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Developmental Biology 275 (2004) 143–157

DEVELOPMENTAL BIOLOGY

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Hedgehog signaling is required for commitment but not initial induction of slow muscle precursors

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Received for publication 5 April 2004, revised 22 July 2004, accepted 26 July 2004
Available online 1 September 2004

Abstract

In zebrafish, skeletal muscle precursors can adopt at least three distinct fates: fast, non-pioneer slow, or pioneer slow muscle fibers. Slow muscle fibers develop from adaxial cells and depend on Hedgehog signaling. We analyzed when precursors become committed to their fates and the step(s) along their differentiation pathway affected by Hedgehog. Unexpectedly, we find that embryos deficient in Hedgehog signaling still contain postmitotic adaxial cells that differentiate into fast muscle fibers instead of slow. We show that by the onset of gastrulation, slow and fast muscle precursors are already spatially segregated but uncommitted to their fates until much later, in the segmental plate when slow precursors become independent of Hedgehog. In contrast, pioneer and non-pioneer slow muscle precursors share a common lineage from the onset of gastrulation. Our results demonstrate that slow muscle precursors form independently of Hedgehog signaling and further provide direct evidence for a multipotent muscle precursor population whose commitment to the slow fate depends on Hedgehog at a late stage of development when postmitotic adaxial cells differentiate into slow muscle fibers.

Keywords: Adaxial cells; Commitment; Fate map; Hedgehog; Slow muscle; Fast muscle; Muscle pioneer; Zebrafish; Smoothened; Cyclopamine

Introduction

Vertebrate skeletal muscles contain two major fiber types, slow and fast, that have distinct physiological, biochemical, morphological, and developmental properties. In zebrafish, several subtypes of slow and fast muscle fibers differentiate before the end of the segmentation period. Regulation of these different muscle cell fates is only partially understood (Stickney et al., 2000).

By the onset of gastrulation, the cells that ultimately give rise to skeletal muscle occupy the marginal zone of the zebrafish embryo (Kimmel et al., 1990). These cells then undergo involution and convergence–extension movements that position them on either side of the nascent notochord in the segmental plate. During gastrulation, they begin myogenesis as indicated by activation of the myogenic factors, *myod* and *myf5* (Coutelle et al., 2001; Weinberg et al., 1996). Before they are incorporated into a somite, muscle precursors adjacent to the notochord adopt a pseudo-epithelial morphology, forming a monolayer of adaxial cells (Devoto et al., 1996; Thisse et al., 1993); cells lateral to the adaxial cells retain their loose mesenchymal morphology. After incorporation into a somite, most of the adaxial cells leave the pseudo-epithelium and migrate to the lateral surface of the somite where they differentiate into the non-pioneer slow muscle fibers. A subset of adaxial cells, the pioneer slow muscle fibers, differentiates next to the notochord. Lateral, non-adaxial muscle precursors in the segmental plate differentiate into fast muscle fibers (Devoto et al., 1996).

By the end of the segmentation period, when embryonic muscle fibers are terminally differentiated, pioneer and non-pioneer slow fibers as well as fast muscle fibers can be identified by their distinct positions, morphologies, and gene expression patterns (Stickney et al., 2000). Each

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0012-1606/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2004.07.030

somite contains about 20 mononucleated superficial non-pioneer slow muscle fibers and two to six pioneer slow fibers that span the mediolateral width of the somite in the region where the horizontal myoseptum forms. Pioneer and non-pioneer slow fibers express slow-specific markers and some fast markers (Devoto et al., 1996). Multinucleated fast muscle fibers are more numerous (about 80 fibers per somite) and constitute the majority of cells within the somite. They are located throughout the somite except at its surface and express fast-specific markers (Devoto et al., 1996; Roy et al., 2001). A subset of the fast fibers near the horizontal myoseptum and the pioneer slow muscle fibers express Engrailed proteins (Ekker et al., 1992; Hatta et al., 1991; Roy et al., 2001; Wolff et al., 2003).

The time at which cells become committed to a specific fiber type identity is still controversial and difficult to determine because muscle precursors can become committed early in development, maintaining this commitment through many cell divisions (Stockdale et al., 2002). In chick, slow and fast myoblasts enter the limb bud at different developmental times and constitute different pools of precursors (Van Swearingen and Lance-Jones, 1995), suggesting that fiber type identity has already been specified. Similarly, in quail-chick chimeras, the patterning of slow and fast muscles in the limb bud is determined by the transplanted myoblast population and not the lateral plate from which the limb bud stroma develops (Nikovits et al., 2001), suggesting that the local environment cannot overcome an earlier commitment to a fiber type identity. In contrast, retrovirus-based lineage analysis shows that in chicken (Kardon et al., 2002) and mouse (Hughes and Blau, 1992) limbs, slow and fast muscle fibers derive from common precursors whose fates are determined after they migrate into the limb. Such muscle precursors are uncommitted to a fiber type identity; single myoblasts can give rise to both slow and fast fibers when transplanted into muscles (Hughes and Blau, 1992), favoring the hypothesis that local cues determine muscle fate. Moreover, in vitro studies of fetal chick muscle development suggest that the *slow myosin heavy chain 2* gene is regulated both by early specification of myoblasts and by later influences of innervation (DiMario and Stockdale, 1997). Even after terminal differentiation of muscle fibers, specific stimulation of slow or fast motor nerves induces activity-dependent remodeling in the adult (see Lin et al., 2002, for references).

Hedgehog signaling is implicated in multiple developmental processes including cell survival, proliferation, patterning, and differentiation (McMahon et al., 2003), although its precise role in muscle development and fiber type specification is unclear. Hedgehog enhances chick slow muscle viability in vitro (Cann et al., 1999) and acts as a survival factor for mouse limb bud muscle (Kruger et al., 2001). Hedgehog induces proliferation of committed muscle cells in the chick limb bud (Duprez et al., 1998) and maintains chick myoblasts in a proliferative state while delaying terminal differentiation (Bren-Mattison and Olwin, 2002). In vitro experiments show that Hedgehog can increase the number of zebrafish slow muscle cells (Norris et al., 2000). Hedgehog has also been linked to regulation of the cell cycle in *Drosophila* (Duman-Scheel et al., 2002). Hedgehog is essential for the differentiation of subsets of muscle. In the zebrafish myotome, overexpression of Hedgehog is sufficient to induce probably all muscle precursors to form slow muscle (Blagden et al., 1997; Currie and Ingham, 1996; Du et al., 1997). In *Sonic hedgehog* (*Shh*; Chiang et al., 1996; Kruger et al., 2001) or *Smoothened* (Zhang et al., 2001) knockout mice, epaxial muscle is absent. Similarly, in *sonic hedgehog* (*syu*, *sonic-you*; Coutelle et al., 2001; Lewis et al., 1999), *gli2* (*yot*, *you-too*; Lewis et al., 1999), and *smoothened* (*smu*, *slow-muscle-omitted*; Barresi et al., 2000) zebrafish mutants, slow muscle fibers are absent or reduced in number. Therefore, different subtypes of muscle require distinct doses and timing of Hedgehog in zebrafish (Wolff et al., 2003). In mice, epaxial *Myf5* expression is abolished in *Shh* and *Smoothened* knockout mice, although *Myf5* continues to be expressed in hypaxial muscle (Chiang et al., 1996; Kruger et al., 2001; Zhang et al., 2001). It is not resolved whether epaxial *Myf5* expression is a direct or indirect result of Hedgehog signaling (Gustafsson et al., 2002; Teboul et al., 2003). In zebrafish, early activation of *myod* in *smu* (*smoothened*) mutants and *myf5* in *syu* (*shh*) mutants occurs in cells around the tail bud that in wild-type embryos presumably form slow muscle (Barresi et al., 2000; Coutelle et al., 2001; Lewis et al., 1999).

We analyzed muscle development in wild-type and mutant zebrafish embryos and unexpectedly find that adaxial cells, the slow muscle precursors, form in the absence of zygotic Hedgehog signaling. To determine the time at which muscle precursors become committed to a fiber type identity and to understand the differentiation steps regulated by Hedgehog, we identified the origins of pioneer and non-pioneer slow muscle and fast muscle populations, analyzed their lineage relationships, and used transplantation to test their commitment. We find that although slow and fast muscle precursors occupy distinct domains in the gastrula, they do not become committed until after they enter the segmental plate and are exposed to Hedgehog. Further, we find that in the absence of Hedgehog signaling, adaxial cells form but later adopt an alternate fate and differentiate into fast muscle. We suggest that Hedgehog function is required to commit muscle precursors to a slow muscle fate but not to induce formation of the adaxial cells.

Materials and methods

Fish strains

Wild-type AB, *smoothened* (*smu*) alleles, smu^{b577} (Barresi et al., 2000) and smu^{b641} (Varga et al., 2001), and

yot-too (*yot*^{ty119}; van Eeden et al., 1996) embryos were obtained from zebrafish (*Danio rerio*) lines maintained with standard procedures (Westerfield, 2000). Animal use protocols are approved by University of Oregon IACUC, A-3009-01. We obtained similar results with both *smu* (*smoothened*) alleles. Embryos were staged by hours postfertilization (h) and by standard staging criteria (Kimmel et al., 1995).

Cyclopamine treatment

We exposed wild-type embryos in their chorions to 100 μM cyclopamine (Toronto Research Chemicals, C988400; dissolved in embryo medium and 0.5% ethanol) at various times between sphere stage and Prim-5 (24 h). Cyclopamine readily crosses membranes and binds with high affinity to Smoothened, inhibiting its signaling activity (Chen et al., 2002). Transcription of the *ptc1* gene is presumed to be a direct response to elevated Hedgehog signaling (Concordet et al., 1996). We found that *ptc1* mRNA levels declined within an hour of cyclopamine treatment (data not shown), consistent with a very rapid down-regulation of Hedgehog signaling by cyclopamine.

Adaxial cell observations

Embryos were generated by intercrossing *smu*$^{b577/+}$, *smu*$^{b641/+}$, or *yot*$^{ty119/+}$ fish. Cyclopamine-treated embryos were also used. At 5- and/or 15-somite stage, live embryos were mounted with the anterior segmental plate in dorsal view and photographed individually using Nomarski optics (Fig. 1). At Prim-15 (30 h), mutant embryos were identified by their somite phenotype.

Antibodies, immunolabeling, and fiber-type identification

F59 (Crow and Stockdale, 1986), an IgG1 mouse monoclonal antibody, differentially recognizes slow and fast muscle fibers in zebrafish (Devoto et al., 1996). S58 (Crow and Stockdale, 1986), an IgA mouse monoclonal antibody, specifically recognizes slow muscle fibers in zebrafish (Devoto et al., 1996). We performed immunohistochemistry as previously described (Devoto et al., 1996) and examined slides by epifluorescence microscopy. Images were acquired using a Zeiss Axiocam digital camera mounted on a Zeiss Axioplan 2 microscope (40× objective, 0.75 N.A.) and processed with Photoshop (Adobe). The identities of progeny derived from injected and transplanted cells were assessed by morphology, position, and F59 or S58 labeling in whole-mount or transverse sectioned embryos. Both slow and pioneer muscle fibers are superficial and parallel to the anteroposterior axis in a whole-mount lateral view. In sections, slow fibers are located superficially and show a flattened morphology. Muscle pioneers are located at the dorsoventral middle of the somite and, unlike the non-pioneer slow fibers, span the mediolateral width of the somite. For both cell types, the F59 and S58 labeling is bright and fills the fiber cytoplasm. In contrast, fast fibers are deep in the somite and most often oriented obliquely to the anteroposterior axis in lateral views of whole-mount embryos. In sections, fast fibers lie medial to the slow fibers and have a round cellular morphology.

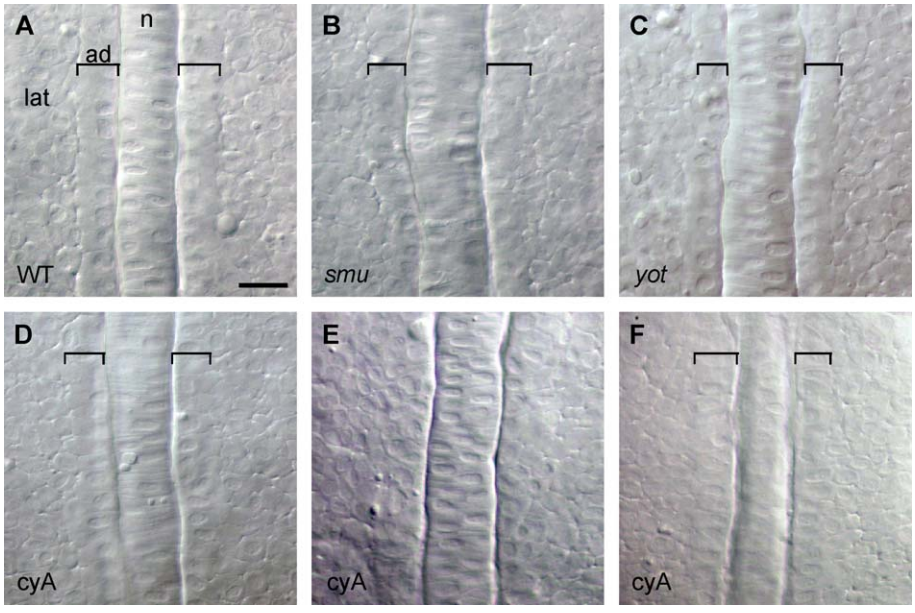

Fig. 1. Adaxial cells form independently of Hedgehog signaling. Adaxial cell morphology at 5- and 15-somite stages in wild-type, mutant, and cyclopamine-treated embryos. (A–F) Nomarski images of dorsal views of 5-somite (A–E) and 15-somite (F) stage embryos at the level of the anterior segmental plate, with anterior to the top. The brackets indicate the width of the adaxial cell pseudo-epithelium. In wild-type (WT, A), *smu* (*smoothened*) mutant (B), *yot* (*gli2*) mutant (C), and cyclopamine-treated embryos (D), adaxial cells are visible. Adaxial cells are not visible at 5-somite stage (E) but are visible at 15-somite stage (F) in this cyclopamine-treated embryo. Abbreviations: ad, adaxial cells; cyA, cyclopamine; lat, lateral cells; n, notochord. Scale bar, 20 μm.

Compared to slow fibers, fast fibers show lower levels of F59 labeling that is organized in a ring around the fiber cytoplasm. The S58 antibody does not label fast fibers.

Intracellular injections of tracer dye

We injected single cells with 3–5% rhodamine dextran, 3000 MW (Molecular Probes, D3308), as previously described (Varga et al., 1999). Embryos were fixed at 24–30 h in 4% paraformaldehyde, cryosectioned, immunolabeled, and analyzed individually as described above. Homozygous mutant embryos were identified at 24–30 h, based on their somite phenotype.

For shield stage injections, the positions of the injected cells were recorded as follows, using the shield as a landmark for the dorsal side. In lateral view, the number of cell diameters between the injected cell and the margin and the injected cell and the lateral edge of the shield were measured. In animal pole view, the depth of the injected cell from the surface and its radial distance from the center of the shield were measured (Figs. 2A and C). Typically, injected cells were 1–3 cell diameters from the margin and one to two cell layers deeper than the enveloping layer (EVL).

For 3-somite stage injections, the identities of the injected cells were determined by position and morphology, using Nomarski optics following injection (Fig. 6A). Typically, one to three cells were injected per embryo.

Generation of the shield stage fate map

Our injections labeled 89 pioneer and slow fibers and 83 fast fibers. At Prim-5 (24 h), there are on average 22 slow muscle fibers per somite (Barresi et al., 2001), including two to six muscle pioneers (Felsenfeld et al., 1991; Hatta et al., 1991). One side of the trunk (18 somites) contains around 400 slow fibers and 1500 fast fibers. Thus, the fate-mapped fibers represent 22% of the slow population and 5% of the fast population. The average pioneer/slow fiber ratio is 1:5.5. In our experiments where no cell death could be detected by residual-labeled cellular debris, 38 precursors gave rise to 81 slow fibers (19 pioneers and 62 non-pioneer slow fibers). This corresponds to a pioneer/slow fiber ratio of 1:4.2, close to the expected average ratio of 1:5.5. Therefore, our sample appears representative of the muscle population.

Only precursors that exclusively gave rise to muscle fibers are reported in the fate map. Cells are positioned in the fate map according to their angular distance from the center of the shield. This distance is independent of the size and curvature of the embryo and thus provides an accurate and reproducible measurement of cell position. Occasionally, two cells instead of one were injected; these were

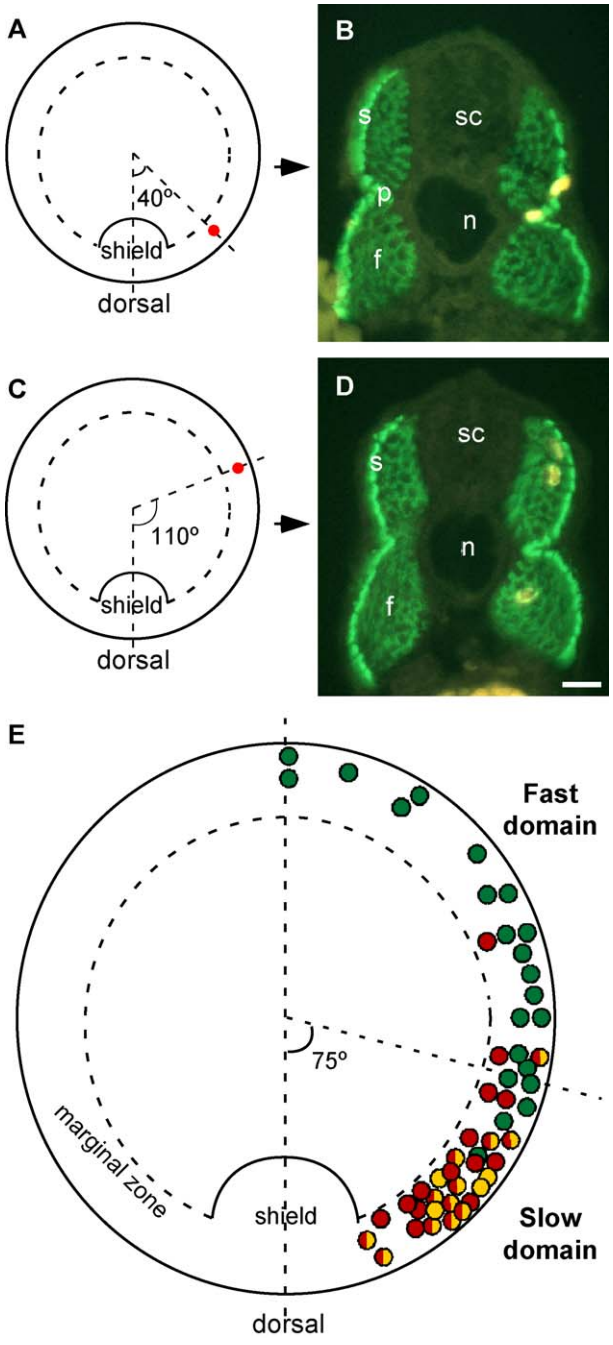

Fig. 2. Slow and fast muscle precursors are spatially segregated in the gastrula. Shield stage slow and fast muscle fate map. Slow and pioneer precursors share a common lineage and occupy an overlapping domain in the marginal zone, whereas slow and fast precursors have distinct origins and occupy different domains in the marginal zone. (A and C) Animal pole view of shield stage embryo diagrams at the level of the marginal zone. The positions of the injected cells are indicated in red with their angular distances in degrees measured from the center of the shield (future anteroposterior axis). (B and D) Cross-sections of Prim-15 (30 h) stage embryos with dorsal to the top. Immunolabeling with F59 in green, lineage tracer dye revealed in red, double-labeled cells appear yellow. One slow and one pioneer fiber are shown in B and four fast muscle fibers are shown in D. (E) Summary fate map. The locations of precursors of slow fibers are shown in red, pioneers in yellow, and fast fibers in green. The locations of precursors that give rise to slow/pioneer progeny are indicated by red/yellow. Abbreviations: f, fast muscle fiber; n, notochord; p, pioneer slow muscle fiber; s, non-pioneer slow muscle fiber; sc, spinal cord. Scale bar, 20 μm.

included in the fate map only if they gave rise to one type of progeny. In agreement with previous fate maps, cells giving rise to other deep tissues, such as hypochord, endoderm, and head mesenchyme, were also labeled, although at a much lower frequency (approximately 10% of injected embryos; data not shown).

Transplantations

We transplanted cells essentially as described by Ho and Kane (1990). After transplantations, host embryos were incubated and analyzed individually after fixation at 24–30 h in 4% paraformaldehyde, cryosectioned and immunolabeled as described above.

For shield stage transplants, micropipettes (VWR, 53508-400; Flaming Brown puller) were broken at an angle to an outer diameter of approximately 40 μm and then tooled into a sharp 'spear-tip' with a microforge. The positions of the transplants were recorded as described for the shield stage intracellular injections (Figs. 3A and C).

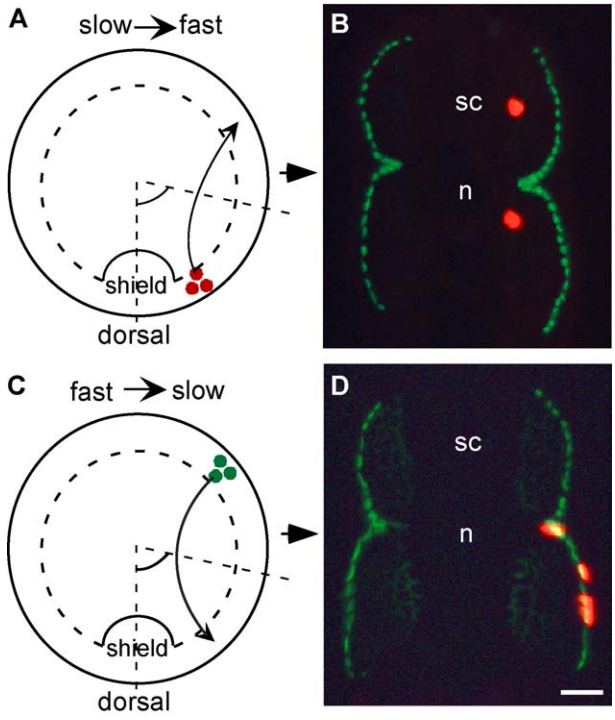

Fig. 3. Slow and fast muscle precursors are uncommitted at shield stage. Slow and fast muscle precursor transplantations at shield stage. (A and C) Animal pole view of shield stage embryo diagrams at the level of the marginal zone. The initial positions of the transplanted cells are indicated in red for cells transplanted from the slow domain (A) or green for cells transplanted from the fast domain (C). The arrows indicate the locations into which the cells are transplanted. The straight broken lines indicate the boundary between the slow and fast domains (see Fig. 2). (B and D) Cross-sections of Prim-15 (30 h) stage embryos with dorsal to the top. Immunolabeling with S58 (B) and F59 (D) in green, lineage tracer dye revealed in red, double-labeled cells appear yellow. Two fast fibers are seen in B and three slow muscle fibers are seen in D. Abbreviations: n, notochord; sc, spinal cord. Scale bar, 20 μm.

For 3-somite stage transplants, micropipettes (Garner Glass Company, PO B390422; Flaming Brown puller) were broken flat to an outer diameter of approximately 15 μm for transplanting adaxial cells and approximately 20 μm for transplanting lateral cells. The positions of the transplants were recorded as described for the 3-somite stage intracellular injections (Figs. 4A and E).

Results

Adaxial cells are morphologically identifiable in embryos with compromised Hedgehog signaling

Hedgehog signaling is required for slow muscle development; slow muscle cells ultimately fail to differentiate in embryos with compromised Hedgehog signaling (Barresi et al., 2000, 2001; Du and Dienhart, 2001; Lewis et al., 1999). Slow muscles derive from adaxial cells (Devoto et al., 1996). To learn whether adaxial cell formation also requires Hedgehog signaling, we assessed the formation of adaxial cells in live embryos at 5- and 15-somite stages and subsequent formation of fast and slow muscle cells in three experimental conditions where Hedgehog signaling is compromised: in *smu* (*smoothened*; Varga et al., 2001) and *yot* (*gli2*; Karlstrom et al., 1999) mutant embryos and in wild-type embryos treated with cyclopamine. Cyclopamine is a plant-derived alkaloid that binds to Smoothened and blocks Hedgehog signaling (Chen et al., 2002; Frank-Kamenetsky et al., 2002). Consequently, at high doses, cyclopamine completely inhibits slow muscle development (Barresi et al., 2001). We find a concentration-dependent reduction in the number of slow muscle fibers following cyclopamine treatment; 100 μM cyclopamine is sufficient to eliminate all slow muscle fibers (X. Feng and S. H. Devoto, unpublished).

Surprisingly, *smu* (*smoothened*; $n = 27$) and *yot* (*gli2*; $n = 9$) mutant embryos form morphologically identifiable adaxial cells (Figs. 1B and C), similar to wild-type embryos (Fig. 1A). As in wild-type embryos, adaxial cells form a highly regular one-cell-thick sheet adjacent to the notochord. Borders between adjacent adaxial cells are barely distinguishable whereas the border between adaxial and lateral cells is distinctively sharp. In dorsal view, adaxial cells and their nuclei in the same dorsoventral row along the anteroposterior axis are aligned in the same focal plane. Each cell is rectangular with its longer axis perpendicular to the midline. Adaxial cell nuclei are eccentrically positioned closer to the midline and aligned along the anteroposterior axis. Adaxial cells in mutant embryos sometimes develop slightly more irregular morphologies than in wild types. Among nine wild-type embryos treated with cyclopamine from sphere or shield stage onward, four embryos formed normal looking adaxial cells by the 5-somite stage (Fig. 1D), and all of the other embryos with less well-formed adaxial cells at the 5-somite stage (Fig. 1E) developed

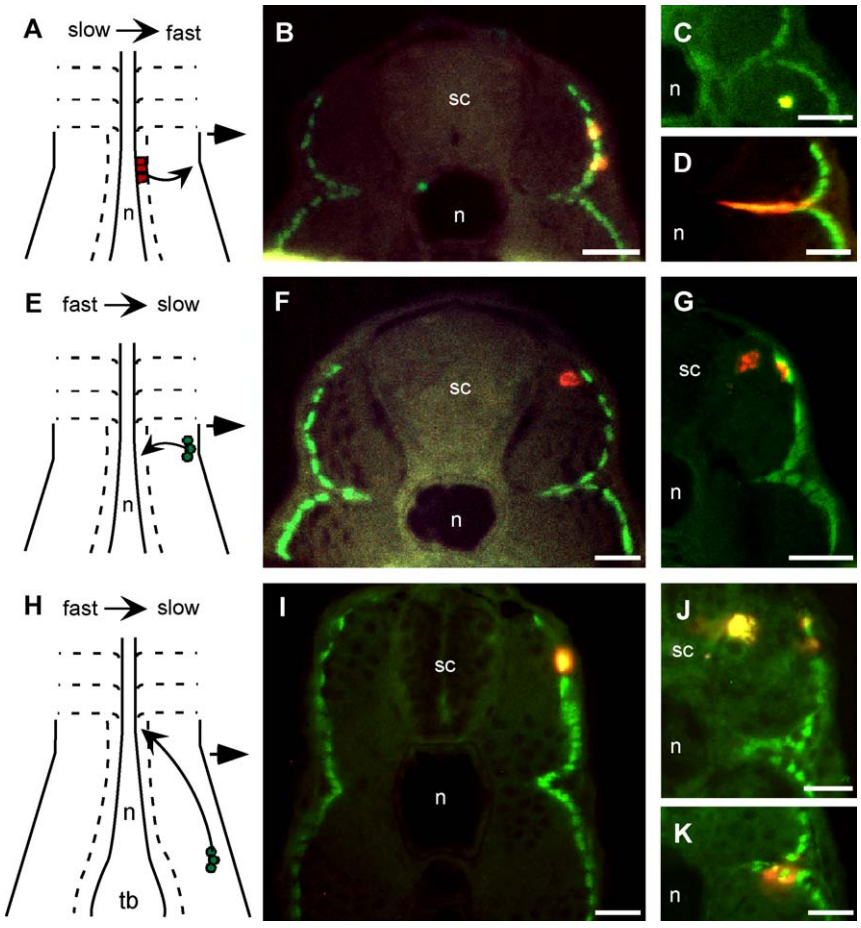

Fig. 4. Slow muscle precursors are committed in the anterior segmental plate; fast muscle precursors are committed in the anterior but not the posterior segmental plate. (A, E, and H) Dorsal views of 3-somite stage embryo diagrams at the level of the segmental plate. The initial locations of transplanted adaxial cells are indicated in red and of lateral cells in green. The arrows indicate the locations into which the cells are transplanted. (B–D, F, G, and I–K) Transverse sections of Prim-15 (30 h) stage embryos with dorsal to the top. Immunolabeling with S58 in green, lineage tracer dye revealed in red, double-labeled cells appear yellow. Slow fibers are shown in B and I, ectopic slow fibers in C and J, pioneers in D and K, one or two fast muscle fibers in F, and three fast muscle fibers with one very superficial in G. Abbreviations: n, notochord; sc, spinal cord; tb, tail bud. Scale bar, 20 μm.

normal looking adaxial cells later (Fig. 1F). Thus, adaxial cells also form in cyclopamine-treated embryos, although their development can be slightly delayed. To ensure that Hedgehog signaling was effectively compromised in these experiments, we also examined slow muscle differentiation. In *smu* (*smoothened*; $n = 8$) and *yot* (*gli2*; $n = 7$) mutant embryos, we found zero to two slow muscle fibers per embryo at Prim-5 (24 h) and in cyclopamine-treated embryos ($n = 9$), less than 10 slow muscle fibers formed in the anterior trunk, compared to approximately 800 slow muscle fibers in wild-type embryos (Barresi et al., 2001). Together, these results suggest that adaxial cells, the precursors of slow muscle, initially form independently of Hedgehog signaling.

Slow and fast muscle precursors are spatially segregated at the onset of gastrulation

To learn whether the segregation of slow and fast muscle precursors also occurs independently of Hedgehog signaling, we fate mapped the presumptive domain of somitic muscle. We previously showed that precursors of slow and pioneer muscle fibers, the adaxial cells, are spatially segregated in the segmental plate from precursors of the fast muscle fibers, the lateral presomitic cells (Devoto et al., 1996). To learn when these muscle precursor lineages diverge, we fate mapped the muscle domain at shield stage during early gastrulation when cells are first restricted to tissue-specific fates (Kimmel and Warga, 1986) and before activation of myogenic factors (Coutelle et al., 2001; Weinberg et al., 1996). We injected lineage tracer dye into individual cells at various dorsoventral locations around the marginal zone (Figs. 2A and C). We analyzed the positions and fates of labeled progeny at late segmentation stages (24–30 h), after muscle fibers have terminally differentiated. We assessed the identity of the labeled cells by morphology, position, and labeling with the F59 antibody (Devoto et al., 1996).

We find that even by early gastrulation stages, slow and fast muscle precursors are spatially segregated and occupy

distinct domains (Fig. 2). Muscle precursors located close to the shield always give rise to slow muscle fibers (n = 30/30 embryos), some of which are pioneer slow muscle cells (Figs. 2A and B). Muscle precursors located farther ventral around the margin give rise to fast muscle fibers (n = 22/22 embryos; Figs. 2C and D in green). Thus, two distinct muscle producing domains can be distinguished in the marginal zone of the shield stage embryo (Fig. 2E). In the "slow" domain, close to the shield, precursors give rise to muscle pioneer cells (n = 4/30 embryos; Fig. 2E in yellow), slow muscle cells (n = 14/30 embryos; Fig. 2E in red), or a mixture of pioneer and slow muscle cells (n = 12/30 embryos; Fig. 2E in yellow and red). Muscle pioneer precursors occupy the entire extent of the slow domain and are apparently evenly distributed among the slow muscle precursors.

These results show that by early gastrulation, before activation of myogenic factor expression (Coutelle et al., 2001; Weinberg et al., 1996), slow and fast precursors arise from different regions of the embryo and thus are exposed to different signaling environments. In contrast, non-pioneer and pioneer slow muscle precursors share a common lineage at shield stage and are mixed together spatially, although some slow precursors give rise exclusively to non-pioneer or pioneer slow muscle progeny.

Muscle precursors are not yet committed to slow or fast fates at shield stage

To learn whether these spatially segregated populations of muscle precursors are committed to give rise exclusively to slow or fast muscle fibers at shield stage, we transplanted cells from the slow domain into the fast domain and vice-versa (Figs. 3A and C). As controls, we transplanted cells homotopically back into their original domains. We assessed the final fates of the transplanted cells after terminal differentiation of muscle fibers at 24–30 h by morphology, position, and expression of fiber type-specific markers (Table 1).

We find that during gastrulation, muscle precursors change their fates when transplanted to a new location. Muscle precursors transplanted from the slow domain into the fast domain of the gastrula margin develop as fast muscle fibers (n = 134/135; one cell developed as a slow fiber), as illustrated in Figs. 3A and B. Similarly, when we transplant muscle precursors from the fast domain into the slow domain, a large proportion of them form slow or pioneer fibers (n = 89/133), as illustrated in Figs. 3C and D, although a small proportion of them forms fast fibers (see below). We obtain similar results regardless of whether we transplant groups of cells (2–20 cells) or individual cells, indicating no apparent community or cell autonomous effects on muscle cell fate determination.

In control experiments, when we transplant muscle precursors from the slow domain back into the slow domain, the majority of the transplanted cells adopt a slow fate (n = 51/65; Table 1), although a small proportion gives rise to fast fibers (n = 14/65; see below). In the reciprocal control experiments, muscle precursors transplanted from the fast domain back into the fast domain adopt a fast fate (n = 237/237; Table 1). Some cells transplanted into these regions at this stage also adopt non-muscle fates, including neurons, neural crest, head mesenchyme, and endodermal derivatives (approximately 78% of the embryos; data not shown). Endodermal and head mesodermal precursors, are thought to invaginate slightly earlier than muscle precursors and neurons and neural crest derive from the more superficial embryonic cell layer (Kimmel et al., 1990). Thus, slight differences in timing or location of our transplants could explain the appearance of these non-muscle cell types in the labeled clones. A small percentage (7%) of the cells we transplant die.

A minority of fast precursors transplanted into the slow domain as well as a minority of control slow precursors

Table 1
Muscle precursors are uncommitted at Shield stage and committed at 3-somite stage

Resulting fates	Transplant types				
	Slow into slow	Fast into slow	Fast into fast	Slow into fast	Heterochronic fast into slow
Shield stage					
Slow fibers	46 (71%)	84 (63%)	0	1 (1%)	
Pioneers	5 (8%)	5 (4%)	0	0	
Fast fibers	14 (21%)	44 (33%)	237 (100%)	134 (100%)	
Total	65 (100%)	133 (99%)	237 (100%)	135 (100%)	
No. of embryos	13	21	17	15	
3-somite stage					
Slow fibers	19 (79%)	0	0	20 (74%)	13 (39.5%)
Pioneers	5 (21%)	0	0	2 (7.5%)	3 (9.5%)
Fast fibers	0	17 (100%)	4 (100%)	5 (18.5%)	17 (51%)
Total	24 (100%)	17 (100%)	4 (100%)	27 (100%)	33 (100%)
No. of embryos	10	8	3	12	11

Terminally differentiated muscle fibers were sorted by fiber type (slow, pioneer, and fast fibers) after each type of transplantation and counted. Percentages are calculated relative to the total number of muscle fibers counted for a particular type of transplantation. See Figs. 2 and 3 for visual representation of the table data.

transplanted back into the slow domain develops as fast fibers (n = 44/133; n = 14/65, respectively). In contrast, all cells transplanted into the fast domain form fast cells. This apparent difference between transplants into the slow and fast domains is likely to arise because the slow domain is a significantly smaller target. Cells transplanted into the slow domain thus have a greater chance to leave this domain and move into the fast domain shortly after transplantation or during gastrulation.

These results demonstrate that although slow and fast muscle precursors occupy distinct locations at shield stage, they are not yet committed to their fates; when transplanted, they usually adopt the fate corresponding to their new environment. Our results are consistent with a previous study (Ho and Kimmel, 1993) that showed that cells are not committed to a hypoblast-derived fate until 2 h after involution.

Muscle precursors are committed to slow or fast fates before the somite forms

To learn when muscle precursors become committed to forming slow or fast muscle fibers, we transplanted groups of one to five adaxial cells into the lateral segmental plate domain of 3-somite stage embryos and vice-versa (Figs. 4A, E, and H). We transplanted cells back into their original domains as controls and analyzed the embryos as described above (Table 1).

We find that by the 3-somite stage, muscle precursor cells in the anterior segmental plate are committed to form either slow or fast muscle fibers. When we transplant adaxial cells into the lateral segmental plate, they differentiate into slow muscle fibers (n = 22/27; Figs. 4A and B). Only a small fraction of transplanted adaxial cells gives rise to fast fibers (n = 5/27), which suggests that few adaxial cells remain uncommitted at this stage. The slow muscle fibers derived from the transplanted adaxial cells are located either in their normal superficial position (Fig. 4B) or ectopically at other locations throughout the myotome (n = 4; Fig. 4C). In these ectopic positions, the slow fibers are surrounded by fast muscle fibers but can still be identified as slow, because they express the slow muscle-specific marker recognized by the S58 antibody. Some of the transplanted adaxial cells develop into muscle pioneer cells despite their transient detachment from the notochord (n = 2; Fig. 4D). Conversely, when we transplant lateral cells into the adaxial domain, they all form fast muscle fibers (n = 17/17; Figs. 4E and F). These transplanted fast fibers adopt a variety of positions throughout the somite, including superficial positions, slightly deeper than the superficial slow muscle layer (Fig. 4G). This result suggests that lateral cells in the anterior segmental plate are committed to the fast fate but does not rule out the alternate interpretation that the transplanted cells are insufficiently exposed to slow inducing signals. To distinguish between these possibilities, we transplanted developmentally younger lateral cells from the posterior part of the segmental plate into the adaxial position in the anterior segmental plate (Fig. 4H). In about half of the cases (Table 1), transplanted posterior lateral cells change fate and differentiate into slow muscle fibers (n = 16/33; Figs. 4H and I). Some of these slow fibers are located ectopically (n = 2; Fig. 4J) or even differentiate into muscle pioneer cells (n = 3; Fig. 4K). We conclude that the adaxial environment retains slow-inducing activity in the anterior segmental plate.

In control experiments, cells transplanted back into their original locations retain their fates in 100% of the embryos; adaxial cells differentiate into slow muscle fibers (n = 24/24) and lateral cells into fast muscle fibers (n = 4/4; Table 1). In contrast to the shield stage transplants, the cells transplanted at the 3-somite stage often die (66% of the embryos).

These results show that slow and fast muscle precursors become committed to their respective fates during a 5-h period between shield and early somite stages, when muscle precursors complete their gastrulation movements but before they integrate into a somite and terminally differentiate. When transplanted at the later stage, they develop according to their original identities, even though they may be in ectopic positions. Expression of the myogenic factors, *myod* and *myf5*, is activated during this period (Coutelle et al., 2001; Weinberg et al., 1996), and by the 3-somite stage, slow precursors start expressing myosins (Stickney et al., 2000).

Slow and fast muscle precursors have different cell division patterns

We compared the cell division patterns of slow and fast muscle precursors by analyzing the sizes of clones at late segmentation stages (24–30 h) resulting from cell injections at shield and 3-somite stages in which no cell death was detected.

We find that slow muscle precursors divide fewer times than fast muscle precursors (Table 2). When injected at shield stage, individual slow precursors (n = 13) give rise to two slow muscle fibers on average, whereas fast precursors (n = 8) give rise to four fast muscle fibers on average. The difference in the clone sizes of these two populations is significant (likelihood ratio test, df = 13, $P < 0.0001$). Unlike slow fibers, fast muscle fibers result from fusion of several fast muscle precursors (Roy et al., 2001). Thus, the

Table 2
Slow and fast precursors have different cell division patterns

	Slow precursors	Fast precursors
Shield stage	2.6 ± 0.11 (n = 13)	4.0 ± 0.00 (n = 8)
3-somite stage	1.1 ± 0.00 (n = 73)	1.1 ± 0.02 (n = 30)

Precursors were injected at shield or 3-somite stages and the number of labeled cells at 24–30 h was counted. Only clones where no cell death could be observed are taken into account. Numbers represent averages ± SEM, n is the number of injected precursors.

number of fast daughter fibers provides an underestimate of the number of fast muscle precursor cell divisions.

Slow and fast precursors in the anterior segmental plate are postmitotic by the 3-somite stage (Table 2). After injection at 3-somite stage, each labeled adaxial cell ($n = 73$) gives rise on average to one slow muscle fiber, in agreement with previous studies (Coutelle et al., 2001; D'Angelo et al., 2001; Devoto et al., 1996). Similarly, labeled lateral cells ($n = 30$) each give rise to one fast muscle fiber on average. Thus, the terminal cell division of both slow and fast muscle precursors occurs between shield and 3-somite stage. Similar to both adaxial and lateral cells in wild-type embryos, *smu* (*smoothened*) adaxial cells ($n = 10$) on average do not divide after 3-somite stage (data not shown), despite their alternate fate.

These results show that between shield and 3-somite stages, slow muscle precursors divide once and fast muscle precursors divide at least twice; both precursor types have had their terminal cell division by 3-somite stage and are committed to their respective fates. These results also show that postmitotic adaxial cells are produced in the absence of Hedgehog signaling.

Previous analyses of gene expression patterns suggest that the Hedgehog signaling pathway first becomes functional in the notochord and floorplate during the 5-h period between Shield and 3-somite stages (Concordet et al., 1996; Currie and Ingham, 1996; Ekker et al., 1995; Krauss et al., 1993). Slow precursors begin to respond to Hedgehog during this period by activating expression of the Hedgehog receptor complex protein Patched1 (Ptc1; Concordet et al., 1996; Lewis et al., 1999). Our results further demonstrate that muscle precursors become committed during this same period, thus demonstrating a spatial and temporal correlation between the activation of Hedgehog signaling and the onset of muscle precursor commitment to specific fiber types.

Slow muscle precursors become independent of Hedgehog signaling in the segmental plate before the somite forms

Our results show that slow muscle precursors in the anterior segmental plate no longer require Hedgehog. We used cyclopamine treatments to determine more precisely when slow muscle precursors are dependent on Hedgehog signaling. We treated embryos with 100 μM cyclopamine at various times and assessed the number of slow muscle fibers formed.

The developmentally more mature slow muscle precursors in anterior somites become resistant to cyclopamine (or Hedgehog independent) before the less mature muscle precursors in more posterior somites (Figs. 5A–C). On average at any given developmental stage, slow muscle precursors become independent of Hedgehog 3.5–6 h before incorporation into a somite. On average, muscle pioneers become independent of Hedgehog 2 h later than non-pioneer slow precursors; the average distance between the last somite with muscle pioneer slow fibers and the last somite that has formed any slow muscle fibers is 3.5 somites (±1.9 SEM). For example (Fig. 5D), when we apply cyclopamine at the 10-somite stage, the earliest decrease in slow muscle fiber formation is visible in somite 16 (that forms 3 h later); no muscle pioneers develop in somite 18 (that forms 4 h after drug application) and no slow muscle fibers develop in somite 22 (that forms 6 h after drug application). These data also show that muscle precursors in a single somite become Hedgehog-independent asynchronously over a 3.5-h period. For example, none of the slow muscle precursors in somite 17 is resistant to cyclopamine

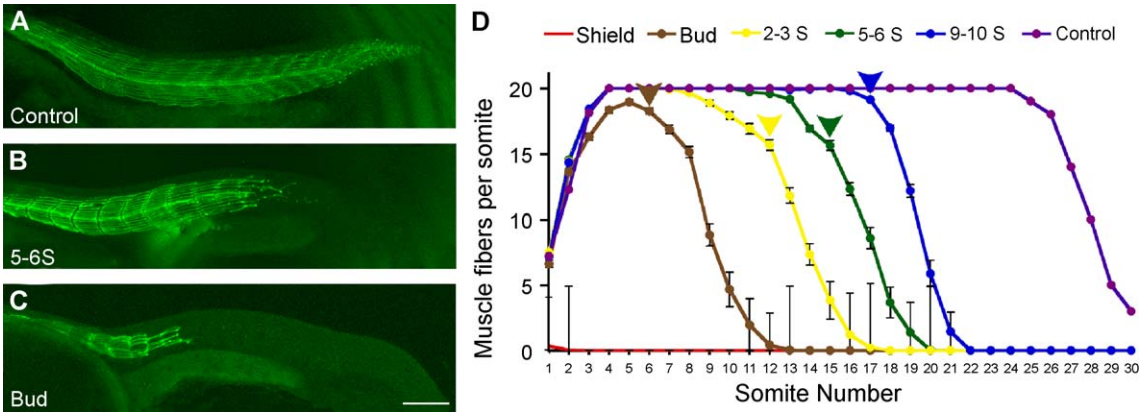

Fig. 5. Slow muscle precursors become independent of Hedgehog signaling in the posterior segmental plate. (A–C) Side views of Prim-5 (24 h) whole-mount embryos labeled with S58 in green, anterior to the left, and dorsal to the top. Representative embryos treated with ethanol (control, A) or cyclopamine (B and C) beginning either at 5–6 somite stage (B) or Bud stage (C) and ending at Prim-5 (24 h) stage. (D) Slow muscle fiber counts per somite along the embryonic axis in embryos treated with ethanol or cyclopamine, beginning at various times and ending at Prim-5 (24 h) stage. Each colored-coded curve represents the counts from embryos treated beginning at a single stage of drug application: Shield (red), Bud (brown), 2- to 3-somite (yellow), 5- to 6-somite (green), 9- to 10-somite (blue) stages; the ethanol (control) is in purple. The arrowheads indicate the average most posterior somite that contained muscle pioneers following each treatment. Treatment beginning at shield stage abolished slow muscle fibers in all somites, whereas treatment beginning at progressively later stages spared slow muscle fibers in progressively more posterior somites. In all treatments, muscle pioneers were consistently missing in more anterior somites than non-pioneer slow cells (an average of four somites more anterior). The error bars represent the standard error of the mean for each point ($n = 24$). Scale bar, 100 μm.

applied at the 2- to 3-somite stage, about 45% are resistant when cyclopamine is applied at the 5- to 6-somite stage, and 95% are resistant when cyclopamine is applied at the 9- to 10-somite stage (Fig. 5D). Paraxial mesodermal cells spend about 6 h in the segmental plate (data not shown), suggesting that slow muscle commitment begins shortly after cells leave the tail bud.

These data show that slow muscle precursors become independent of Hedgehog signaling while they are in the posterior segmental plate and that pioneer slow muscle fibers require a 2-h longer exposure to Hedgehog signaling than non-pioneer slow muscle cells. Our transplantation data show that slow muscle precursors are committed by the time they reach the anterior segmental plate. Taken together, these results are consistent with the hypothesis that Hedgehog signaling is required for the onset of commitment to the slow muscle fate.

Adaxial cells differentiate as fast muscle fibers in smu (smoothened) mutant embryos

Because adaxial cells normally differentiate into slow muscle, we followed the fates of the adaxial cells that form in *smu* (*smoothened*) mutant embryos to learn what they become in the absence of Hedgehog signaling. We injected tracer dye into adaxial cells in the anterior segmental plate of 3-somite stage *smu* (*smoothened*) and wild-type sibling embryos (Fig. 6A). We analyzed the fates of injected cells at late segmentation stages (24–30 h) using the F59 antibody that labels slow and fast muscle fibers differentially.

We find that mutant adaxial cells adopt a fast muscle fate. In wild-type embryos, adaxial cells form pioneer or non-pioneer slow muscle fibers (n = 80/80; Figs. 6A and B), as we previously reported (Devoto et al., 1996). In *smu* (*smoothened*) mutant embryos, adaxial cells form muscle fibers, but unlike wild-type cells they develop into fast muscle fibers (n = 19/19; Figs. 6A and C), as indicated by the F59 labeling pattern.

As further confirmation of this Hedgehog-dependent change in fate of adaxial cells, we labeled cells in the slow domain of shield stage *smu* (*smoothened*) mutant and wild-type sibling embryos (Fig. 6D). Consistent with our results of adaxial cell injections, injected slow precursors differentiate into slow and pioneer fibers (n = 28/28; Figs. 6D and E) in wild-type embryos, whereas muscle precursors in the same domain of *smu* (*smoothened*) shield stage embryos differentiate into fast fibers (n = 6/6; Figs. 6D and F).

Together, these results show that although adaxial cells form independently of Hedgehog signaling, Hedgehog is subsequently required for their differentiation into slow muscle fibers.

Adaxial cells adopt random positions throughout the somite in absence of Hedgehog signaling like wild-type fast muscle precursors

Lateral cells transplanted into the adaxial position adopt a variety of positions throughout the somite (Fig. 4), unlike normal adaxial cells that migrate superficially (Devoto et al., 1996). To learn whether this behavior is characteristic of fast

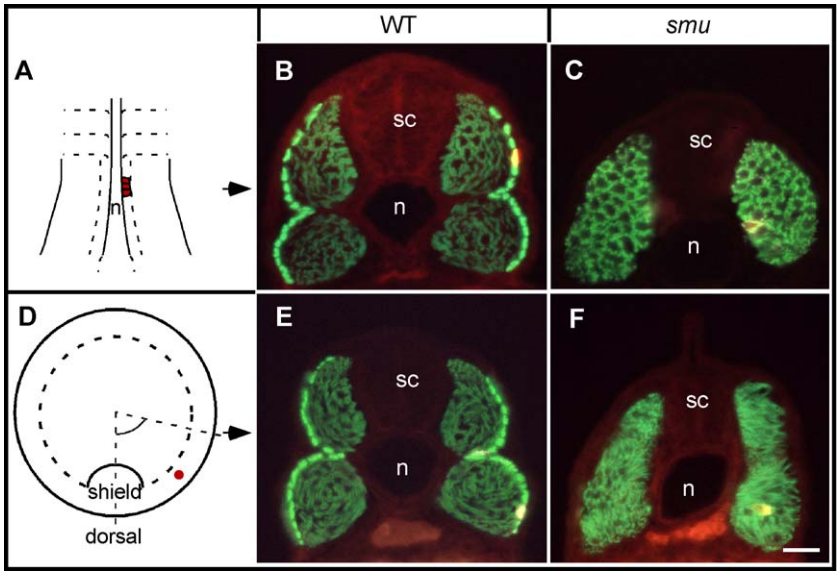

Fig. 6. Muscle precursors in the slow domain adopt a fast fate in the absence of Hedgehog signaling. The 3-somite and Shield stage fate maps from *smu* (*smoothened*) mutant embryos. (A) Dorsal view of a 3-somite stage embryo diagramed at the level of the notochord in the anterior segmental plate. The locations of injected adaxial cells are indicated in red. (D) Animal pole view of shield stage embryo diagram at the level of the marginal zone. The position of the injected cell is indicated in red; the straight broken line indicates the boundary between the slow and fast domains (see Fig. 2). (B, C, E, and F) Cross-sections of Prim-5 (24 h) stage embryos with dorsal to the top. Immunolabeling with F59 in green, lineage tracer dye revealed in red, double-labeled cells appear yellow. One slow fiber is shown in the wild-type embryo (B); one fast muscle fiber is shown in the *smu* (*smoothened*) mutant embryo (C). One slow and one pioneer fiber are shown in the wild-type embryo (E); two fast muscle fibers are shown in the *smu* (*smoothened*) mutant embryo (F). Abbreviations: n, notochord; sc, spinal cord. Scale bar, 20 μm.

precursors, we examined the movements of normal lateral cells.

We injected tracer dye into the most medial non-adaxial cells in the anterior segmental plate of 3-somite stage wild-type embryos (Fig. 7A) and analyzed the positions of resulting labeled fast fibers at late segmentation stages (24–30 h). We find that these medial (non-adaxial) cells can adopt a variety of positions throughout the mature somite. Medially derived fast fibers are later found next to or one cell away from the notochord (medial position; n = 24/63; Fig. 7B), they are also found deep within the somite (central position; n = 11/63; Fig. 7C) and next to or one cell away from the superficial slow muscle fibers (lateral position; n = 28/63; Fig. 7D). The labeled fibers occupy these different positions at similar frequencies suggesting that their relocation is random.

Fast precursors transplanted into the adaxial position behave in the same way (Fig. 7E); they adopt medial (n = 3/17), central (n = 4/17), and lateral positions (n = 10/17). Thus, transplanted lateral cells adopt the fate and final position of their closest new neighbor, the medial non-adaxial cells, and differentiate as fast muscle fibers located throughout the somite.

To learn whether adaxial cells in *smu* (*smoothened*) mutant embryos behave similarly to normal lateral cells, we compared the mediolateral locations of *smu* (*smoothened*) adaxial cell-derived fast muscle fibers to the medially derived fast muscle fibers in wild-type embryos (Fig. 7E). We find that like wild-type fast muscle precursors, *smu* (*smoothened*) adaxial cells adopt random locations throughout the somite. Out of 19 adaxial cells labeled at the 3-somite stage in *smu* (*smoothened*) mutant embryos, 9 were located medially, 3 lay centrally, and 7 were lateral by the Prim-5 (24 h) stage.

These results show that in the absence of Hedgehog signaling, both adaxial cells and cells in the slow domain of shield stage embryos survive, differentiate as muscle, but adopt an alternate fate, the fate of their lateral neighbors. Like adjacent fast muscle precursors, adaxial cells in *smu* (*smoothened*) mutant embryos differentiate into fast muscle fibers that are later found throughout the somite.

Discussion

We previously showed that slow and fast skeletal muscle fibers arise from distinct populations of precursors in the segmental plate (Devoto et al., 1996) and, in agreement with other studies (Blagden et al., 1997; Coutelle et al., 2001; Currie and Ingham, 1996; Lewis et al., 1999; Norris et al.,

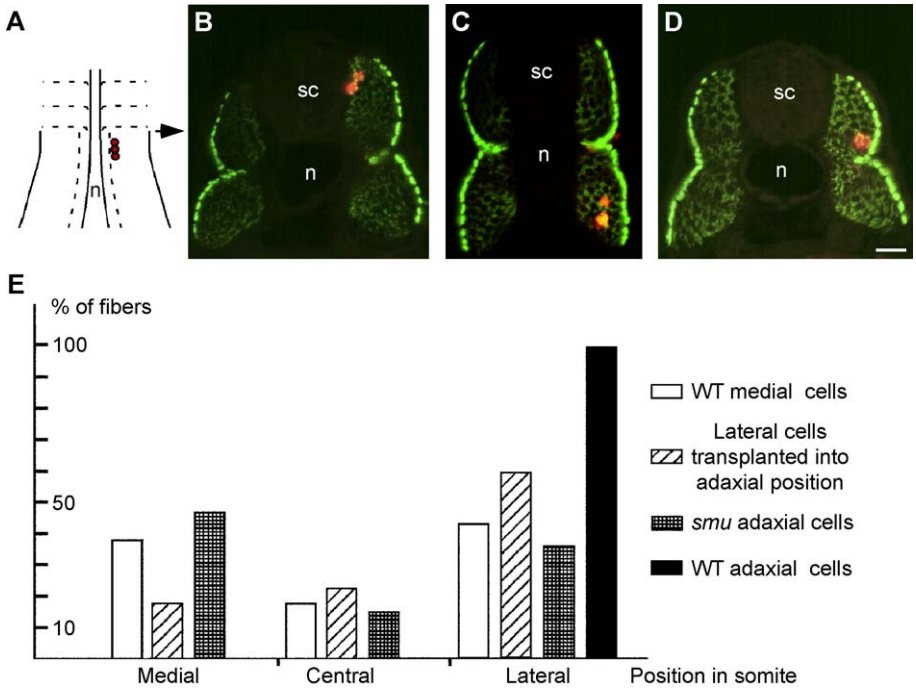

Fig. 7. Hedgehog is required for the migration of slow muscle precursors to the surface of the somite. (A–D) Fast precursors at the medial edge of the segmental plate adopt a variety of mediolateral positions in the mature somite. (A) Diagram of dorsal view of a 3-somite stage embryo at the level of the anterior segmental plate. The initial locations of injected cells are indicated in red. (B–D) Transverse sections of Prim-15 (30 h) stage embryos with dorsal to the top. Immunolabeling with F59 in green, lineage tracer dye revealed in red. Precursors initially located medially adopt medial locations next to or one cell away from the notochord (B), central locations (C), or lateral locations next to or one cell away from the superficial slow muscle cells (D). (E) Medially transplanted lateral cells and *smu* (*smoothened*) mutant adaxial cells adopt the same distribution of mediolateral positions as wild-type lateral cells. The percentage of total labeled muscle fibers is plotted as a function their final mediolateral positions in the somite assayed at Prim-15 (30 h) stage. Wild-type adaxial cells (black) all migrate to a lateral position whereas wild-type medial cells (white), medially transplanted lateral cells (oblique stripes), and *smu* (*smoothened*) mutant adaxial cells (dots) have an essentially equal probability of adopting any position in the somite. Abbreviations: n, notochord; sc, spinal cord. Scale bar, 20 μm.

2000), that Hedgehog signaling is both necessary (Barresi et al., 2000) and sufficient (Du et al., 1997) for induction of the slow muscle fate. Hedgehog is required at different doses and times by different cell types (Wolff et al., 2003). These previous studies, however, did not allow conclusions to be drawn about when muscle precursors become committed to form a particular fiber type or how Hedgehog affects specification of the slow muscle fate. The new results we report here demonstrate that slow and fast muscle precursors occupy distinct locations in the presumptive muscle domain in the gastrula, even before formation of the segmental plate, and give rise exclusively to slow or fast muscle fiber lineages (Fig. 2) that exhibit distinct cell division patterns (Table 2). Despite this lineage restriction, however, individual muscle precursors in the gastrula are not yet committed; they can adopt either slow or fast fates depending upon their local environment (Fig. 3). A few hours later, after muscle precursors have joined the segmental plate as adaxial or lateral cells, they become committed to their particular fates (Fig. 4). As slow muscle precursors commit to their fate, they also become independent from Hedgehog signaling for their development (Fig. 5). Surprisingly, we find that formation of postmitotic adaxial cells occurs independently of Hedgehog (Fig. 1). In the absence of Hedgehog signaling, adaxial cells still form but differentiate into fast muscle fibers (Fig. 6). Similar to wild-type fast fibers, these adaxial cell-derived fast fibers adopt a variety of positions throughout the somite (Fig. 7). Together, these data are consistent with a model where formation of adaxial cells and their subsequent pseudo-epithelial morphology occurs independently of Hedgehog. Hedgehog signaling then presumably acts relatively late in the muscle differentiation pathway, either to commit muscle precursor cells to a slow muscle fate or to maintain the slow fate (Fig. 8).

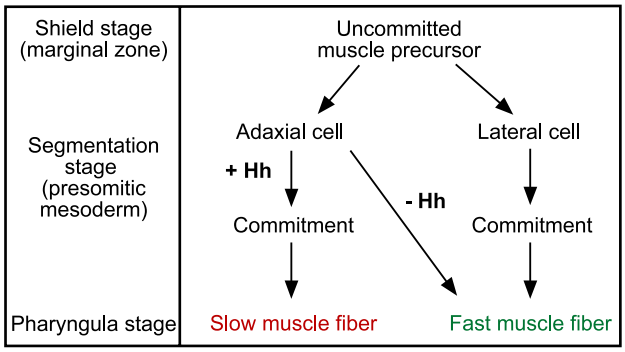

Fig. 8. At shield stage, uncommitted muscle precursors, despite their lineage restrictions, can alternatively adopt slow or fast fates depending upon their local environment. Five hours later, these precursors have developed into adaxial or lateral cells and are committed to their particular fates. In the absence of Hedgehog signaling, adaxial cells still form but ultimately differentiate as fast muscle fibers. In the presence of Hedgehog signaling, adaxial cells differentiate as slow muscle fibers. This suggests that Hedgehog signaling is important at a relatively late step in the muscle differentiation pathway, either to commit adaxial cells stably to their slow fate or to maintain the adaxial cell slow fate.

Distinct slow and fast muscle lineages

We find that zebrafish slow and fast muscle progenitors have distinct origins during gastrulation (Fig. 2), similar to epaxial and hypaxial muscle progenitors in chick and mouse (Eloy-Trinquet and Nicolas, 2002; Pasteels, 1937; Psychoyos and Stern, 1996; Selleck and Stern, 1991; Wilson and Beddington, 1996). Development of fish slow and fast muscle shares characteristics of amniote epaxial and hypaxial muscle development, even though they are not homologous muscle types. In fish, slow muscle precursors are the early differentiating medial cells, similar to the epaxial cells of the myotome in chick and mouse, and fast muscle precursors are the late-differentiating lateral cells, similar to hypaxial cells of the myotome. Slow precursors initially develop as mononucleated fibers adjacent to the notochord and are dependent upon Hedgehog signals (Barresi et al., 2000; Blagden et al., 1997; Currie and Ingham, 1996; Du and Dienhart, 2001; Du et al., 1997; Lewis et al., 1999; Schauerte et al., 1998) similar to epaxial myoblasts (Hirsinger et al., 2000). Fast muscle fibers in fish develop later as multinucleated fibers at a distance from and independently of Hedgehog signaling (Barresi et al., 2000; Blagden et al., 1997; Du and Dienhart, 2001), similar to hypaxial precursors (Hirsinger et al., 2000). In both zebrafish and tetrapods, the two types of progenitors occupy different environments throughout development; thus, their differentiation is likely to rely on distinct cues. In both systems, medially located muscle precursors depend upon Hedgehog signaling (Barresi et al., 2000; Borycki et al., 1999) and the role of Hedgehog signaling in slow muscle differentiation in fish is well established (reviewed by Pownall et al., 2002). No signaling pathway specifically required for fast muscle development has yet been described. Several lines of evidence suggest that members of the Wnt and Bmp signaling families may be candidates for specifying fish fast muscle. Zebrafish fast muscle precursors express the *doublesex*-related gene, *terra*, which is regulated by Bmps (Meng et al., 1999). Fast muscle precursors activate MyoD later than slow muscle precursors. The same asynchrony in MyoD activation is found in mouse and chick myotomes (Buckingham, 1992; Pownall and Emerson, 1992) where the delay in hypaxial MyoD expression is mediated by BMP signaling (Cossu et al., 1996; Pourquié et al., 1996). We previously provided evidence that zebrafish Bmps can inhibit formation of muscle pioneers (Du et al., 1997). However, there is no direct evidence for a role of Wnt or Bmp signaling pathways in fast muscle development.

Zebrafish slow and fast fibers derive from different precursors at shield stage (Fig. 2). Our transplantation experiments, however, show that although muscle precursors are lineage restricted by shield stage, they are not actually committed to forming either slow or fast progeny (Fig. 3). This result may suggest that the bias to produce a particular lineage can be overridden by local cues at this

stage. Alternatively, there may be no lineage bias and the apparently restricted fates of shield stage muscle precursors may simply reflect their restricted and stereotyped morphogenetic movements; muscle precursors close to the shield converge toward the midline first and form adaxial cells, whereas muscle precursors far from the shield remain farther lateral and do not mix significantly with the adaxial precursors. In support of this interpretation, time-lapse recordings have shown limited mixing of mesodermal cells during late gastrula and early segmentation stages (Glickman et al., 2003). Later in development, adaxial and lateral cells are committed and, when transplanted, retain their fates (Fig. 4). Therefore, by this stage, local environmental cues no longer affect the fates of muscle precursors. Fast precursors, like slow precursors, become committed asynchronously while they travel through the segmental plate. Half of the cells in the posterior segmental plate are committed, and essentially all are committed by the time they form a somite. Even in the anterior segmental plate, some adaxial cells do not appear committed. This result agrees with the previous study of Williams and Ordahl (1997) that suggested a progressive increase in the number of committed muscle cells along the anteroposterior axis of the paraxial mesoderm and persistence of only a small population of multipotent cells in the somites.

Postmitotic adaxial cells develop independently of Hedgehog signaling

Previous studies showed that Hedgehog pathway mutants have reduced or absent *myod* and *ptc1* expression adjacent to the notochord in the segmental plate where adaxial cells normally form reduced or absent slow muscle cells (Barresi et al., 2000; Coutelle et al., 2001; Lewis et al., 1999; van Eeden et al., 1996). Coutelle et al. (2001) showed that adaxial cells were identifiable morphologically when Hedgehog was reduced but not absent in *syu* mutants that have significant residual Hedgehog function. These observations have led to the commonly held view that formation of the adaxial cell morphology is linked to the slow muscle fate and that adaxial cell formation is dependent upon Hedgehog signaling (reviewed by Pownall et al., 2002). Surprisingly, we find that postmitotic adaxial cells form quite normally when Hedgehog signaling is disrupted (Fig. 1), but subsequently they differentiate into fast muscle fibers (Fig. 6). The persistence of adaxial cells is unlikely to be due to residual maternal Hedgehog function. Cyclopamine treatment blocks maternal and zygotic Hedgehog activities as early as sphere stage, before any myogenic induction; *yot* (*gli2*) is a dominant-negative mutation that blocks both Gli1 and Gli2 activities (Karlstrom et al., 2003); and adaxial cells are observed as late as the 15-somite stage in *smu* mutant embryos, probably well after maternal Hedgehog function is exhausted. Thus, it is unlikely that Hedgehog plays a significant role in the induction of adaxial cells. The close apposition of adaxial cells to the notochord suggests that a non-Hedgehog, notochord-derived signal could be responsible, an idea that could be tested by examining adaxial cell formation in notochord deficient mutants. Alternatively, adaxial identity may be induced earlier, perhaps during mesoderm induction.

Our results raise new questions about the role of adaxial cells as slow muscle precursors. In the absence of Hedgehog signaling in *smu* (*smoothened*) mutant embryos, formation of adaxial cells can be uncoupled from adoption of a slow muscle fate because mutant adaxial cells form fast muscle fibers (Fig. 6). Adaxial cells form a transient pseudo-epithelium (Devoto et al., 1996), similar to the transient formation of the dermomyotome in chick and mouse somites. The role of this pseudo-epithelium is unknown. An epithelium might be required for Hedgehog to act as a slow muscle-promoting signal. For example, Hedgehog is known to mediate both short- and long-range patterning in a variety of systems (Ingham and McMahon, 2001), and the slow muscle fate may require short range signaling by direct contact. Alternatively, the adaxial cell epithelium may limit diffusion, thus ensuring the high level of Hedgehog required for slow muscle induction (Lewis et al., 1999; Wolff et al., 2003). We and others previously showed that essentially all muscle precursors can be converted into slow muscle fibers when exposed to ectopic Hedgehog (Blagden et al., 1997; Currie and Ingham, 1996; Du et al., 1997). Thus, formation of the adaxial epithelium may also prevent Hedgehog from diffusing into the lateral segmental plate where it would inappropriately affect fast precursors. Our transplantation experiments show that posterior lateral cells are not all committed to their fast fate (Fig. 4). Therefore, the Hedgehog barrier function of adaxial cells may well be required early in the posterior segmental plate where fast muscle precursors are still responsive to Hedgehog signal.

Hedgehog regulates commitment to the slow muscle fate

Our results demonstrate that Hedgehog acts downstream of adaxial cell formation (Fig. 8). In zebrafish embryos, MyoD is expressed initially by mesodermal cells around the tail bud and by adaxial cells in the segmental plate (Weinberg et al., 1996). In wild-type zebrafish, anterior adaxial cells commit to their slow fate while becoming independent from Hedgehog signaling; they maintain MyoD expression and differentiate into slow muscle fibers. In embryos with decreased Hedgehog signaling, MyoD expression is induced normally around the tail bud (Barresi et al., 2000; Coutelle et al., 2001; Lewis et al., 1999; van Eeden et al., 1996), and although adaxial cells form (Fig. 1), they fail to maintain MyoD expression and later develop into fast fibers (Fig. 6). These data suggest that Hedgehog is required after the initial induction of MyoD expression and adaxial cell formation. For example, MyoD expression may be activated in prospective adaxial cells and Hedgehog may subsequently induce those naive MyoD-expressing adaxial cells to adopt the slow muscle fate and to maintain MyoD

expression. Alternatively, MyoD-expressing adaxial cells may already be specified to form slow muscle but require Hedgehog to maintain MyoD expression and their slow precursor identity.

Together with data from previous studies, our results provide new insight into the mechanisms that regulate the specification of skeletal muscle cell fates. During gastrulation, mesodermal cells located near the dorsal midline express MyoD, converge early, and form adaxial cells. After this initial Hedgehog-independent cell specification, the adaxial cells differentiate into slow muscle fibers and migrate to the lateral surface of the nascent somite where they form a monolayer of slow muscle. Hedgehog signaling from midline cells maintains MyoD expression in the adaxial cells and commits them to the slow muscle lineage. The longest exposure to Hedgehog signaling is required for differentiation of muscle pioneer slow cells, shorter exposure is sufficient for differentiation of non-muscle pioneer slow cells. Future genetic and embryological experiments should provide information about the signals that induce MyoD and adaxial cell identity in zebrafish as well as whether Hedgehog also functions in maintenance and/or induction during amniote myogenesis.

Acknowledgments

We are grateful to K. Lewis, E. Melançon, J.F. Nicolas, and C. Ordahl for their constructive critical reading of the manuscript. We thank colleagues at the UO for fruitful discussions and scientific interactions and Anni O'Shea and Judy Pierce, the UO Histology and Zebrafish Facilities, and the UO Bio-Optics Laboratory for their excellent technical assistance. We are indebted to Frank Stockdale for the generous gift of S58 and F59 antibodies. This work was supported by NIH AR45575 and HD22486. E. H. was supported by Human Frontier and ARC postdoctoral fellowships and the CNRS. E. H. would like to acknowledge particularly Olivier Pourquié, Jacques Pradel, and the CNRS for their collaboration and support.

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