Expression of multiple slow myosin heavy chain genes reveals a diversity of zebrafish slow twitch muscle fibres with differing requirements for Hedgehog and Prdm1 activity

Stone Elworthy, Murray Hargrave, Robert Knight, Katharina Mebus and Philip W. Ingham*

The zebrafish embryo develops a series of anatomically distinct slow twitch muscle fibres that characteristically express genes encoding lineage-specific isoforms of sarcomeric proteins such as MyHC and tropinin. We show here that different subsets of these slow fibres express distinct members of a tandem array of slow MyHC genes. The first slow twitch muscle fibres to differentiate, which are specified by the activity of the transcription factor Prdm1 (also called Ubo or Blimp1) in response to Hedgehog (Hh) signalling, express the smyhc1 gene. Subsequently, secondary slow twitch fibres differentiate in most cases independently of Hh activity. We find that although some of these later-forming fibres also express smyhc1, others express smyhc2 or smyhc3. We show that the smyhc1-positive fibres express the ubo (prdm1) gene and adopt fast twitch fibre characteristics in the absence of Prdm1 activity, whereas those that do not express smyhc1 can differentiate independently of Prdm1 function. Conversely, some smyhc2-expressing fibres, although independent of Prdm1 function, require Hh activity to form. The adult trunk slow fibres express smyhc2 and smyhc3, but lack smyhc1 expression. The different slow fibres in the craniofacial muscles variously express smyhc1, smyhc2 and smyhc3, and all differentiate independently of Prdm1.

KEY WORDS: Zebrafish, Myotome, Fibre type, Slow myosin heavy chain, Troponin, Prox1, Shh, Blimp1, u-boot, prdm1, Craniofacial

INTRODUCTION

The formation and growth of skeletal muscles in vertebrates proceeds via successive waves or phases of myogenesis that occur during embryonic and foetal development under the influence of various intrinsic and extrinsic signals (reviewed by Buckingham, 2007; Ponnall et al., 2002; Shi and Garry, 2006). Vertebrate muscles contain a spectrum of fibre types varying between fatigue-resistant slow twitch fibres with oxidative metabolism and fast twitch fibres with glycolytic metabolism for brief powerful activity. The myosin heavy chain (MyHC) isotype is a major determinant of these contrasting contractile properties (Bottinelli, 2001; Weiss et al., 2001). In a wide range of vertebrate genomes, numerous MyHC genes are organised as members of tandem arrays or as individual genes. Mammals have a tandem array with fast MyHC isoform genes organised as members of tandem arrays or as individual isoform for skeletal as well as cardiac muscle (Lompré et al., 1984; Mahdavi et al., 1984; Mahdavi et al., 1984). MyHC genes elsewhere in the mammalian genome provide fast isoforms for particular craniofacial muscles (Korfage et al., 2005a). During development and regeneration, mammalian muscle fibres express different MyHC isoforms in a specific temporal sequence (Agbulut et al., 2003). Once fibres are formed, neural activity can remodel fibre type to suit the induced contraction behaviour (Buller et al., 1960; Buller et al., 1969).

In contrast to the single MyHCβ gene in the mammalian genome, the chick has a tandem array of three slow MyHC genes with differing developmental expression patterns (Chen et al., 1997; Page et al., 1992; Sacks et al., 2003). Analysis of chick MyHC isoforms demonstrated that embryonic muscle fibres are initially patterned as slow or fast twitch independently of neural activity (Crow and Stockdale, 1986).

In teleost fish, such as carp and medaka, different MyHC genes are expressed during different developmental stages and at different water temperatures (Liang et al., 2007; Nihei et al., 2006; Ono et al., 2006). The previously described zebrafish smyhc1 gene (Bryson-Richardson et al., 2005; Nakano et al., 2004; Rauch et al., 2003) is within a tandem array of five MyHC genes that has been extensively studied on a genomic sequence basis as a classic example of gene conversion events (McGuigan et al., 2004).

In the zebrafish embryo, the first muscle fibres to form are of the slow twitch type (Devoito et al., 1996; van Raamsdonk et al., 1978). These fibres derive from precursors known as adaxial cells, so called because they lie adjacent to the axial midline structures that secrete Hedgehog (Hh) family proteins (Devoito et al., 1996). Adaxial cells respond to Hh signalling by expressing the Prdm1 transcription factor, the activity of which is both necessary and sufficient for adaxial cells to adopt the slow twitch fibre identity (Baxendale et al., 2004) and express slow MyHC (Bryson-Richardson et al., 2005; Devoto et al., 1996), and the homeodomain protein Prox1 (Glasgow and Tomarev, 1998; Roy et al., 2001). Once specified, the cells undergo a radial migration from their original medial location to form a superficial monolayer of slow twitch muscle fibres covering the embryonic myotome and subsequently a lateral wedge-shaped strip of slow muscle called the lateralis superficialis in juveniles (Devoto et al., 1996).

Following the initial wave of primary fibre differentiation in the zebrafish, further slow twitch fibres differentiate in a variety of locations. The specification of some of these secondary slow twitch fibres has been shown to be independent of Hh signalling (Barresi et al., 2001). Here, we have investigated the molecular identity of these secondary slow fibres and explored the role of Prdm1 in their specification. We show that Prdm1 activity is
required only by a specific subset of these slow fibres that are distinguished from other secondary slow fibres by their expression of the *smyhc1* gene.

**MATERIALS AND METHODS**

Zebrafish, mutants, genotyping and cycloplamine treatment

Zebrafish embryos were kept and staged following standard methods (Kimmel et al., 1995; Westerfield, 1995). Pigmentation was inhibited by immersion in 0.2 mM N-phenylthiourea. Zebrafish mutants *ubo*, *nrd* and *smyhc1* were cloned by cDNA IMAGE_7041248.5 (Accession Number BK006465) is represented by the existing cDNA IMAGE_7433531, whereas primer tagtggggaacgacccttta+ggcctatttccaagtgtagtgc followed by digestion with *AciI*, which cuts at the site of the *ubo* point mutation but not the wild-type allele. Cycloplamine treatment followed a standard method with immersion in 50 μM cycloplamine (Toronto Research Chemicals) from the four-cell stage or 20–somite stage (Hirsinger et al., 2004; Wolff et al., 2003).

**RESULTS**

The expression of different slow MyHC genes defines distinct groups of slow twitch muscles

The slow MyHC specific antibodies F59 and S58 (raised against chicken myosin) have been widely used as markers for investigations of muscle fibre type specification in zebrafish embryos (Devoto et al., 1996; Hernandez et al., 2005). Expression of a slow MyHC gene, designated *smyhc1*, has previously been reported in F59-positive muscle fibres in early embryos, suggesting that it may encode the epitope recognised by F59 and S58 in zebrafish (Bryson-Richardson et al., 2005; Nakano et al., 2004; Rauch et al., 2003). Using in situ hybridisation, we confirmed that a probe complementary to the coding sequence of this gene (*smyhc1-CDS*) detects slow fibres in a diverse set of muscles (Fig. 1A) (Rauch et al., 2003). To investigate the expression of this gene further, we generated gfp reporter constructs; surprisingly, we found that transgenic animals carrying these constructs expressed GFP in only a subset of the S58-positive muscles (Fig. 1A). This could imply that the expression pattern is not fully recapitulated by our reporter genes; alternatively, it may be that the *smyhc1-CDS* probe detects...
transcripts from more than one Smyhc gene. Consistent with this latter hypothesis, genomic analysis has shown that zebrafish smyhc1 resides within a tandem array of five genes that have a remarkable level of DNA sequence identity (Fig. 1C) (McGuigan et al., 2004). The long stretches of DNA sequence identity between smyhc1 and the other genes in the tandem array, including a 270 bp stretch of 100% nucleotide identity between smyhc1 and the adjacent gene in the tandem array cause the CDS probe to cross-hybridise, confounding expression analysis of the individual genes. We therefore synthesised gene-specific 5′/H11032 UTR probes to resolve the expression of these genes in different slow fibres by in situ hybridisation (Fig. 1; see Fig. S1 and Table S1 in the supplementary material; Fig. 3).

From 48 hpf, a series of muscles differentiate with slow twitch fibres that express smyhc2 but not smyhc1 (Fig. 1D-G, see Fig. S1 and Table S1 in the supplementary material). The third gene in the tandem array, which we call smyhc3, has very short UTR sequences, making detection by in situ hybridisation difficult. Nevertheless, we could observe at the limit of detection smyhc3 expression in some of the smyhc2-positive trunk muscles (Fig. 1H; see Table S1 in the supplementary material).

In contrast to the differential expression of the Smyhc genes, slow troponin C is expressed in all slow MyHC-expressing fibres throughout the embryo (see Fig. S2 in the supplementary material) (Thissie et al., 2001). Expression is restricted to S58-positive fibres except in the pectoral fin muscle that is S58 negative but strongly expresses slow troponin C (see Fig. S2 in the supplementary material); the physiological status of this muscle is unclear.

There is expression of smyhc2 at 72 hpf and smyhc3 at 96 hpf in a few fibres lateral to the horizontal myoseptum that we have called the embryonic lateralis superficialis (Fig. 1D,F,H; see Fig. S1 in the supplementary material). These few fibres may later become

---

**Fig. 1. Different Smyhc genes have distinct expression patterns.** (A) At 96 hpf, the smyhc1:gfp reporter gene is expressed in a subset of the slow fibres labelled with the S58 anti slow MyHC Ab or the smyhc1-CDS in situ hybridisation (ISH) probe. The iob is a major muscle that does not express smyhc1:gfp in its slow fibres. (B) High magnification lateral view of smyhc1-CDS in situ hybridisation in posterior trunk showing transcript is concentrated at the ends of the superficial slow fibres. (C) A diagram illustrating the tandem array of Smyhc genes as described by McGuigan et al. (McGuigan et al., 2004) together with the gene names used here. We followed the Bryson-Richardson et al. (Bryson-Richardson et al., 2005) renaming of myhcE as smyhc1 and similarly renamed the adjacent genes smyhc2 and smyhc3. (D) Differential expression of Smyhc genes at 96 hpf, as shown by in situ hybridisation using smyhc1 (1 to 249 bp) or smyhc2 (1 to 324 bp) probes. The sca, els, iob, sh, scp and icp somite-derived muscles, and dorsal and ventral craniofacial muscles express smyhc2. (E) smyhc1:gfp together with smyhc2 (1 to 324 bp) in situ hybridisation at 96 hpf. (F) At 96 hpf, smyhc2 (1 to 324 bp) in situ hybridisation shows colocalisation with slow MyHC S58 antigen but not with smyhc1:gfp in the sca muscle (dorsal view anterior trunk), els or iob muscles (lateral view anterior trunk) or scp or icp muscles (lateral view posterior tail). (G) A deep focus ventral view shows smyhc2 (1 to 324 bp) expression in the oesophagus at 96 hpf. (H) The low sensitivity smyhc3 (1 to 129 bp) in situ hybridisation weakly detects expression in the sca and els muscles at 96 hpf, as shown by dorsal and lateral views of the anterior trunk. Scale bars: 25 μm.

Abbreviations: dm, head dorsal muscles; els, embryonic lateralis superficialis; icp, infracarinalis posterior; iob, inferior obliquus; oes, oesophagus; sca, suprarcarinalis anterior; scp, suprarcarinalis posterior; sh, sternohyoideus; vm, head ventral muscles. Muscle nomenclature is taken from previous work (Schilling and Kimmel, 1997; Stiassny, 2000; Winterbottom, 1974).
incorporated into the lateralis superficialis muscle that forms at this location in juveniles predominantly from adaxially derived fibres (Devoto et al., 1996). At 32 dpf, smyhc1 and smyhc2 are co-expressed in the lateralis superficialis (Fig. 2A). By 42 days post fertilisation (dpf), the trunk lacks smyhc1 expression (data not shown), and smyhc2 and smyhc3 are expressed in the lateralis superficialis muscles (Fig. 2C). GFP is known to persist long after reporter transcription has ceased (Tallafuss and Bally-Cuif, 2003). This presumably explains why lateral fibres of the 42 dpf lateralis superficialis lack expression (Fig. 2B). The adult lateralis superficialis lacks smyhc1 expression (data not shown) and has a complex complementary expression of smyhc2 and smyhc3 (Fig. 2D).

From 48 hpf, a series of craniofacial muscles develop from cranial paraxial mesoderm (Schilling and Kimmel, 1997). Most of these muscles comprise both slow fibres and fast fibres (Hernandez et al., 2005). As in the trunk and tail, slow troponin C is expressed in all SS8-positive craniofacial muscles (Fig. 3A,B,E). Most of these slow fibres express both smyhc1 and smyhc2 (Fig. 3A-D; see Table S1 in the supplementary material). Strikingly, however, smyhc1 expression is absent from the slow fibres of a disparate subset of the craniofacial muscles (Fig. 3; see Table S1 in the supplementary material; Fig. 1D). Furthermore, some muscles contain both clusters of fibres that co-express smyhc1 with smyhc2, and also clusters that just express smyhc2 (Fig. 3D).

Despite the limitations of the smyhc3 probe, intense smyhc3 expression was detected in the levator arcus palatini muscle that lacks smyhc1 expression (Fig. 3A). Weaker smyhc3 expression was detected in certain other craniofacial muscles (Fig. 3A,B).

Using SS8 staining, Barresi et al. (Barresi et al., 2001) showed that slow fibres are added all along the dorsal and ventral margins of the primary superficial slow fibre layer after 24 hpf. As most of this region is devoid of smyhc2 and smyhc3 (Fig. 1D; see Fig. S1 in the supplementary material) it seems probable that these secondary fibres express smyhc1. To distinguish such fibres from their primary counterparts, we adopted the BrdU labelling technique used by Barresi et al. (Barresi et al., 2001). Exposure of smyhc1:gfp embryos to BrdU at 18 hpf, resulted in extensive labelling of fast muscle and external cell nuclei across all dorsal ventral levels of the myotome at 48 hpf, in accordance with the recent report of Stellabotte et al. (Stellabotte et al., 2007) (Fig. 4A,B). By contrast, only smyhc1:gfp-positive slow fibres at the dorsal and ventral margins of the layer of superficial slow fibres were BrdU labelled (Fig. 4B-D; see Movie 1 in the supplementary material). Exposure to BrdU at 35 hpf similarly led by 96 hpf to BrdU-labelled, smyhc1:gfp positive, fibres only at the dorsal and ventral margins (Fig. 4C,D). These findings establish that these fibres do indeed express smyhc1. We refer to these fibres as ‘secondary superficial slow fibres’ to distinguish them from the series of smyhc2-expressing secondary slow fibres.

**smyhc1 and smyhc2 expressing secondary fibres differ in their requirement for Hedgehog signalling**

Previous studies have shown that some secondary slow fibres can differentiate normally in the absence of Hh signalling activity, in contrast to their primary counterparts (Barresi et al., 2001). We investigated the identity of these Hh-independent fibres using the gene-specific Smyhc probes. Embryos either mutant for the Hh signal transducer Smoothened (smo) or treated with the Smo antagonist cyclopamine, have extensive secondary superficial slow fibres at the dorsal and ventral margins of the posterior trunk and the tail that express smyhc1 and not smyhc2 (Fig. 5A,C,D). Expression of smyhc2 in smo mutants is similar to that in wild type; however, the embryonic lateralis superficialis fibres are absent, as are the smyhc2-expressing fibres of the tail (Fig. 5D; see Table S1 in the supplementary material). This lack of the smyhc2-expressing fibres of the tail is strikingly similar to the loss of third wave slow muscle reported for cyclopamine-treated Xenopus embryos (Grimaldi et al., 2004). We treated embryos with cyclopamine from the 20-somite stage to determine whether zebrafish have a similar requirement for Hh signalling during slow fibre myogenesis late in embryogenesis. These treated embryos had a normal pattern of smyhc1:gfp and smyhc2 expression except for the lack of the tail smyhc2-expressing slow fibres (Fig. 5E). The posterior tails of these embryos had a narrower dorsal-ventral extent and lacked any muscle dorsal or ventral of the smyhc1-expressing layer (Fig. 5F), suggesting that Hh signalling is required for the formation of this muscle. This contrasts with its role in specifying the fibre type of adaxial cells but is strikingly similar to the generation of third wave slow muscle in Xenopus (Grimaldi et al., 2004).
Hedgehog independent smyhc1 expressing secondary superficial slow fibres require prdm1

Using the highly sensitive smyhc1-CDS in situ hybridisation probe, we have compared the ontogeny of slow twitch muscle fibres in prdm1 mutant, smo mutant and wild-type embryos (Fig. 6A; we use ‘Smyhc’ to mean transcripts detected with the smyhc1-CDS probe). Consistent with the report of Barresi et al. (Barresi et al., 2001), we found that in smo mutant embryos, slow fibres are virtually absent at 24 hpf, but subsequently appear at the dorsal and ventral margins of the somites at 36 hpf, increasing in number by 48 hpf. By contrast, in prdm1 mutants at 24 hpf, we found that many fibres scattered through the myotome show low levels of Smyhc expression, in line with previous descriptions of the ubo(prdm1)p39 mutant phenotype (Roy et al., 2001). This Smyhc expression was observed not only in embryos homozygous for the ubo(prdm1)p39 mis-sense allele but also in those homozygous for nrd(prdm1)m805, which has a stop codon at amino acid 154 and is thus a presumptive null allele (Hernandez-Lagunas et al., 2005) (Fig. 6A). Notably, a few fibres have a less dramatic reduction in expression and these tend to be more dorsal and more numerous in posterior somites. By 48 hpf, the scattered expression of Smyhc throughout the myotome is greatly diminished, but expression persists in fibres at the dorsal margin. The location of these latter fibres is similar to that of the secondary superficial slow fibres, raising the possibility that at least some secondary superficial slow fibres can form in the absence of prdm1 function. Alternatively, these could correspond to adaxially derived, primary slow fibres that escape the requirement for wild-type levels of prdm1 function.

Fig. 3. Differential expression of Smyhc genes in craniofacial muscles. (A) Lateral and ventral views of the dorsal group of craniofacial muscles at 96 hpf with slow troponin C (stnnC), smyhc1 (1 to 249 bp), smyhc2 (1 to 324 bp) or smyhc3 (1 to 129 bp) in situ hybridisation (see Fig. 1A,D,E,G for gross location of these muscles). The lap and do lack smyhc1 expression while smyhc3 expression is restricted to the lap. (B) Ventral views showing ventral craniofacial muscles at 96 hpf with slow troponin C (stnnC), smyhc1 (1 to 249 bp), smyhc2 (1 to 324 bp) or smyhc3 (1 to 129 bp) in situ hybridisation. The ima, sh, iob and posterior tv lack smyhc1 expression. (C) Lateral and ventral views of the dorsal group of craniofacial muscles at 96 hpf showing colocalisation of slow MyHC S58 antigen with smyhc2 (1 to 324 bp) in situ hybridisation and, in the ao and lo, with smyhc1:gfp. (D) Ventral view at 96 hpf showing colocalisation of smyhc1:gfp and smyhc2 (1 to 324 bp) in situ hybridisation with slow MyHC S58 antigen. In the am, some fibres express smyhc2 and S58 antigen but not smyhc1:gfp (blue arrows). Some fibres express S58 antigen but neither smyhc2 nor smyhc1:gfp (white arrows). The extraocular muscles have zones of S58 antigen-expressing fibres that co-express smyhc1:gfp and zones that do not (pink arrows). (E) Ventral view at 96 hpf showing expression of slow troponin C in situ hybridisation (stnnC) in all slow MyHC S58 antigen-expressing fibres. Scale bars: 25 μm. Abbreviations: abh, abductor hyoideus; am, adductor mandibulae; ao, adductor operculi; do, dilator operculi; hh, hyohyoidus; ih, interhyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; io, inferior oblique; iob, inferior obliquus; ir, inferior rectus; lap, levator arcus palatini; lo, levatator operculi; sh, sternohyoides; tv, transversus ventralis.
To distinguish between these possibilities, we eliminated primary slow muscle fibres from ubo\(prdm1\)^{m39} mutant embryos by treating them with the Smo antagonist cyclopamine (Hirsinger et al., 2004; Wolff et al., 2003). Such embryos are essentially devoid of both primary and secondary superficial slow fibres at 48 hpf, as indicated by the absence in the tail and posterior trunk of both Smyhc and slow troponin C expression (Fig. 6B). It follows from this finding that the persistent dorsal slow fibres seen in ubo\(prdm1\)^{m39} mutants are adaxially derived primary slow fibres and that prdm1 function is essential for the differentiation of the secondary superficial slow fibres that form normally in the absence of Hh signalling.

By contrast, the Smyhc2-expressing fibres are largely unaffected by the loss of prdm1 activity, though the embryonic lateralis superficialis muscles are lost in nrd\(prdm1\)^{m805} mutants as in smo mutants (Fig. 6C; see Table S1 in the supplementary material). This may be a secondary consequence of the aberrant somite morphology caused by lack of the horizontal myoseptum.

The craniofacial muscles generally have wild-type levels of expression of both smyhc1 and smyhc2 in nrd\(prdm1\)^{m805} mutants (see Fig. S3B and Table S1 in the supplementary material; Fig. 6C). The only perturbations to the craniofacial slow fibres in prdm1 mutants are associated with the major loss of skeletal elements in the posterior head (see Fig. S3A in the supplementary material) (see also Wilm and Solnica-Krezel, 2005).

**prdm1:gfp expression in primary and secondary slow fibres**

We next investigated whether prdm1 is expressed in the precursors of the secondary superficial (smyhc1^{+}ve) slow fibres. Low-level prdm1 expression can be detected in situ hybridisation at 35 hpf in cells tentatively identified as newly forming secondary superficial slow fibres in smo mutants (Fig. 7A). To characterise this expression further, we took advantage of the stability of GFP transcript and protein (Tallafuss and Bally-Cuif, 2003) to monitor slow fibres in prdm1:gfp reporter lines. The expression of GFP in these lines (Fig. 7B) recapitulates the pattern of endogenous prdm1 transcription in adaxial cells (Baxendale et al., 2004) but persists as the cells migrate and differentiate into the superficial layer of mononucleated slow fibres that covers the myotome at 24 hpf (Fig. 7C). As expected, in smo mutants carrying the prdm1:gfp transgene, no such labelled fibres are present at 24 hpf (Fig. 7C). At 48 hpf, however, prdm1:gfp-positive fibres are present at the dorsal and ventral margins of the myotome, locations consistent with their being secondary superficial slow fibres. Simultaneous staining for Smyhc or slow troponin C expression confirmed that the prdm1:gfp-positive fibres are indeed secondary superficial slow fibres (Fig. 7D).

In addition, we also found that these fibres accumulate Prox1 in their nuclei (although at lower levels than their primary counterparts) (Fig. 7E) and lack expression of the fast muscle F310 antigen (Fig. 7F). Intriguingly, prdm1:gfp is specifically expressed not only in the smyhc1 expressing slow fibres but also in the slow fibres of muscles that express smyhc2 and not smyhc1 (Fig. 7G, see also Fig. 1D,F).

**Transformation of secondary superficial slow fibres to a fast twitch character in ubo (prdm1) mutants**

We next analysed the fate of primary and secondary superficial slow fibre precursors in ubo\(prdm1\)^{m39} mutants using the prdm1:gfp transgene as a lineage marker. At 24 hpf, prdm1:gfp colocalises with the scattered weakly Smyhc-positive cells typical of ubo\(prdm1\)^{m39} mutants, indicating that these are derived from adaxial cells that have failed to migrate properly owing to the loss of prdm1 function (Fig. 8A). Although by 48 hpf Smyhc expression is lost from all but the most dorsal fibres, the scattered prdm1:gfp positive cells persist (Fig. 8A) and differentiate into fibres that are multinucleate and F310 positive, characteristics that are typical of fast twitch fibres (Hamade et al., 2006; Roy et al., 2001) (Fig. 8B,C). Similarly, even the dorsal fibres in which slow MyHC expression persists are marked by both prdm1:gfp and the fast muscle F310 antigen (Fig. 8D) and lack expression of Prox1 (Glasgow and Tomarev, 1998) (Fig. 8E).

At 48 hpf, cyclopamine-treated transgenic ubo\(prdm1\)^{m39} mutant embryos have the normal complement of prdm1:gfp-positive fibres at the location of the secondary superficial slow fibres in their genetically wild-type siblings. However, unlike those in the latter, these fibres do not express slow MyHC and do express the fast fibre-specific F310 antigen (Fig. 8F).
The zebrafish embryo develops a series of distinct muscle types with slow twitch fibres. We show here that these different slow fibres can be distinguished by the expression of the Smyhc1, Smyhc2 and Smyhc3 genes that form a tandem array in the genome. During the juvenile period, there is an apparent transition from Smyhc1 to Smyhc2 and Smyhc3 expression in the lateralis superficialis. This is reminiscent of the developmental transitions in MyHC isoform expression in amniotes. The chick also has three tandemly arrayed slow MyHC genes that are expressed with differing developmental control, timing and subcellular transcript localisation (Sacks et al., 2003). We do not know whether this arrangement of slow MyHC genes is evolutionarily ancient or arose independently in each lineage. A molecular phylogenetic assessment of this issue is hampered by gene conversion events within the zebrafish Smyhc tandem array (McGuigan et al., 2004).

DISCUSSION
The zebrafish embryo develops a series of distinct muscle types with slow twitch fibres. We show here that these different slow fibres can be distinguished by the expression of the Smyhc1, Smyhc2 and Smyhc3 genes that form a tandem array in the genome. During the juvenile period, there is an apparent transition from Smyhc1 to Smyhc2 and Smyhc3 expression in the lateralis superficialis. This is reminiscent of the developmental transitions in MyHC isoform expression in amniotes. The chick also has three tandemly arrayed slow MyHC genes that are expressed with differing developmental control, timing and subcellular transcript localisation (Sacks et al., 2003). We do not know whether this arrangement of slow MyHC genes is evolutionarily ancient or arose independently in each lineage. A molecular phylogenetic assessment of this issue is hampered by gene conversion events within the zebrafish Smyhc tandem array (McGuigan et al., 2004).

The expression of slow troponin C indicates that pan slow lineage expression can be driven by elements associated with a single transcription unit. This raises the issue as to what evolutionary constraints have produced the Smyhc tandem array genomic structure. McGuigan et al. have previously described the extraordinary level of DNA sequence conservation between the genes in the Smyhc tandem array (McGuigan et al., 2004). Although frequent and recent gene conversion events have acted to maintain this sequence conservation, it is conceivable that the remaining amino acid differences between the different gene products may have functional significance. The regions of most amino acid sequence divergence correspond to the 25-50 junction and 50-20 junction. These regions show low sequence conservation across the myosin classes (Cope et al., 1996), although there is evidence that their sequence can influence the enzymatic activity of myosins (reviewed by Murphy and Spudich, 2000). Establishing whether these differences confer functional changes will require biochemical and physiological analyses.

Fig. 5. Hedgehog dependent and independent Smyhc1 and Smyhc2 expression. All panels show lateral views. (A) Smyhc1 (1 to 249 bp) in situ hybridisation at 48 hpf showing expression in secondary superficial slow fibres at the dorsal and ventral edges of the somites of a smo mutant. (B) Smyhc2 (1 to 155 bp) in situ hybridisation at 48 hpf showing wild-type levels of expression in the iob of smo mutant (see Fig. 51 in the supplementary material for comparison with wild type). (C) Untreated and cyclopamine treated 55 hpf embryos showing Hh-independent Smyhc1:gfp in the secondary superficial slow fibres and Hh-independent Smyhc2 (1 to 324 bp) in situ hybridisation signal in the iob. (D) 96 hpf wild-type sib and smo mutant. The smo mutant has slow MyHC S58 antigen in iob, sca and secondary superficial slow fibres; Smyhc2 (1 to 324 bp) in situ hybridisation signal in iob and sca; but lacks scp and icp Smyhc2 (1 to 324 bp) in situ hybridisation signal. (E) Untreated embryo and embryo exposed to cyclopamine from the 20-somite stage. This late cyclopamine exposure does not eliminate the Smyhc1:gfp surface slow fibres but does eliminate Smyhc2 (1 to 324 bp) in situ hybridisation signal in the scp and icp, as well as causing a dorsoventral narrowing of the posterior tail. Fifteen out of 15 embryos with this treatment showed this phenotype. (F) High-magnification views of posterior tail in untreated embryo and embryo exposed to cyclopamine from the 20-somite stage. In untreated embryos, Smyhc2 (1 to 324 bp) in situ hybridisation signal colocalises with the general muscle MF20 antigen in the scp and icp dorsal and ventral to the Smyhc1:gfp surface slow fibre layer. The treated embryos lack scp and icp Smyhc2 (1 to 324 bp) in situ hybridisation signal and general muscle MF20 antigen dorsal or ventral to the Smyhc1:gfp surface slow fibre layer. Scale bars: 25 μm. Abbreviations: icp, infracarinalis posterior; iob, inferior obliquus; sca, supracarinalis anterior; scp, supracarinalis posterior.
The evolution of the tandem array genomic structure may have been driven by transcriptional control constraints. The fact that our 9.7 kb promoter smyhc1:gfp reporter gene recapitulates the expression of the endogenous gene, indicates that, for smyhc1 expression, at least, the adjoining parts of the tandem array are not required in cis for transcriptional control. The tandem arrangement of mammalian MyHC genes is constrained by bidirectional expression of MyHC genes and antisense transcripts required for transcriptional control of adjacent MyHC genes (Haddad et al., 2003; Pandorf et al., 2006). We do not know whether such a mechanism exists in zebrafish.

The mammalian craniofacial muscles express an especially diverse selection of MyHC genes. This is thought to reflect intricate functional requirements in these muscles (Korfage et al., 2003a; Korfage et al., 2003b; Noden and Francis-West, 2006). We do not know whether such a mechanism exists in zebrafish.

There are two additional predicted genes [called myhA and myhB by McGuigan et al. (McGuigan et al., 2004)] in the same tandem gene array as the three Smyhc genes we describe here. Although myhA and myhB are not represented in current EST databases, we isolated 5’ RACE products for two alternative 5’UTRs of myhA using RNA from 120 hpf embryos. We were unable to detect any expression in embryos by in situ hybridisation. Possibly, the predicted genes are expressed principally at later stages of the life cycle or under different growth conditions.

Prdm1 acts as a transcriptional repressor to drive the differentiation of adaxial cells into primary slow twitch fibres in response to Hh signalling (Baxendale et al., 2004; von Hofsten et al., 2008). Our analysis shows that prdm1 is also required for secondary superficial slow fibres to adopt a slow twitch character independently of Hh signalling. How expression of prdm1 is activated in the progenitors of these secondary superficial slow fibres remains to be determined.
Intriguingly, the specific expression of prdm1:gf in slow fibres extends beyond the smyhc1-positive fibres and includes the smyhc2-positive fibres for which we could discern no prdm1 requirement. We consider it likely that this prdm1:gf expression reflects the expression of the endogenous gene, as we have observed it in three independent prdm1:gf reporter gene lines. It remains possible that prdm1 acts redundantly with some other gene in these fibres or has a subtle role we failed to discern.

Although specification of slow fibre type shows diversity in its requirement for Hh signalling and prdm1 activity, it is possible that there is a common basis for slow fibre type determination at a more downstream step. The specific expression of slow troponin C across all slow fibres could result from a shared transcriptional program specifying slow fibre type that is common to different slow muscles. Alternatively, the slow troponin C expression may be determined by a composite of independent transcriptional control elements for each of the different types of slow fibre. The complex transcriptional control of mouse Myf5 in myogenic cells provides an example of numerous independent control elements acting in distinct muscle types (Carvajal et al., 2001; Hadchouel et al., 2003).

The origin of the progenitor cells for the various secondary slow fibres and their relationship to the recently identified, Pax7-positive, secondary fast muscle progenitors is currently unclear (Hammond et al., 2007; Hollway et al., 2007; Stellabotte et al., 2007). An additional issue is the mechanism for myogenic induction versus progenitor maintenance for secondary slow fibres. Hh signalling induces the myogenesis of primary slow fibres and also of lateral fast fibres in the primary myotome (Feng et al., 2006; Hammond et al., 2007; Lewis et al., 1999). Our data suggest that Hh signalling is also required for myogenesis of the smyhc2-positive slow fibres of the posterior tail. This shows a striking similarity to third wave slow fibre myogenesis in Xenopus, suggesting that it is an evolutionarily ancient mechanism (Grimaldi et al., 2004).

It is unknown how various slow fibres are specified to express particular Smyhc genes. This question possibly relates to the more general issue of muscle identity determination. Muscle identity may be influenced by intrinsic cues, as well as signalling from other cell types. In zebrafish, anterior trunk somites have an intrinsic axial identity that enables them to produce appendicular muscle (Haines et al., 2004). Earlier in development, paraxial mesoderm for different axial levels is specifically determined by different
combinations of Nodal, Bmp and Fgf signals (Szeto and Kimelman, 2006). Numerous Hox genes are expressed in particular regions of the paraxial mesoderm (Prince et al., 1998). Possibly, certain paraxial mesoderm cells are predisposed to form certain types of slow fibre by such intrinsic cues. Intrinsic cues may also influence whether muscle has a fast twitch or slow twitch character (Nikovits, Jr et al., 2001).

In the head in particular, the different Smyhc genes reveal an unprecedented detail of molecular diversity in the slow fibres. A single Engrailed-expressing condensation of first arch mesoderm splits to form the adjacent levator arcus palatini and dilator operculi (Hatta et al., 1990), and yet the levator arcus palatini expresses high levels of smyc3 while the dilator operculi has barely detectable levels. The differential expression of Smyhc genes between different fibres within craniofacial muscles such as the adductor mandibulae is equally remarkable.

The different Smyhc genes described here will provide valuable late differentiation markers for experimental studies required to understand the specification of muscle identity.

We thank L. Gleaddall, M. Green, F. Browne and S. Surfleet for expert zebrafish husbandry; J. Sanderson for imaging advice; K. Ohymama for cryosection in situ hybridisation advice; K. Berry, C. Moore, A. Burguiere, A. Taylor and C. Davison for generating and characterising reagents; anonymous reviewers for helpful suggestions; H. Roehl, S. Baxendale and F. van Eeden for helpful discussions; N. Copeland for the EL250 strain; and K. Artinger for nrdm805 zebrafish. This research project was funded by a UK Medical Research Council (MRC) Programme Grant (G0100151) and by the EU MYORES Network of Excellence. The CDBG Zebrafish Aquaria was supported by an MRC Centre Development Grant (G0400100) to P.W.I. Imaging facilities were funded by the MRC,Wellcome Trust and Yorkshire Cancer Research.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/12/2115/DC1

Fig. 8. The prdm1:gfp transgenic reporter provides a lineage tracer to follow the fate of prdm1-dependent muscle precursors in ubo (prdm1) mutants. (A) Transverse sections show colocalisation prdm1:gfp with smyc1-CD5 in situ hybridisation in ubo (prdm1)tp39 mutants and wild-type siblings at 24 hpf. At 48 hpf, the prdm1:gfp marked muscle fibres are still present in ubo (prdm1)tp39 mutants, although Smyhc expression is reduced. Cell boundaries are marked with anti-β-catenin 1588. (B) Lateral views showing that muscle fibres with prdm1:gfp form a superficial layer of horizontal, mononucleate fibres in 48 hpf wild-type embryos but are among the fast fibres in ubo (prdm1)tp39 mutants and like fast fibres are frequently multinucleate (TOTO stain) and orientated diagonally. (C) Transverse sections at 48 hpf show colocalisation of F310 fast muscle antigen with prdm1:gfp in ubo (prdm1)tp39 mutants but not in wild-type siblings. (D) In posterior trunk at 48 hpf, in ubo (prdm1)tp39 mutants, the dorsal fibres with persistent slow MyHC S58 antigen are marked with prdm1:gfp and fast muscle F310 antigen. (E) In posterior trunk at 36 hpf, in ubo (prdm1)tp39 mutants, even the most dorsally positioned fibres marked with prdm1:gfp lack Prox1 (arrows). Note the intense Prox1 staining in isolated cells external to the myotome (arrowheads). (F) In posterior trunk of cyclopamine-treated embryos, prdm1:gfp marked secondary fibres colocalise with slow MyHC S58 antigen but not fast muscle F310 antigen in genotyped wild-type siblings and vice versa in genotyped ubo (prdm1)tp39 mutants. Scale bars: 25 μm.
Pdrm1 and zebrafish slow muscle fibres


