Distinct mechanisms regulate slow-muscle development

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Vertebrate muscle development begins with the patterning of the paraxial mesoderm by inductive signals from midline tissues [1, 2]. Subsequent myotome growth occurs by the addition of new muscle fibers. We show that in zebrafish new slow-muscle fibers are first added at the end of the segmentation period in growth zones near the dorsal and ventral extremes of the myotome, and this muscle growth continues into larval life. In marine teleosts, this mechanism of growth has been termed stratified hyperplasia [3]. We have tested whether these added fibers require an embryonic architecture of muscle fibers to support their development and whether their fate is regulated by the same mechanisms that regulate embryonic muscle fates. Although Hedgehog signaling is required for the specification of adaxial-derived slow-muscle fibers in the embryo [4, 5], we show that in the absence of Hh signaling, stratified hyperplastic growth of slow muscle occurs at the correct time and place, despite the complete absence of embryonic slow-muscle fibers to serve as a scaffold for addition of these new slow-muscle fibers. We conclude that slow-muscle-stratified hyperplasia begins after the segmentation period during embryonic development and continues during the larval period. Furthermore, the mechanisms specifying the identity of these new slow-muscle fibers are different from those specifying the identity of adaxial-derived embryonic slow-muscle fibers. We propose that the independence of early, embryonic patterning mechanisms from later patterning mechanisms may be necessary for growth.

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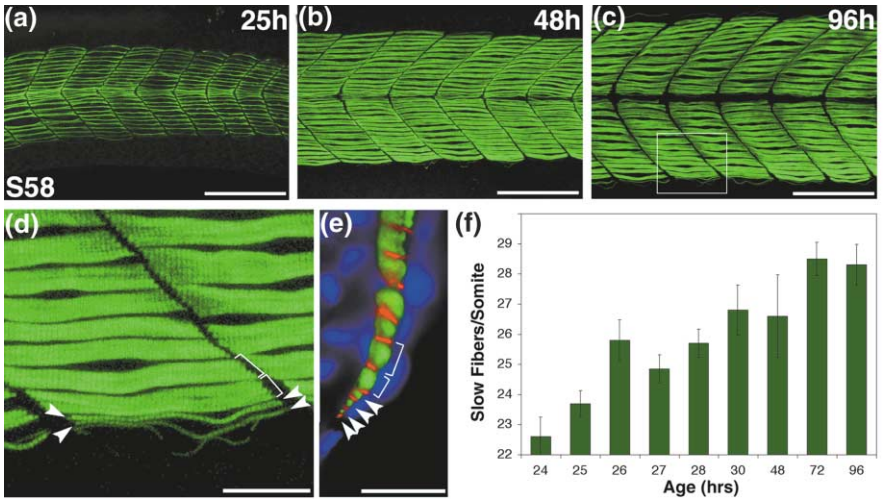
Results and discussion

Vertebrate embryonic muscle development requires inductive signals from midline tissues [1, 2]. It is unknown whether these same inductive signals regulate muscle development during later growth. In zebrafish, embryonic slow-muscle precursors, known as adaxial cells, are induced before the 12h stage of development by Hedgehog (Hh) proteins secreted from the notochord and floorplate [4, 5]. Adaxial cells then migrate radially from their initial positions adjacent to the notochord to form a superficial monolayer of about 20 slow-muscle fibers on the lateral surface of the myotome [4–6]. The number and size of slow-muscle fibers dramatically increases between the embryonic and adult stages [5]. Hyperplasia, an increase in cell number, occurs in the larval stage of most teleosts, while hypertrophy, an increase in the size of existing fibers, is the dominant mechanism of growth in the juvenile and adult stages [3, 7, 8]. During larval stages, new fibers are initially added in the dorsal and ventral regions of the myotome, and these regions have been termed growth zones [9–13]. This type of localized growth, also called stratified hyperplasia, is reminiscent of the growth that occurs in the chick myotome [3, 14]. We wished to determine whether stratified hyperplastic muscle growth occurs in zebrafish and to test whether fiber type identity during growth is determined by the same developmental mechanisms that govern the initial development of embryonic muscle fiber types.

Growth of slow muscle in zebrafish began at 24h, just after the end of the segmentation period (Figure 1). Between 24h and 96h, the height of the slow-muscle monolayer increased 2-fold, and the length increased 1.5-fold (Figure 1a–c). The fibers in the central portion of both the epaxial and the hypaxial domains of the monolayer increased in size, while smaller diameter fibers appeared at the dorsal and ventral extremes (Figure 1d,e). These new slow-muscle fibers were labeled by general muscle- and slow muscle-specific antibodies (S58, F59, N1.551, zn5, 9D10, and MF20) but were not labeled by fast muscle-specific antibodies (zm4 and 12/101; see Figures S1 and S2 in the Supplementary material available with this article online; data not shown). None of the many antibodies we tested distinguished between adaxial-derived fibers and these new slow fibers. zn5, which demarcates individual cells by labeling slow-muscle cell membranes [15], labeled membranes of all slow-muscle fibers, including the smaller fibers located in the dorsal and ventral extremes (Figure 1e). The appearance of small-diameter fibers adjacent to larger fibers is the hallmark of hyperplasia [16]. We quantified the number of slow-muscle fibers

Figure 1



Addition of slow-muscle fibers. **(a–c)** Lateral view of S58 labeling of slow-muscle fibers at 25h, 48h, and 96h. New, small-diameter slow-muscle fibers were observed in the dorsal and ventral extremes of the superficial monolayer in the wild-type. **(d)** Enlargement of boxed area in (c). Arrowheads indicate small-diameter fibers, and brackets indicate larger-diameter fibers. **(e)** Transverse section of the lateral, ventral-most hypaxial region of a 96h larva double-labeled for slow-muscle fibers (S58, green) and slow-muscle cell borders (zn5, red); Hoechst labeling for nuclei is in blue. Arrowheads indicate small-diameter fibers, and brackets indicate larger-diameter fibers. **(f)** The number of slow-muscle fibers per somite in somites 16–18 increases from 22 to 28 between 24h and 96h (see also Figure S3 in Supplementary material). Anterior is to the left and dorsal is to the top in all lateral views. Scale bars equal 100 μm in (a)–(c) and 25 μm in (d) and (e).

per somite in somites 16–18 and found an average increase of 5.7 fibers between 24h and 96h (Figure 1f).

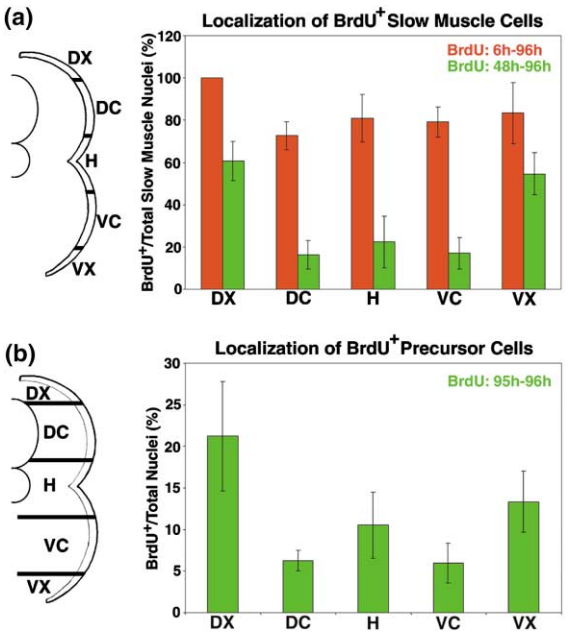
To test whether slow-muscle fibers forming in the dorsal and ventral extremes of the superficial monolayer are younger than cells in the central portion of the monolayer, we used two BrdU labeling protocols to determine when slow-muscle fibers are born (Figure 2; Figure S2 in the Supplementary material). Slow-muscle precursors that entered S phase between 6h and 96h gave rise to fibers distributed evenly thoughout the slow-muscle monolayer (Figure 2a, red bars), whereas slow-muscle precursors that entered S phase after 48h gave rise to fibers preferentially at the dorsal and ventral extremes of the slow-muscle monolayer (Figure 2a, green bars). Together, our results suggest that in zebrafish, stratified hyperplasia of slow-muscle fibers begins in the dorsal and ventral extremes of the monolayer soon after 24h, long before the transition from embryo to larva.

The number of slow-muscle fibers continues to increase after 96h (data not shown; [17]), and slow fibers remain in a superficial monolayer until the larva is about 10 mm (3 weeks; data not shown). In other teleost species, stratified hyperplasia persists well into larval life [9–13]. Therefore, it is likely that in zebrafish, stratified hyperplasia of slow muscle continues to generate new fibers during the larval period. After they differentiate, slow-muscle fibers added during stratified hyperplasia are indistinguishable from adaxial-derived slow-muscle fibers with respect to their position on the surface of the myotome, their expression of molecular markers, and their ultrastructure [18]. Most larval fishes and amphibians have slow-muscle cells in a superficial monolayer [3, 7, 19], where they all function as a physiologically homogeneous group of fibers to generate lateral oscillations of the trunk [20].

The source of precursor cells for new slow-muscle fibers during stratified hyperplasia is unlikely to be adaxial cells because these all differentiate during the segmentation period [6, 21, 22] and have not been observed to divide after being incorporated into a somite [6, 22]. In order to identify a possible source of new slow-muscle fibers, we have used a BrdU labeling protocol to identify proliferating cells in the somite at 96h. We found that the highest percentage of cells in S phase were in the dorsal and ventral extremes of the myotome at 96h (Figure 2b; see also [10]). If the myogenic precursors of these slow-muscle fibers are in fact in these regions, then muscle regulatory transcription factors such as *myoD* should be expressed there. We have found this to be the case. At 24h, *myoD* transcripts were detected thoughout the entire myotome by in situ hybridization (Figure 3a; [23]). However, by 48h, high levels of *myoD* were observed only in the dorsal and ventral extremes of the myotome (Figure 3b). This pattern was also seen at 72h and was maintained to at least 120h (Figure 3c,d; data not shown). Taken together, our results suggest that after 24h, there are proliferating myogenic precursor cells in the dorsal and ventral extremes of the myotome that give rise to new slow-muscle fibers in the dorsal and ventral extremes of the superficial monolayer.

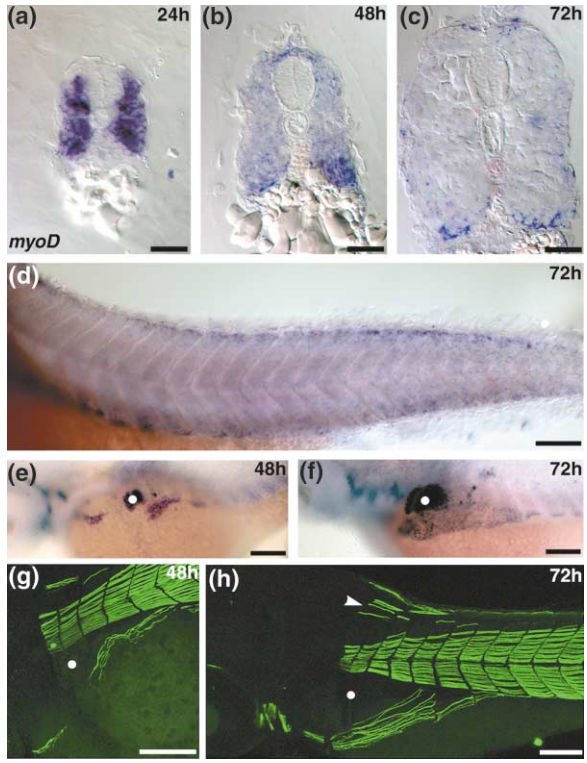
Slow-muscle fibers added after 24h were in the same dorsal and ventral regions as the *myoD*-expressing cells (Figure 3a–d, Figure 1). In somites 1–7, these slow-muscle fibers and *myoD*-expressing cells were displaced dorsally and ventrally, away from the embryonic slow-muscle fi-

Figure 2



Dividing cells in the dorsal and ventral extremes of the myotome give rise to slow-muscle fibers after 48h. **(a)** Slow-muscle fibers in the dorsal and ventral extremes of the superficial monolayer are born after 48h. Embryos incubated in BrdU from 6h to 96h showed an even distribution of BrdU-positive nuclei thoughout the slow-muscle monolayer (red bars). However, larvae that were incubated in BrdU from 48h to 96h showed a higher percentage of $BrdU^+$ slow-muscle nuclei at the dorsal and ventral extremes of the slow-muscle monolayer (green bars). **(b)** The dorsal and ventral extremes of the somite are regions of high cell proliferation. Larvae of 95h were incubated in BrdU until fixation at 96h. The percent of $BrdU^+$ nuclei was highest at the dorsal and ventral extremes of the myotome. (a,b) The schematics to the left represent one half of a 96h transverse section. The horizontal lines demarcate the five regions that were used to count (a) slow-muscle nuclei or (b) all nuclei within the myotome (Figure S2 in Supplementary material). Abbreviations: DX, dorsal extreme; DC, dorsal central; H, horizontal septum area; VC, ventral central; VX, ventral extreme.

Figure 3

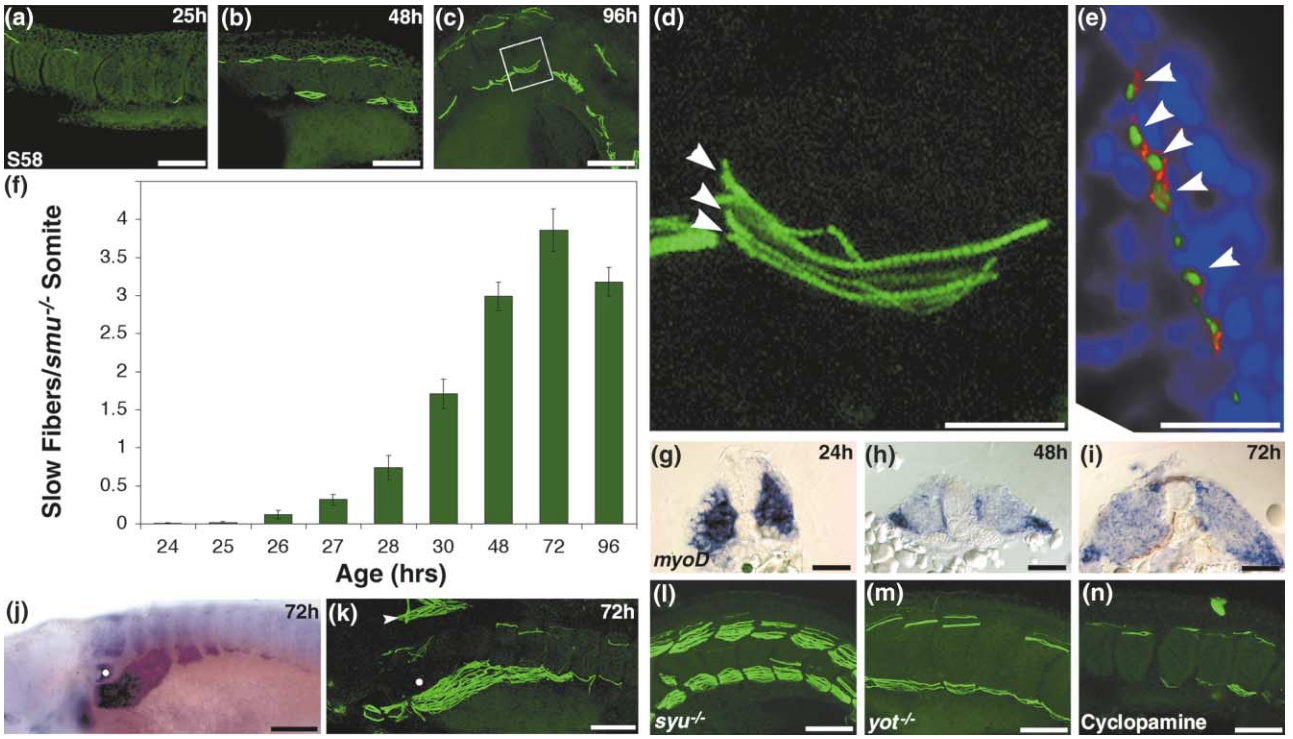


Dorsal and ventral growth zones. **(a–c)** Transverse sections of embryos probed for *myoD* expression at 24h, 48h, and 72h of development**.** *myoD* expression was maintained in the extreme dorsal and extreme ventral regions of the myotome at 48h and 72h. **(d)** Lateral view of whole-mount *myoD* expression in the somites at 72h. *myoD* was expressed in every somite. **(e,f)** Lateral view of whole-mount *myoD* expression in the ventral-most hypaxial regions of somites 1–7 at 48h and 72h. A band of *myoD* expression extended ventrally to the pectoral fin bud (white dot). **(g,h)** Lateral view of S58 labeling of slow-muscle fibers at 48h and 72h. S58 labeling, like *myoD,* was ventral to the pectoral fin (white dot) and expanded anteriorly between 48h and 72h. (h) In addition, S58-labeled slow-muscle fibers in the dorsal-most epaxial region became more dorsal over time (arrowhead). Scale bars equal 50 μm in (a)–(c) and 100 μm in (d)–(h).

bers. In the ventral-most hypaxial region, *myoD* was expressed in a band posterior to the pectoral fin bud at 48h; this band of *myoD* expression enlarged and extended anteriorly to become ventral to the fin bud at 72h (Figure 3e,f). This anterior extension of *myoD* expression corresponded to a similar anterior extension of both slow- and fast-muscle fibers (Figure 3g,h; Figure S1 in Supplementary material). Similarly, in the dorsal-most epaxial region, *myoD* expression as well as slow- and fast-muscle fibers extended dorsally (Figure 3h; Figure S1 in Supplementary material; data not shown). The coincident movement of *myoD*-expressing cells and new slow-muscle fibers in somites 1–7 suggest that cells expressing *myoD* in the dorsal and ventral extremes of the somite are the precursors to the new slow-muscle fibers at the extremes of the slow-muscle monolayer.

We next addressed whether the genetic mechanisms regulating slow-muscle fiber development during stratified hyperplasia are the same as those regulating slow-muscle fiber development during early embryonic patterning. The *slow-muscle-omitted* gene *(smu)*, which encodes zebrafish Smoothened [24], is necessary for Hh signaling and for the development of adaxial-derived, embryonic slow muscle [25]. $smu^{-/-}$ larvae live to about 5 days and thus provide an opportunity to test whether slow-muscle fiber type fate during stratified hyperplasia is also dependent on Hh signaling and, if not, whether it depends on the presence of embryonic slow muscle. At 24h in *smu* mutants, there was an average of less than 0.01 slow-muscle fibers per somite as determined by morphology or by labeling with slow-muscle antibodies such as S58, F59, 4D9, or zn5 [25]. After 24h, a small number of slow-

Figure 4

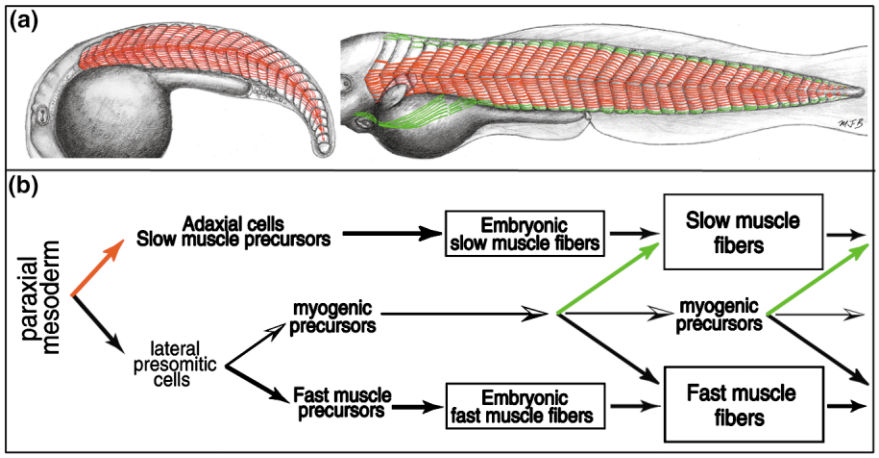


Slow-muscle fiber addition does not require either embryonic slow muscle or Hedgehog signaling. **(a–c)** Lateral views of S58 labeling of *smu* mutants at 25h, 48h, and 96h. Slow-muscle fibers developed after 24h in the most dorsal and ventral extremes of the myotome in *smu* mutants. **(d)** Enlargement of boxed area in (c). Arrowheads indicate small-diameter fibers. Fiber morphology is similar to that seen in wild-type (compare with Figure 1d). **(e)** Transverse section of the lateral, ventral-most hypaxial region of a 96h $smu^{-/-}$ larva double-labeled for slow-muscle fibers (S58, green) and slow-muscle cell borders (zn5, red); Hoechst labeling for nuclei in blue. Arrowheads indicate small diameter fibers (green) surrounded by plasma membrane (red). **(f)** The number of slow-muscle fibers per somite in somites 16–18 increases from zero to three between 24h and 96h. **(g–i)** *myoD* expression in 24h, 48h, and 72h *smu* mutants. *myoD* expression was maintained in the extreme dorsal and ventral regions of the myotome at 48h and 72h. **(j)** Lateral view of *myoD* expression in the ventral-most hypaxial regions of the anterior somites of 72h *smu* mutants (white dot is fin bud). **(k)** Lateral view of S58 labeling of slow-muscle fibers in the anterior somites of a 72h *smu* mutant (white dot is fin bud). The most ventral band of S58 labeling (k) was similar to the expression of *myoD* (j). Slow-muscle fibers were also present in the dorsal-most epaxial region (arrowheads). **(l–n)** Lateral views of S58 labeling of *syu* and *yot* mutants and a cyclopamine-treated fish at 72h. Slow-muscle fibers were seen in the dorsal and ventral extremes of the myotome in all cases. Scale bars equal 100 μm in (a)–(c), (f), and (j)–(n); 25 μm in (d) and (e); and 50 μm in (g)–(i).

muscle fibers were present in *smu* mutants (Figure 4a–c). These slow-muscle fibers were in the most dorsal and ventral extremes of the myotome and had a similar morphology to the slow-muscle fibers in the growth zones of wild-type larvae (Figure 4a–e; compare with Figure 1a–e; see also Figure S1 in Supplementary material). As in wild-type fish, there was a significant increase in the number of slow-muscle fibers in mutant animals between 24h and 96h. More than three fibers per somite were added in somites 16–18; in the entire trunk an average of 160 slow-muscle fibers were present at 96h (Figure 4f).

The spatial and temporal expression pattern of *myoD* in *smu* mutants was also similar to that seen in wild-type embryos (Figure 4g–j; compare with Figure 3a–d). In 24h *smu* mutants, *myoD* was expressed thoughout the entire myotome, but at 48h and 72h, expression was maintained only in the dorsal and ventral extremes (Figure 4g–i). In somites 1–7, slow-muscle fibers and *myoD*-expressing cells were displaced dorsally and ventrally. In the ventral-most hypaxial regions of *smu* mutants, as in wild-type fish, *myoD* was expressed in a band posterior to the pectoral fin bud at 48h; this band of *myoD* expression enlarged and extended anteriorly to become ventral to the fin bud at 72h (Figure 4j; data not shown). As in wild-type, this anterior extension of *myoD* expression corresponded with a similar anterior extension of both slow- and fast-muscle fibers (Figure 4k; see also Figure S1 in Supplementary material). Similarly, in the dorsal-most epaxial region, *myoD* expression as well as slow- and fast-muscle fibers extended dorsally (data not shown; Figure 4k, arrowheads; Figure S1 in Supplementary material). The morphology, position, and generally similar time course of development suggest that the slow-muscle fibers developing after 24h in $smu^{-/-}$ em-

Figure 5



Summary of embryonic and larval muscle development. **(a)** Drawings of a 23h embryo and a 72h larva (embryonic slow-muscle fibers, red; slow-muscle fibers added during stratified hyperplasia, green). Slow-muscle fibers are added in the dorsal and ventral extremes of the myotome after 24h. **(b)** Schematic flow chart of muscle development. Early patterning events establish two populations of muscle precursors, i.e., adaxial and lateral presomitic cells. Adaxial cells give rise to embryonic slow-muscle fibers, while lateral presomitic cells give rise to embryonic fast-muscle fibers (and sclerotome cells, not shown; [5]). We propose that the lateral presomitic cells also give rise to a population of myogenic precursor cells. Some of these cells later differentiate into slow-muscle fibers. Hedgehog signaling is required for the specification of embryonic slow-muscle precursors (red arrow); however, it is not required for the specification of slow-muscle fibers during stratified hyperplastic growth (green arrow).

bryos are the same as those added in wild-type embryos. We conclude that during the period of stratified hyperplasia, slow-muscle specification is not critically dependent on Smu-mediated Hh signaling. However, although wild-type *smu* function is not required for the establishment of slow-muscle cell identity, it is required for the development of the correct number of newly added slow-muscle fibers and for the normal hypertrophy of these fibers that occurs before 96h. The quantitative and qualitative deficits present in *smu* mutants may be an indirect effect of the loss of other cell types dependent on Hh signaling—Hh signaling is required for the development of not only embryonic slow-muscle fibers, but also the horizontal myoseptum, motor neurons, the dorsal aorta and circulation, and a variety of other tissues in the trunk [24–26].

If slow muscle fate during stratified growth does not depend on Hh signaling, other deficiencies in Hh signaling should not affect it. Mutations in either *sonic you* (*syu*) or *you too* (*yot*), which encode the Hh pathway components Sonic Hh and Gli2, respectively [27, 28], lead to deficiencies in adaxial-derived embryonic slow muscle [5, 29]. However, as in $smu^{-/-}$ individuals, new slow fibers were added in the dorsal and ventral extremes of the myotome by 72h in larvae mutant for either gene (Figure 4l,m). Moreover, wild-type embryos treated with cyclopamine (a plant-derived alkaloid that inhibits Hh signaling; [30]) had no slow-muscle fibers at 23h; however, slow-muscle fibers developed after 24h in the dorsal and ventral extremes of the myotome and increased in number over time (Figure 4n; data not shown). We combined cyclopamine treatment with mutations in *smu* to maximally reduce Hh signaling. There were no additive effects of this combination; cyclopamine-treated *smu* mutant embryos and their wild-type siblings were indistinguishable from each other and from untreated *smu* mutants (data not shown). These results suggest that Hh signaling is not required for the induction or maintenance of slow-muscle fibers added during stratified hyperplasia.

Our results demonstrate that the mechanisms regulating the development of slow muscle in the early embryo are distinct from the mechanisms regulating the development of slow muscle during stratified hyperplastic growth. In zebrafish, after adaxial cells are induced and embryonic slow-muscle fibers have migrated, new slow-muscle fibers are added in new locations within the myotome by a process that is governed by different molecular mechanisms (Figure 5a). We propose that these new slow fibers derive from cells that remain undifferentiated during the segmentation period (Figure 5b). After the end of the segmentation period and through the process of stratified hyperplasia, some of these cells, in the dorsal and ventral extremes of the myotome, may proliferate in response to growth signals from neighboring tissues and ultimately generate new muscle fibers. We speculate that those cells directly adjacent to the surface ectoderm will be specified as slow-muscle fibers, while those farther away will develop into fast-muscle fibers.

Other vertebrates may also have a distinction between the mechanism(s) that generates muscle fibers in the embryo and the mechanism(s) that generates more of those muscle fibers during growth. The earliest muscle fibers in quail are mononuclear, slow myosin-expressing fibers on the surface of the myotome [31, 32]. Growth then

occurs as new fibers are added at the dorsal and ventral extremes of the myotome [14]; this growth is regulated by signals from the overlying surface ectoderm [33]. Myogenesis in mouse can also be separated into distinct waves, and as in zebrafish these waves have distinct genetic requirements [34–36]. Thus, control of cell fate by different mechanisms during different periods of development may be a common feature of muscle development.

During growth, some cell types may develop from progenitor cells that are in an environment far removed from the original source of signaling molecules that regulated the development of cell identity in the early embryo. If proper patterning of the tissue is to occur during growth, either the original signaling molecule must be expressed in this new environment or the progenitors must respond to novel signals in their new environment. The expression of a signaling molecule such as Hedgehog in a new environment may disrupt the patterning of many other cell types that are differentiating in this environment. Therefore, the evolution of two independent mechanisms for cell fate patterning may have been a necessary innovation for growth to occur.

Materials and methods

Animals

Wild-type animals were obtained from the Oregon AB line and maintained in the Wesleyan University zebrafish colony. The mutant alleles used were *smu*^{b641}, *syu*^{tbx392}, and *yot*^{ty119}. Embryos were staged by counting somite numbers and converting to hours (h) postfertilization at 28.5°C [37]; after 24h, animals were kept in 28.5°C embryo medium until the indicated stage. In order to reduce pigmentation, embryos were kept in 0.003% 1-phenyl-2-thiourea (PTU) starting at the 23h stage according to published procedures [38]. There were no differences in muscle growth between PTU-treated and untreated embryos.

Antibodies

S58 is an IgA monoclonal antibody specific for slow isotypes of myosin heavy chain in chicken [39] and slow-muscle fibers in zebrafish [6]. F59 is an IgG1 monoclonal antibody raised against chicken myosin [39] that labels slow muscle strongly and fast muscle faintly in zebrafish [6]. Tissue culture supernatants of S58 and F59 were generously provided by Frank Stockdale at Stanford University and used at a dilution of 1:10. The IgG1 zn5 monoclonal antibody recognizes the antigen DM-GRASP and labels secondary motor neurons as well as slow-muscle cell membranes in zebrafish [15]; supernatant was obtained from the University of Oregon monoclonal antibody facility and used at a dilution of 1:5. The IgG1 monoclonal antibody zm4 is specific for fast-muscle fibers in zebrafish; supernatant was generously provided by Monte Westerfield at the University of Oregon. N1.551 is an IgM monoclonal antibody that labels neonatal fast-muscle IIa in mouse [40] and labels slow-muscle fibers strongly and fast muscle fibers very faintly in zebrafish (data not shown). 9D10 is an IgM monoclonal antibody that recognizes bovine titin [41] and labels slow- and fast-muscle membranes in zebrafish larvae (Figure S2 in Supplementary material). MF20 is an IgG2b monoclonal antibody that labels slow- and fast-muscle fibers in all species that have been examined. The IgG1 monoclonal antibody 12/101 specifically labels fast muscle fibers in zebrafish [6]. G3G4 is an IgG monoclonal antibody that recognizes BrdU. Supernatants of N1.551, 9D10, MF20, 12/101, and G3G4 were obtained from the Developmental Studies Hybridoma Bank and used at 5 μg/μl. Secondary antibodies FITC-conjugated goat anti-mouse IgA and IgM were obtained from Sigma and used at a dilution of 1:100 and 1:200 respectively; and Texas Red-conjugated goat anti-mouse IgG1 and Cy5-conjugated goat anti-mouse IgM were obtained from the Jackson Immuno Research Laboratories and both used at a dilution of 1:200.

Immunocytochemistry and in situ hybridization

Antibody labeling with S58 was carried out as previously described [25] with minor modifications. In brief, Carnoys-fixed animals were rehydrated in an ethanol series and then treated with 10 μg/ml proteinase K to facilitate antibody penetration. Treatment times varied according to age: 25h–26h were treated for 10 min, 27h–28h for 20 min, 30h–48h for 30 min, and 72h–120h for 60 min. The proteinase K was washed out with PBS-Tween 3 × 5′. Double antibody labeling with S58 and zn5 was performed on sections of Carnoys-fixed embryos according to protocols previously described [6, 25]. Antibody labeling with F59, N1.551, 9D10, MF20, zm4, and 12/101 were also performed on sections of Carnoys-fixed embryos.

In situ hybridization was performed using published procedures [42] with modifications made to the duration of proteinase K treatment, according to age: 24h were treated for 10 min, 48h for 40 min; 72h for 75 min, 96h for 90 min, and 120h for 105 min.

Birthdating of muscle cells with 5-Bromo-2′-deoxyuridine

Embryos were incubated in embryo medium with 10 mM BrdU for the appropriate time, preserved in Carnoys fixative, and sectioned on a cryostat. Section staining was carried out as previously described [6] with minor modifications. In brief, after sections were rehydrated to PBS-Tween, they were washed in 2 N HCl for 20 min. After blocking in 5% NGS, sections were treated with the primary monoclonal antibodies S58 (slow muscle), 9D10 (muscle membranes), and G3G4 (anti-BrdU). Sections were then treated with appropriate secondary antibodies. See Figure S2 in Supplemental material for details on the quantification of BrdU-positive nuclei.

Cyclopamine treatment

Beginning at 5.5h, wild-type and *smu* mutant embryos were treated with 50 μM cyclopamine dissolved in embryo medium and 0.5% ethanol. Controls consisted of corresponding incubations in embryo medium alone or 0.5% ethanol in embryo medium. There were no differences between controls. Cyclopamine was very generously provided by William Gaffield (Western Regional Research Center, Albany, California, USA) as well as purchased from Toronto Research Chemicals.

Quantification of slow-muscle fiber numbers

Fibers were counted on whole-mount wild-type and *smu*$^{-/-}$ fish stained with S58. Wild-type fibers were counted only on one side of somites 16, 17, and 18. To reduce potential bias in counting, we conducted wild-type counts on graphical representations of fibers. Confocal images were acquired, and fluorescence intensity plots were generated (Figure S3 in Supplemental material). These plots were coded for age and counted blindly. *smu*$^{-/-}$ fibers were counted by eye with a Zeiss Axioskop under 20× magnification. Any slow-muscle fiber obviously within the somite of the embryo/larva along the entire trunk was counted. This method excluded all head muscles, fin muscles, and the more anterior, ventral band of S58 expression seen in Figure 3h. Fiber numbers per time point were averaged, and standard errors were generated based on sample size per time point. There were approximately 15 mutants counted per time point, which were derived from two independent samples counted by two different people.

Imaging

Whole-mount S58 labeling was captured using a Zeiss confocal microscope at 25× magnification. Images of in situ hybridization and sections were captured with the Zeiss Axiocam and Axiovision software on a Zeiss Axioplan compound microscope. Nomarski (DIC) optics was used on all bright-light images. Fluorescent overlays were done with Adobe Photoshop 5.5.

Supplementary material

Three supplementary figures are available with the electronic version of this article at http://images.cellpress.com/supmat/supmatin.htm.

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