-representation of knockdown cells in muscle in chimaeras. We conclude that normal levels of Mef2c and Mef2d are not required for muscle fibre terminal differentiation.

Could the *mef2a* gene, which is expressed in fast muscle after differentiation (Hammond et al., 2007; Ticho et al., 1996; Wang et al., 2006) (www.zfin.org), compensate for loss of other Mef2s? Our *mef2* morphants lack Mef2 immunoreactivity, even though the anti-Mef2 serum was raised against human MEF2A and detects zebrafish Mef2a protein in the brain (Y.H. and S.M.H., unpublished). So Mef2a is unlikely to provide significant compensation. Our data show that Mef2c or Mef2d is required for thick filament mRNA and protein accumulation in fast fibres. Interestingly, body curvature is seen after *mef2a* knockdown in older embryos (Wang et al., 2006). We suggest that Mef2a contributes to myofibrillogenesis at later stages.

The failure of fibre maturation and growth in *mef2* morphants fits well with the suggested roles of Mef2 in a late differentiation step and in the adult fibre response to electrical activity and physical load (Jordan et al., 2005; Nakagawa et al., 2005; Penn et al., 2004; Wu et al., 2001). In cultured myotubes, Mef2 binds to many genes, and functional targets are likely to be diverse (Black and Olson, 1998; Nakagawa et al., 2005). Our data indicate that the defects in sarcomere assembly result from a requirement for Mef2 in the activation of various thick filament genes. However, Mef2 might not act directly on all thick filament genes, although many contain Mef2 sites and E boxes, through which Mef2 can act (Beylkin et al., 2006; Molkentin et al., 1995). Mef2 might also act elsewhere, for example on genes required for muscle fibre attachment, which also appears defective (Y.H. and S.M.H., unpublished). In *Drosophila*, Mef2 binds to and is required for correct quantitative expression of hundreds of genes (Sandmann et al., 2006), but we see no contradiction to our finding that Mef2 is not essential for terminal differentiation in fish. One explanation, given that *Drosophila* and *C. elegans* Mef2 differ greatly in their importance for myogenesis (Dichoso et al., 2000), is that the role of Mef2 has evolved after divergence of vertebrates and invertebrates. Alternatively, the first essential role of *Drosophila* Mef2 in somatic muscle might be similar to that in fish: *Mef2*-null flies still form nascent myofibres, but fail to express much myosin or form proper attachments (Lilly et al., 1995; Bour et al., 1995; Ranganayakulu et al., 1995; Prokop et al., 1996; Gunthorpe et al., 1999). Indeed, muscle defects in adult flies lacking Mef2 arise in the absence of changes in myofibrillar actin expression (Baker et al., 2005). Our analysis is restricted to the earliest stages of skeletal muscle formation, but reveals the importance of Mef2d and Mef2c in turning the nascent myotube into a mature fibre.

The finding of a new regulatory step in embryonic myofibrillogenesis prompts further analysis of the role of Mef2 in fetal and adult animals. Mef2 has been implicated in regulating size, metabolism and type of adult muscle cells (Kolodziejczyk et al., 1999; Liu and Olson, 2002; Wu et al., 2000). Our work raises the possibility that myofibrillogenetic thick filament protein turnover might control muscle strength and character in the adult. Interestingly, the defects we observe in nascent fibres are reminiscent of those in some forms of human acute quadriplegic myopathy, in which thick filament gene expression can be specifically depleted leading to catastrophic paralysis in a significant fraction of critical care patients (Larsson et al., 2000). Although Mef2 target genes are likely to have diversified as complex muscle evolved, it will be interesting to determine which aspects of Mef2 function in nascent fibres persist in the adult.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/13/2511/DC1>

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