Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 300 (2006) 736–746
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Hedgehog acts directly on the zebrafish dermomyotome to promote myogenic differentiation

Xuesong Feng, Eric G. Adiarte, Stephen H. Devoto *

Department of Biology, Wesleyan University, Middletown, CT 06459, USA

Received for publication 19 May 2006; revised 23 August 2006; accepted 24 August 2006
Available online 30 August 2006

Abstract

Vertebrate myogenesis is regulated by signaling proteins secreted from surrounding tissues. One of the most important, Sonic hedgehog, has been proposed to regulate myogenic precursor cell survival, proliferation, and differentiation in a variety of vertebrates. In zebrafish, Hedgehog signaling is both necessary and sufficient for the development of embryonic slow muscle fibers—the earliest differentiating muscle fibers. Here we investigated the function of Hedgehog signaling in another zebrafish myogenic lineage, a dermomyotomal population of cells defined by somitic *pax3/7* expression. We found that Hedgehog negatively regulates the number of myogenic precursors expressing *pax3/7*. Hh also positively regulates the growth of embryonic fast muscle. Unlike Hedgehog's function in regulating the elongation of fast muscle fibers, this regulation is not mediated by embryonic slow muscle fibers. Instead, it is a direct Hedgehog response, cell autonomous to myogenic precursors. The regulation of myogenic precursors and their differentiation into fast fibers have a different critical time period for Hh signaling, and different requirements for specific *gli* gene family members of Hh activated transcription factors from the earlier promotion of embryonic slow muscle fiber differentiation. We propose that Hedgehog signaling acts at multiple times on different lineages, through different downstream pathways, to promote myogenic differentiation.

Keywords: Myogenesis; Dermomyotome; Hedgehog; Zebrafish; *pax3*; *pax7*; Fast muscle; Adaxial; Slow muscle

Introduction

Vertebrate trunk and limb skeletal muscle develops from somites, repeated structures that segment from the paraxial mesoderm in a rostral to caudal progression during early embryogenesis. After their formation, somites undergo a series of cellular and morphological changes to generate the precursors for skeletal muscle, cartilage, axial skeleton, ribs, tendon, dermis, and vascular endothelia. In most vertebrates that have been studied, somites form as uniform epithelial balls. In amniotes, as somites mature, the ventro-medial part of the somite undergoes an epithelial to mesenchymal transition to form the sclerotome, precursors of axial skeleton and ribs. The dorso-lateral somite remains epithelial and forms the dermomyotome, which gives rise to the dermis, trunk, and limb muscles and mesodermal vascular endothelia (Brent et al., 2003; Christ and Ordahl, 1995; Huang and Christ, 2000).

The dermomyotome contains mitotic myogenic precursors expressing transcription factors *pax3* and *pax7* (Amthor et al., 1999; Goulding et al., 1994; Williams and Ordahl, 1994). At non-limb levels, myogenic precursors from the dermomyotome are specified into myoblasts. This involves the down-regulation of the *pax3* and *pax7* genes, and the up-regulation of myogenic transcription factors such as *Myf5* and *MyoD*. Myoblasts disassociate from the dermomyotome and translocate into the myotome, the embryonic structure beneath the epithelial dermomyotome that gives rise to deep trunk and body wall muscle (Denetclaw et al., 1997; Gros et al., 2004; Kahane et al., 1998a,b; Kalcheim et al., 1999). At limb levels, a subset of myogenic precursors in the ventro-lateral lip (VLL) migrate laterally to the limb bud, where they differentiate into limb muscles and vascular cells (Buckingham et al., 2003).

Zebrafish somite development shares many features with amniote somite development. The first wave of myogenesis also

* Corresponding author. Fax: +1 860 685 3279.
E-mail address: sdevoto@wesleyan.edu (S.H. Devoto).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.08.056

comes from a medial somite population, in teleosts called adaxial cells, and the sclerotome also derives from the ventral part of the somite (Stickney et al., 2000). Adaxial cells are committed to the myogenic fate early and express *myoD* and *myf5* while still in presomitic mesoderm (Hirsinger et al., 2004; Weinberg et al., 1996). After somite formation, adaxial cells move radially away from the notochord and form a layer of embryonic slow muscle fibers (Blagden et al., 1997; Devoto et al., 1996; Roy et al., 2001). The movement of adaxial cells may be driven by differential adhesion between adaxial cells and the initially more lateral fast muscle precursors (Cortes et al., 2003). As these fast muscle precursors become medial to the slow muscle fibers, they begin to differentiate and elongate (Blagden et al., 1997; Henry and Amacher, 2004). By the end of the segmentation period (24 h), a functional embryonic myotome has formed and been innervated.

After the embryonic period, there is tremendous muscle growth by hyperplasia, the increase in muscle fiber number, and hypertrophy, the increase in muscle fiber size. New muscle fibers are added in localized areas of the embryonic myotome, in a process known in teleosts as stratified hyperplasia (Rowlerson and Veggetti, 2001). In zebrafish, new slow fibers develop at the dorsal and ventral extremes of the embryonic myotome (Barresi et al., 2001), and new fast fibers develop at the external surface of existing fast fibers (unpublished, D. Fernandez, SHD). As in amniotes, the continued growth of the myotome likely relies on a myogenic precursor cell type from the zebrafish dermomyotome (Devoto et al., 2006). The zebrafish dermomyotome is a layer of cells expressing *pax3* and *pax7*, external to the newly formed myotome (Devoto et al., 2006; Groves et al., 2005). Some of these cells co-express Pax7 and Myogenin, indicating that they include myogenic precursors (Devoto et al., 2006). By the end of the segmentation period, the dermomyotome has become a very thin layer of Pax7-positive cells on the external surface of the superficial slow muscle fibers (these cells were called “external cells” by Waterman, 1969). The zebrafish dermomyotome resembles that of many other vertebrates, including the tetrapod *Xenopus* (Devoto et al., 2006; Grimaldi et al., 2004).

Signaling molecules from tissues surrounding the somite regulate the balance between proliferation and myogenic differentiation that is necessary for proper development of the embryonic myotome and its growth between embryogenesis and adulthood (Amthor et al., 1999; Buckingham et al., 2003; Tajbakhsh, 2003; Te Kronnie and Reggiani, 2002). These signals include Hedgehog (Hh) family proteins, secreted by the notochord and ventral spinal cord (Johnson et al., 1994), Wnt family members secreted by the surface ectoderm (Munsterberg et al., 1995), BMP family members secreted by the dorsal spinal cord, surface ectoderm, and lateral plate (Amthor et al., 1999; Lyons et al., 1995), and the BMP antagonist Noggin secreted within the DML (McMahon et al., 1998). How these various signaling proteins combine to regulate the development of muscle, dermis, cartilage, and other somitic derivatives is not fully understood.

Hh has been shown to promote myogenesis in both fish and amniotes. Hedgehog protein binds to a cell surface receptor, Patched, which in the absence of Hh blocks signaling by inhibiting another transmembrane protein, Smoothened. When Hh binds to Patched, it relieves the inhibition of Smoothened by Patched, leading to the activation of members of the Gli transcription factor family (for reviews, see Ingham and McMahon, 2001; Lum and Beachy, 2004). Ectopic Hh protein triggers ectopic MyoD and/or Myf5 expression in chick (Borycki et al., 1998), mouse (Borycki et al., 1999), and zebrafish (Blagden et al., 1997; Du et al., 1997). Mutations which disrupt Hh signaling in mouse or zebrafish lead to defects in myogenesis. Multiple cellular effects of Hh proteins have been described, including specification of cell identity (Barresi et al., 2000; Du et al., 1997), cell proliferation (Duprez et al., 1998; Marcelle et al., 1999), differentiation (Bren-Mattison and Olwin, 2002; Marcelle et al., 1999), and cell survival (Cann et al., 1999; Kruger et al., 2001). In zebrafish, Hh signaling cell-autonomously determines the slow muscle fate of adaxial cells (Barresi et al., 2000; Hirsinger et al., 2004), and is required for the development of Engrailed-expressing slow and Engrailed-expressing fast muscle fibers (Wolff et al., 2003). The induction of multiple cell types by a single molecule has been proposed to be a result of differences in the dose of Hh and the time at which somite cells are exposed to Hh (Wolff et al., 2003). Given the wealth of different effects of Hh on myogenic precursors, and the presence of other signaling molecules influencing cell specification, it has proven quite difficult to create a unifying model for the cellular basis of the effect of Hh on myogenesis. By acting at different times during development, Hh may affect several different populations of myogenic precursors, as has been proposed for the neural tube (Blaess et al., 2006; Cayuso et al., 2006).

Here we show that in addition to its early role in inducing embryonic slow muscle identity in the most medial presomitic cells, Hh signaling has a later role in regulating the differentiation of the dermomyotome. We show that Hh signaling is not required for the initial establishment of the dermomyotome but is required for its differentiation into fast muscle fibers. In the absence of Hh signaling, the differentiation of fast muscle fibers is compromised. The effect of Hh on embryonic slow fibers and the effect of Hh on fast muscle fibers can be distinguished genetically and pharmacologically: slow muscle fibers require earlier Hh signaling and are completely absent in embryos containing mutations in the *yot(gli2)* gene, while fast myotome differentiation requires Hh signaling later and depends only partially on the *yot(gli2)* gene. We use genetic mosaics to demonstrate that the requirement for Hh signaling is cell-autonomous to the dermomyotome, and not mediated by other cells that respond to Hh signaling.

Materials and methods

Fish strains and embryos

Wild-type AB, *slow-muscle-omitted* (*smu*) alleles (smu^{b577} and smu^{b641}, Barresi et al., 2000), *you-too* (yot^{ty119}, van Eeden et al., 1996), and *sonic-you* (syu^{tbx392}, van Eeden et al., 1996) embryos were obtained from zebrafish (*Danio rerio*) lines maintained with standard procedures (Westerfield, 1995). We obtained similar results with both *smu*(*smo*) alleles. Embryos were staged by

counting somite numbers (Kimmel et al., 1995). We exposed wild-type embryos in their chorions to 50 μM cyclopamine (Toronto Research Chemicals, C988400), at various times between shield stage and Prim-5 (24 h) stage, as described (Hirsinger et al., 2004). We injected 1–2 cell stage embryos with *in vitro* synthesized *shh* mRNA as described (Barresi et al., 2000).

Antibodies, immunocytochemistry, and in situ hybridization

MF20 is an IgG2b monoclonal antibody that labels differentiated muscle fibers in all species that have been examined, obtained from Developmental Studies Hybridoma Bank (DSHB) (Bader et al., 1982). Pax7 is an IgG1 monoclonal antibody specific for Pax7 in chicken, obtained from DSHB (Kawakami et al., 1997). Pax7 and MF20 antibodies were used at 5 μg/ml. Rabbit antibody against human Prox1 was obtained from Chemicon, and used at a dilution of 1:1000 (Roy et al., 2001).

For Pax7 labeling, Prim-5 (24 h) embryos were fixed in fresh 4%PF (less than a week old) for 2 h at room temperature or over night at 4°C. Fixed embryos were dehydrated with 100% methanol at −20°C at least 30 min or stored for a long period of time at −20°C. When ready to be processed, embryos were rehydrated with a series of methanol (75%, 50%, 25%), each for 5′ and rinsed with PBS-Tween 3×5′. Antibody labeling was carried out as previously described (Barresi et al., 2000; Devoto and Barnstable, 1989; Hernandez et al., 2005). Secondary antibodies conjugated with Alexa 488, Alexa 547, and Alexa 647 (Molecular Probes, Eugene, Oregon) were used at a dilution of 1:800.

In situ hybridization was performed as described (Barresi et al., 2000; Jowett, 1997), using probes for *pax3* and *pax7* (Seo et al., 1998), and a fast muscle specific myosin heavy chain cloned in our lab from zebrafish genome sequences (accession number: AY333450; F. Chan, D. Fernandez, SHD, unpublished).

Transplantations

We transplanted cells as previously described (Barresi et al., 2000). After transplantations, host embryos were fixed at 24 h, and immunolabeled as described above, while donor embryos were grown up to determine whether donor cells were wild type or mutant. Transplanted Pax7$^+$ dermomyotomal cells and muscle fibers were counted under the confocal microscope, as described below.

Imaging and the quantification of Pax7$^+$ myogenic precursors

Immunolabeled embryos were photographed using the Zeiss LSM510 confocal microscope. *In situ* hybridization images were taken on a Zeiss Axioplan compound microscope with Nomarski (DIC) optics. Monochromatic images of fluorescence were pseudo-colored for clarity. All image manipulations were done on the entire image, in Photoshop. The number of Pax7$^+$ nuclei and transplanted cells were counted using the Zeiss LSM confocal microscopy software. Each image in a stack of optical sections from each embryo was used to sequentially inspect each optical section in isolation, to ensure that every cell and every nucleus were counted only once.

Results

Myogenic pax gene expression in the zebrafish dermomyotome

In amniotes, *pax3* and *pax7* genes are expressed in epithelial dermomyotome cells and down-regulated during their differentiation into muscle fibers (Amthor et al., 1999). In zebrafish, *pax3* expression begins in the paraxial mesoderm between the 5- and 10-somite stage. *pax3* is not expressed in adaxial cells, but is found in the anterior portion of the first few somites and throughout the 3–4 recently formed epithelial somites (Fig. 1A, Seo et al., 1998). As somites mature, *pax3* expression becomes restricted to the anterior half of the somite (Fig. 1A). *pax7* expression begins about 2 h after the onset of *pax3* expression (Figs. 1A, B), beginning as segmentally repeated stripes at the anterior border of each somite. The segmented expression pattern persists until the 19-somite stage. *pax3* and *pax7* are then dramatically down-regulated in an anterior to posterior direction, with only the 3–4 most recently formed somites continuing to show expression (Fig. 1C).

We used antibody labeling to chart the number and position of dermomyotomal myogenic precursors present at the end of the segmentation period (24 h). At this time, Pax7 labels two types of cells on the surface of the myotome, migrating neural crest cells, and dermomyotomal cells (Seo et al., 1998). Neural crest cells can be readily distinguished by their higher levels of Pax7 expression, they are also the only dorso-ventrally elongated, bean-shaped nuclei over the central portion of the somite (Fig. 2A). These brightly labeled cells only appear after neural crest begins to migrate (data not shown), and are selectively missing in neural crest mutants (Simon Hughes, personal communication). Pax7$^+$ somitic cells form a monolayer on the external surface of the myotome at the end of the segmentation period (Fig. 2A, Devoto et al., 2006); these cells are part of the dermomyotome and do not express neural crest specific genes such as *crestin* (data not shown, Luo et al., 2001).

Hedgehog signaling negatively regulates the number of Pax7$^+$ myogenic precursors

We tested several components of the Hh signaling pathway for their involvement in the development or differentiation of *pax3/7*-positive dermomyotome cells. *Smu* (*slow muscle omitted*) encodes zebrafish Smoothened, which is required for all Hh signaling (Barresi et al., 2000; Varga et al., 2001). Until the 19-somite stage, *pax3* and *pax7* expression is indistinguishable between wild type and *smu(smo)* mutants (Figs. 1A, B; data not shown). After the 19-somite stage, as wild-type embryos lose the segmented expression pattern, *smu(smo)* mutants maintain strongly segmented expression of both *pax3* and *pax7*, this segmented expression pattern is maintained at least until the end of the segmentation period in *smu(smo)*$^{-/-}$ (Fig. 1C). These results suggest that Hh signaling does not regulate the induction of *pax3/7* expression, but instead affects the down-regulation of *pax3/7* expression. Consistent with these *in situ* hybridization data, we see an elevated number of Pax7-positive dermomyotome cells in *smu(smo)* mutant embryos until at least 24 h. Many of the extra Pax7$^+$ cells remain at the anterior border of each somite (Figs. 2B, C, G see also Figs. 3B, H).

Cyclopamine is a plant alkaloid that specifically binds Smoothened (Chen et al., 2002; Frank-Kamenetsky et al., 2002), and blocks all known Hh signaling in zebrafish (Barresi et al., 2001). Cyclopamine-treated embryos closely resembled *smu(smo)*$^{-/-}$ embryos, with a large increase in the number of Pax7$^+$ myogenic precursors in the anterior part of each somite (Fig. 2C). This is reminiscent of the segmented expression pattern of *pax3* and *pax7* mRNA in *smu(smo)*$^{-/-}$ embryos (Fig. 1C). Embryos mutant in *sonic-you* (*syu*), which encodes Sonic hedgehog, or *you-too* (*yot*), which encodes Gli2, also

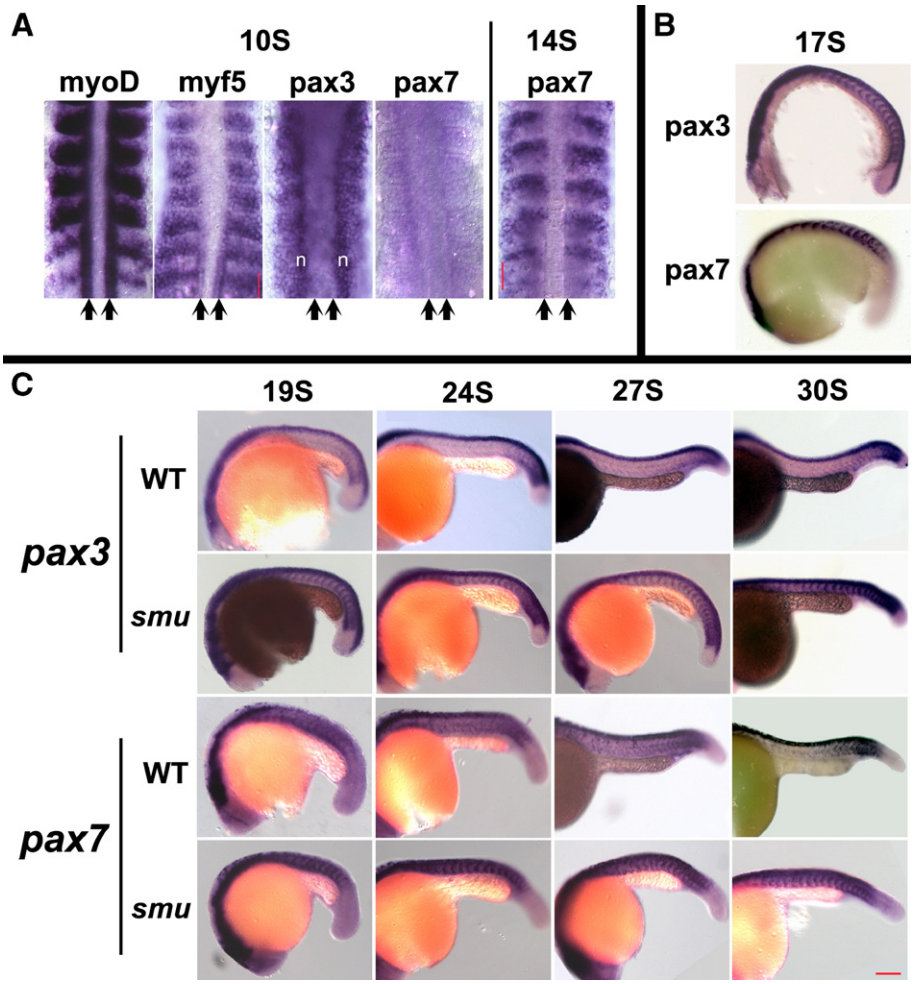

Fig. 1. Hedgehog signaling down-regulates *pax3/7* expression after the 19-somite stage. A clutch of *smu*$^{+/-}$ embryos was hybridized with *pax3* or *pax7* probes during early embryonic stages. (A) Dorsal view of embryos at 10S and 14S stages. At the 10S stage, *myoD* and *myf5* are expressed in adaxial cells (arrows) and the posterior part of newly formed somites. Neither *pax3* nor *pax7* is expressed in adaxial cells (arrows). *pax3* is expressed initially throughout the non-adaxial portion of the somite; *pax3* is also expressed in the neural folds (n). *pax7* is not expressed in the somites. At the 14S stage, *pax7* is expressed in the anterior part of somites, at this stage the somitic expression of *pax3* is very similar but has much higher expression in neural tissue. (B) Lateral view of embryos at 17S stage, the expression of *pax3* and *pax7* continues to be strongest in the anterior part of somites, and is indistinguishable between wild-type and *smu* mutant embryos. (C) After the 19-somite stage, *pax3* expression is down-regulated in wild-type embryos, while the segmented expression of *pax3* is maintained in embryos mutant for *smu(smo)*, at least until the end of segmentation stage (30S). *pax7* expression is also down-regulated during this period in wild-type embryos, while the segmented expression of *pax7* is maintained at high levels in embryos mutant for *smu(smo)*. Scale bar=100 μm.

have more Pax7^{+} myogenic precursors (Figs. 2A, D, E, H), although the effects of mutations in these genes are not as dramatic as mutations in *smu*. Other homologues may provide sufficient Hh signaling for the regulation of these myogenic precursors. Overexpression of Shh by mRNA injection into the 1–2 cell stage embryo transforms the myotome into embryonic slow muscle fibers (Barresi et al., 2000; Du et al., 1997), and dramatically reduces the number of Pax7^{+} myogenic precursors (Figs. 2F, H). We conclude that Hh signaling negatively regulates the number of Pax7^{+} myogenic precursors that remain at the end of the segmentation period (24 h).

Hh signaling promotes myogenic differentiation

The increase in the number of *pax3/7* myogenic precursors in the absence of Hh could be a result of a decrease in cell death, an increase in proliferation, or a decrease in differentiation. We examined cell death and did not observe a decrease in somitic cell death that correlated in time with the increase in the number of Pax7-positive myogenic precursors (data not shown). Hh promotes myogenic differentiation by inducing *Myf5* and *MyoD* expression in myogenic precursors in mouse and chick (Amthor et al., 1999). As myogenic gene expression is up-regulated, *pax3/7* expression is down-regulated (Amthor et al., 1999; Borycki et al., 1999). We surmised that the increase in the number of Pax7^{+} myogenic precursors in Hh deficient mutants reflected a decrease in muscle differentiation. All embryonic slow muscle fibers are derived from early differentiated adaxial cells, which do not express detectable levels of *pax3* or *pax7* (Fig. 1A). Thus, if Pax7^{+} myogenic precursors undergo myogenic differentiation before the end of the segmentation stage, they must develop into fast muscle fibers. We tested whether fast muscle differentiation is dependent on Hh signaling by examining the developmental

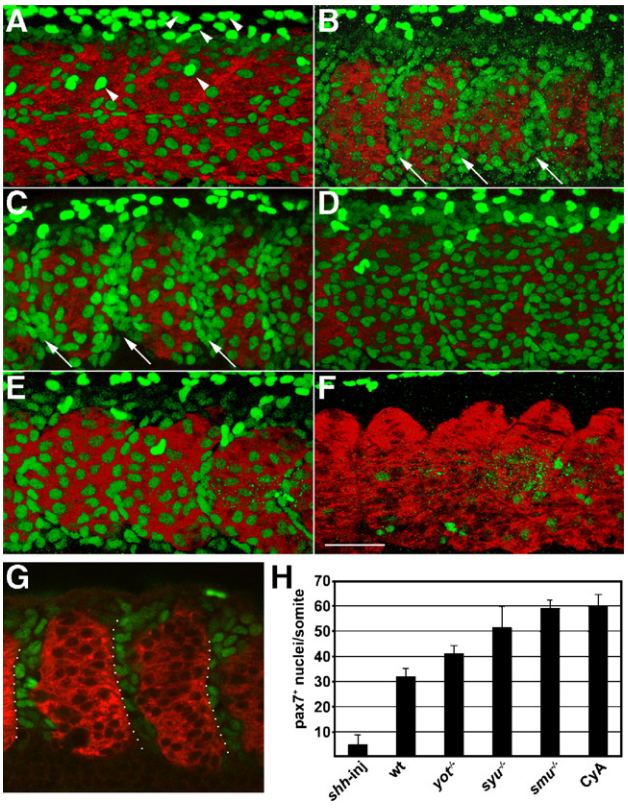

Fig. 2. Hedgehog signaling negatively regulates the number of Pax7$^+$ myogenic precursors. Embryos were labeled at the prim5 (24 h) stage for Pax7 (green) and myosin (MF20, red). (A) In wild-type embryos, the surface of each somite has two types of Pax7$^+$ myogenic precursors, moderately labeled cells that are part of the somite, and more brightly labeled, dorso-ventrally elongated neural crest cell nuclei. (B, C) *smu(smo)*$^{-/-}$ and CyA-treated wild-type embryos have more Pax7$^+$ myogenic precursors than wild-type control embryos, particularly in the anterior part of each somite (arrows). (D, E) In *syu(shh)*$^{-/-}$ and *yot(gli2)*$^{-/-}$ embryos, the number of Pax7$^+$ nuclei is intermediate between untreated wild-type and *smu(smo)*$^{-/-}$ or CyA-treated wild-type embryos. (F) *shh* mRNA-injected wild-type embryos have virtually no Pax7$^+$ nuclei. (G) A single optical slice of a CyA-treated wild-type embryo through the middle part of somites, demonstrating the gaps between myotomes that are filled with Pax7$^+$ cells. Many of the Pax7$^+$ cells are within the anterior of each somite (dotted outline). (H) Quantitation of the number of Pax7$^+$ myogenic precursors per somite in each genotype or treatment (mean of 5 embryos per genotype or treatment, somites 16–18 were counted; error bar = standard deviation). Panels A–F are projections of Z-stack confocal sections (see Materials and methods), panel G is a thin optical slice, all panels are anterior to the left, dorsal up; somite 17 is shown in each panel. Scale bar = 50 μm.

expression of a fast muscle specific Myosin Heavy Chain (*MyHC$_f$*) gene in wild-type and *smu(smo)* mutant embryos. We found that the fast myotome, marked by *MyHC$_f$* expression, extended the full length of the somite by 19S in wild-type embryos (Fig. 3D). In contrast, *smu(smo)* mutant embryos had a smaller fast myotome that did not span the full length of the somite. The gaps between *MyHC$_f$*-positive cells of adjacent myotomes appeared to be at the anterior of each somite, in the same position as the maintained *pax3/7* expression in *smu (smo)* mutants (Figs. 1C, 3B, E). We confirmed this by examining a thin optical slice of double labeling for Pax7 and MyHC (MF20; Fig. 2G). The presence of substantial numbers of myogenic precursors between differentiating myotomes resembles the situation in amniotes, where large numbers of myogenic precursors are found between myotomes, at the rostral and caudal lips of the dermomyotome (cf. Denetclaw et al., 1997).

The effect of Hh signaling on Pax7 down-regulation in myogenic precursors is independent of its effect on embryonic slow fibers

Down-regulation of the *pax3/7* segmental expression begins at about the 16-somite stage in the 8–10 anterior most somites; this is approximately the same time as embryonic slow muscle cells become displaced from the medial to the superficial surface of the somite (Fig. 1, Blagden et al., 1997; Devoto et al., 1996). Embryonic fast muscle fiber elongation also correlates with the timing of slow muscle migration (Cortes et al., 2003). In *smu(smo)* mutant embryos, fast muscle fiber elongation is dramatically delayed, suggesting that Hh signaling is required for the proper timing of fast muscle morphogenesis (Henry and Amacher, 2004). The requirement for Hh in fast fiber *elongation* is mediated by its effect on slow muscle fibers, as transplantation of a few slow muscle fibers into a *smu(smo)* mutant somite can rescue the proper timing of fast fiber elongation (Henry and Amacher, 2004). We wished to test whether slow muscle fibers also mediate the effect of Hh on fast muscle *differentiation*—The down-regulation of *pax3* and *pax7*, and the up-regulation of *MyHC$_f$*. To do this, we compared the phenotypes of *pax3/7*$^+$ myogenic precursors in *smu(smo)* and *yot(gli2)* mutants. Even though embryos mutant in *smu (smo)* and *yot(gli2)* cannot be distinguished by the number of embryonic slow muscle fibers (neither has any), *yot(gli2)* mutants differ from the *smu(smo)* mutant in multiple ways in the dermomyotome. For instance, compared to *smu(smo)* mutants or cyclopamine-treated embryos, *yot(gli2)* mutants have fewer Pax7$^+$ myogenic precursors (Figs. 2B, C, E, H), less *pax3* expression (Figs. 3B, C, H, I), and fast myosin expression more completely spans the somite (Figs. 3D–F, J–L). These varying phenotypes in embryos lacking slow muscle fibers suggest that the effect of a loss of Hh signaling on myogenic precursor differentiation is not mediated solely by embryonic slow muscle fibers.

We created genetic mosaics, with wild-type slow muscle fibers in a *smu(smo)*$^{-/-}$ host, to test if embryonic slow muscle fibers are sufficient to regulate the number of Pax7$^+$ myogenic precursors (Fig. 4). We identified the transplanted slow muscle fibers by the overlapping staining of injected dye from the donor cells and expression of the slow muscle nuclear marker Prox1 (Roy et al., 2001) (Fig. 4A). We found that the number of Pax7$^+$ nuclei in *smu(smo)*$^{-/-}$ somites was unaffected by the presence of transplanted wild-type slow muscle fibers (~60 Pax7$^+$ nuclei/somite) and far more than the number in wild-type embryos (~30 Pax7$^+$ nuclei/somite) (Figs. 4B, C, 2H). Thus, wild-type embryonic slow muscle cells are not sufficient to rescue the Pax7 phenotype of otherwise *smu (smo)*$^{-/-}$ somite cells. The requirement for Hh signaling in regulating the number of myogenic precursors cannot be substituted for by the presence of isolated slow muscle fibers.

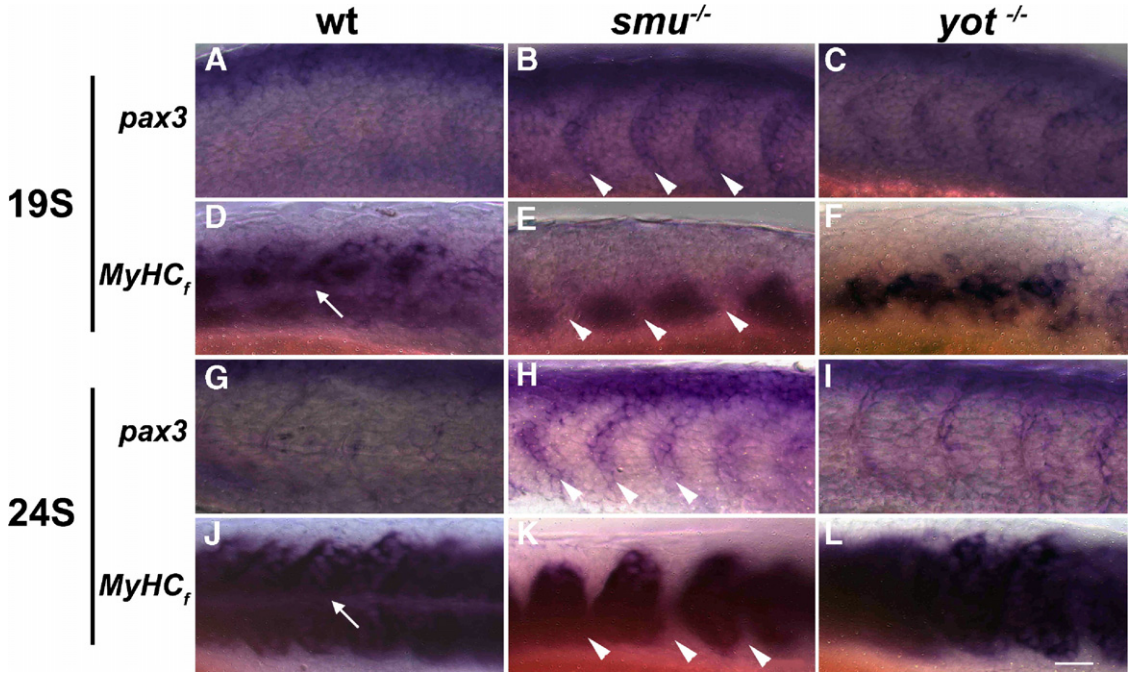

Fig. 3. *pax3* and *fast MyHC* gene expression show reciprocal regulation. Wild-type (A, D, G, J), *smu(smo)*$^{-/-}$ (B, E, H, K), or *yot(gli2)*$^{-/-}$ (C, F, I, L) embryos were analyzed for *pax3* (A, B, C, G, H, I) and *fast Myosin Heavy Chain* (*MyHC$_f$*: D, E, F, J, K, L), expression. By the 19-somite stage (A–F), when *pax3* expression in somite anterior cells is down-regulated in wild-type embryos (A), expression of *MyHC$_f$* begins to span the full anterior–posterior extent of the somite (D). In contrast, segmented expression of *pax3* is maintained in anterior cells within somites of *smu(smo)* mutants (B), while expression of *MyHC$_f$* is only found in the posterior cells of each somite (E). Arrowheads in panels B and E indicate the anterior cells of the somite, which in *smu(smo)* mutants express *pax3* but not *MyHC$_f$*. In *yot(gli2)* embryos (C, F), the segmented expression of pax3 is also down-regulated (C), and *MyHC$_f$* is expressed in the posterior part of the somite. By the 24-somite stage (G–L), expression of MyHC$_f$ has increased in wild-type, *smu(smo)*, and *yot(gli2)* mutants (J–L). However, in *smu(smo)* mutant embryos cells in the anterior of each somite still do not express MyHC$_f$ (K), and the segmental *pax3* expression is still maintained (H). In *yot(gli2)* mutant embryos, anterior cells of each somite down-regulate *pax3* and up-regulate *MyHC$_f$* similar to wild type (I). The arrow indicates the horizontal myoseptum where muscle pioneers, not expressing *MyHC$_f$*, are present in wild type. All panels are anterior to the left, dorsal up; all images are of somites 7–11. Scale bar=100 μm.

Hh is required at two separate times for myogenesis

The requirement for Hh signaling in Pax7 expressing cells can also be distinguished from the requirement for Hh signaling in embryonic slow muscle precursors by the time when cells become resistant to the effects of cyclopamine. We treated wild-type embryos with 50 μM cyclopamine from the 8-somite stage to the 24-h stage. This treatment paradigm has minimal effect on the differentiation of embryonic slow muscle fibers in the anterior somites, as adaxial cells are already committed to a slow muscle fate. More posterior somites show a loss of slow muscle fibers (Hirsinger et al., 2004). In these same embryos, the number of Pax7$^+$ cells was also affected in posterior somites, but not in anterior somites. However, the timing of the requirement for Hh signaling was different for Pax7 than it was for slow muscle fibers. In agreement with previous results (Hirsinger et al., 2004), cells were committed to the slow muscle fate no later than 4–5 h prior to becoming incorporated into a somite (the most anterior affected somite following treatment at the 8-somite stage was somite 16). In contrast, the Pax7 phenotype was resistant to cyclopamine no earlier than 2 h prior to becoming incorporated into a somite (following treatment at the 8-somite stage, somite 12 has an excess of Pax7$^+$ cells). Thus, somite 14 has the same overabundance of Pax7$^+$ myogenic precursors as in *smu (smo)* mutants, despite the fact that this somite has a nearly complete set of slow muscle fibers (Fig. 5). Treatment at earlier or later times showed a similar difference in the time of

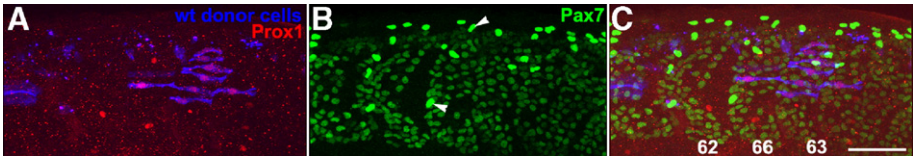

Fig. 4. Wild-type slow muscle fibers do not rescue the number of Pax7+ myogenic precursors in *smu(smo)*$^{-/-}$. A *smu(smo)*$^{-/-}$ host embryo with transplanted wild-type donor cells (blue) was labeled with antibodies for Prox1 (red) and Pax7 (green). (A) A lateral view of 6 mid-trunk somites, some of which contain donor-derived (blue), Prox1-positive (red) embryonic slow muscle fibers. (B) The same embryo labeled for Pax7, to show myogenic precursor nuclei (green), arrowheads point to neural crest cells. (C) The merged image of panels A and B, the numbers on the bottom indicate the number of Pax7$^+$ myogenic precursor nuclei within the corresponding somite, compare to Fig. 2G. Anterior to the left, dorsal up; scale bar=50 μm.

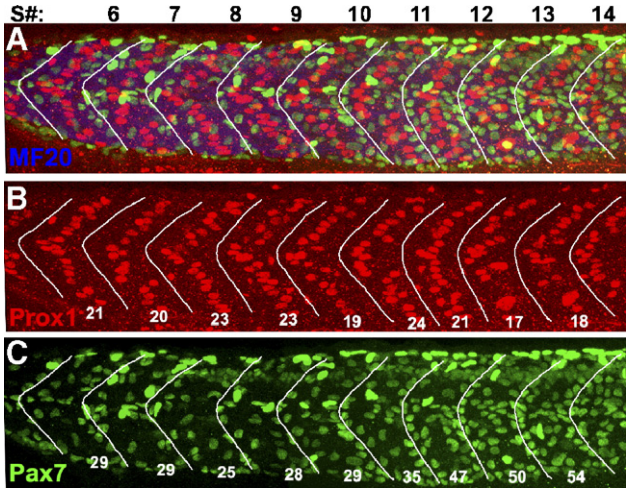

Fig. 5. Hh regulation of Pax7^{+} myogenic precursors occurs later than Hh induction of slow muscle fibers. Wild-type embryos were treated with cyclopamine from the 8-somite stage to the 30-somite (24 h) stage; they were then labeled for differentiated muscle fibers, slow muscle nuclei, and myogenic precursor nuclei. The numbers at the top of panel A indicate somite number. White lines frame the borders of somites: these were drawn solely with the MF20 labeling visible. (A) Merged image showing myosin (MF20, blue), slow muscle nuclei (Prox1, red), and myogenic precursor nuclei (Pax7, green). (B) A single color panel of A, showing Prox1-positive slow muscle nuclei in each somite, the number per somite is shown. All somites have an approximately wild-type number of Prox1-positive nuclei. Somites posterior to somite 18 had a loss of slow muscle fiber nuclei (data not shown, Hirsinger et al., 2004). (C) Single color panel of A, showing the Pax7-positive myogenic precursor nuclei in each somite. The number of Pax7^{+} myogenic precursors in somites 5 to 11 is similar to wild type, while posterior to somite 11, the number of Pax7^{+} myogenic precursors in each somite is increased to the level of *smu*$^{-/-}$; compare to Fig. 2H. Anterior to the left, dorsal up; scale bar=50 μm.

sensitivity to cyclopamine—Pax7 expression but not slow muscle fibers in earlier formed (anterior) somites were sensitive to cyclopamine treatment. In other words, slow fibers become cyclopamine-resistant prior to Pax7^{+} cells. However, the time of commitment relative to somite formation was a little later in the most anterior somites. As with other measures of trunk maturation, anterior somite Pax7 expression became cyclopamine-resistant later, relative to segmentation, than posterior somite Pax7 expression.

The differential timing of the Hh signaling requirement in Pax7^{+} cells and embryonic slow muscle fibers confirms that Hh regulation of the number of pax7^{+} myogenic precursors is not mediated solely through its action in forming embryonic slow muscle fibers. We suspect that the down-regulation of Pax7 in myogenic precursors is a direct response to Hh signaling.

Hh signaling acts directly on myogenic precursors to regulate Pax7 expression

The above results leave open the possibility that the effect of Hh on Pax7 expression is mediated by an Hh-responsive cell type other than embryonic slow muscle fibers. We reasoned that if the effect of Hh on pax7 expression is cell-autonomous to myogenic precursors, cells incapable of responding to Hh would be more likely to remain Pax7-positive than cells that are capable of responding to Hh, when placed in an environment of Hh-responsive cells. In contrast, if the effect of Hh on Pax7 expression is mediated by another tissue, then Hh-unresponsive cells would behave the same as Hh-responsive cells, as long as their surrounding cells could respond to Hh. Therefore, we created mosaics by transplanting either wild-type or Hh unresponsive *smu(smo)*$^{-/-}$ cells into wild-type embryos, and determined the proportion of donor cells expressing Pax7. As we expected, transplanted donor cells gave rise to both muscle fibers and undifferentiated Pax7^{+} myogenic precursors by the end of the segmentation stage (Figs. 6A–F). However, the proportion of cells that maintained Pax7 expression was different depending on whether the donor cells could respond to Hh. *smu(smo)*$^{-/-}$ cells transplanted into wild-type embryos were significantly more likely to retain Pax7 expression at 24 h (Figs. 6A–C) than wild-type cells transplanted into wild-type embryos (Figs. 6D–F, Table 1; chi-square test: $p \leq 0.001$). We

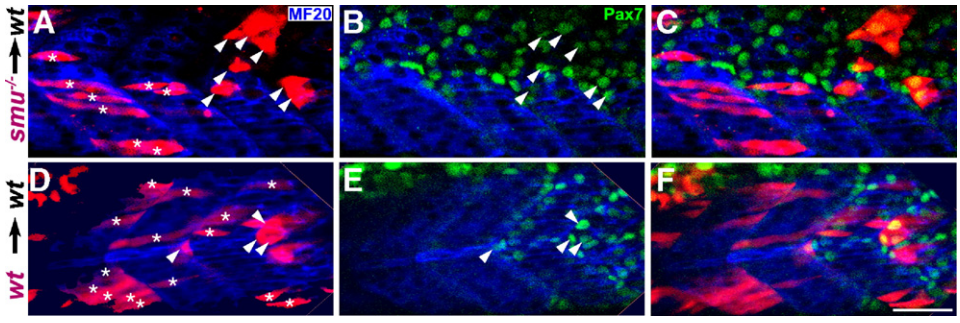

Fig. 6. Analysis of the cell autonomy of Hh signaling requirements for Pax7^{+} myogenic precursor regulation. Genetic mosaics were created by transplanting *smu (smo)*$^{-/-}$ cells (A–C) or wild-type cells (D–E) into wild-type embryos, embryos were labeled for myosin (MF20, blue) and Pax7 (green). Donor-derived somite cells (red) that are elongated and presumed to be muscle are labeled with asterisks; donor-derived somite cells that are Pax7-positive are indicated with arrowheads. Hh-unresponsive cells were more likely than wild-type cells to maintain Pax7^{+} expression (Table I). (A) Transplanted *smu(smo)*$^{-/-}$ somitic cells (red) formed differentiated muscle fibers (arrows) as well patches of cells on the external surface of differentiated muscle fibers (arrowheads) (B) Pax7^{+} myogenic precursor nuclei (green) in the same optical section as panel A. (C) The merged image of panels A and B, showing that patches overlap with several Pax7^{+} nuclei. (D) Transplanted wild-type somitic cells (red) which have differentiated into muscle fibers (arrows) and patches of cells on the external surface of differentiated muscle fibers. (E) Pax7^{+} myogenic precursor nuclei (green) in the same optical section as in panel D. (F) The merged image of panels D and E, showing that the patch of transplanted cells are Pax7^{+} myogenic precursors. All images are single optical sections at the same magnification; anterior to the left, dorsal up; scale bar=50 μm.

Table 1
Hh regulation of the number of myogenic precursors is cell autonomous to muscle precursors

Donor→Host	# embryos	Donor-derived somite cells (total)		
		% Pax7^{+} [a]	# Pax7^{+} [b]	# muscle fibers [c]
wt$^{\pm}$→wt	24	2.3%	21	889
smu$^{-/-}$→wt	21	7.8%	56	666

Every donor-derived somite cell was tested for Pax7 expression using antibody labeling (e.g. Fig. 6). The number of Pax7-positive cells and the number of elongated muscle fibers are shown. The difference between wt and *smu*$^{-/-}$ donor cells is significant (chi-square, $p \leq 0.001$).
wt: wild type. wt$^{\pm}$: includes both *smu(smo)*$^{+/-}$ and *smu(smo)*$^{+/+}$ embryos.
[a] Proportion of total donor-derived somite cells which were Pax7 positive.
[b] Pax7-positive myogenic precursor cells on the external surface of the somite, excludes neural crest cells. These are labeled with arrowheads in Fig. 6.
[c] Elongated cells within the somite, these are asterisked in Fig. 6. This is a conservative estimate of the number of nuclei, each fast fiber likely contains 2–4 nuclei at this stage (Kimmel and Warga, 1987).

conclude that the effect of Hh on the number of Pax7 expressing cells is a direct effect, and not mediated by another signal released by surrounding cells.

Discussion

We have demonstrated that Hh signaling promotes the differentiation of myogenic precursors from the zebrafish dermomyotome. Without Hh signaling, dermomyotome cells do not efficiently differentiate into fast muscle fibers, and instead continue to express high levels of *pax3* and *pax7* and remain in the anterior portion of each somite. We further demonstrated that the regulation of Hh is not mediated by its effects on embryonic slow muscle fibers, but that Hh acts directly on dermomyotome cells.

Myogenic pax3 gene expression and the zebrafish dermomyotome

Pax3 is expressed in the dermomyotome of birds and mammals, and in mouse is required for myogenesis (Tajbakhsh et al., 1997). Pax7 is also expressed in the dermomyotome and is required for the normal development of myogenic precursor cells underlying postnatal growth and injury repair (Relaix et al., 2005). Here we show that, as in amniotes (and for pax3 amphibians, Grimaldi et al., 2004), *pax3* and *pax7* are expressed in a group of lateral somitic cells in zebrafish (Fig. 1, also seen in Devoto et al., 2006; Groves et al., 2005; Seo et al., 1998). As in amniotes, *pax7* is turned on later than *pax3* (Otto et al., 2006). As in amniotes, mesodermal *pax3* and *pax7* expression is down-regulated during somite patterning, and at the end of segmentation persists only in somite cells external to the myotome (Fig. 1, Devoto et al., 2006). We suggest that *pax3/7* expression in the zebrafish somite also defines the zebrafish dermomyotome, which gives rise to fast muscle during the segmentation stage. We suspect that the zebrafish dermomyotome, like that of amniotes, also includes precursors to both fast and slow fibers that are added after the segmentation stage.

Hh signaling acts at multiple times to promote myogenic differentiation

Hh signaling acts at least twice during somite patterning to induce myogenic differentiation. These two effects of Hh can be distinguished in at least three significant ways. First, Hh acts on distinct target cells early than it does later. Early Hh signaling triggers differentiation of embryonic slow muscle fibers from a population of cells adjacent to the notochord (adaxial cells), that do not ever express detectable *pax3/7* (Blagden et al., 1997; Du et al., 1997; Seo et al., 1998). Later Hh signaling acts on non-adaxial somitic cells that likely do express *pax3*, promoting their ultimate differentiation into fast muscle fibers, probably in cooperation with other secreted signaling molecules. Second, the time when Hh signaling is required in each cell type can be experimentally separated. For embryonic slow muscle fibers, Hh signaling is only required very early in paraxial mesoderm maturation, at about the time that cells enter the segmental plate (Hirsinger et al., 2004). For regulation of the dermomyotome and Pax7 expression, Hh signaling is required considerably later, close to the time of somite formation. Third, the requirements for downstream *gli* genes in these two separate populations differ. While both groups of cells require Hh signaling mediated by *smu(smo)*, Pax7 expressing cells are not as severely affected as embryonic slow muscle fibers in *yot (gli2)* mutants. The *yot(gli2)* mutations encode a dominant repressor version of Gli2, which has been shown to block both Gli2 and Gli1 function (Karlstrom et al., 2003). The Pax7 expressing cells may not express the *gli2* gene (and thus would not express the dominant repressor), they may express *gli3* (Tyurina et al., 2005), which may not be as completely repressed by the *yot(gli2)* mutations (Karlstrom et al., 2003; Vanderlaan et al., 2005), or residual Gli activity remaining in *yot(gli2)* mutants may be sufficient for the later population of myogenic precursors, but insufficient for embryonic slow muscle development. In amniotes, *gli* genes are differentially expressed in the somite, and play overlapping but distinct roles in the development of the dermomyotome (Borycki et al., 2000; McDermott et al., 2005).

Hedgehog secreted from midline structures could act on all cells in the zebrafish somite. The *ptc2* Hh receptor gene is Hh responsive and expressed throughout the somite (Lewis et al., 1999). In the chick neural tube, Hh has been demonstrated to diffuse more than 200 μm (Briscoe et al., 2001; Gritli-Linde et al., 2001), the zebrafish somite is no more than 50 μm wide at 24 h. However, we suspect that Hh is not acting on the precursors to fast fibers while those cells are at the periphery of the myotome. The earliest pax3 expression is in most non-adaxial cells, and the earliest pax7 expression is in the anterior, non-adaxial cells (Fig. 1A). The Pax7^{+} cells or their precursors are sensitive to cyclopamine at about the time of segmentation (Fig. 5), which is prior to the displacement of embryonic slow fibers precursors (adaxial cells) away from the notochord. This model is consistent with the results of Wolff et al. (2003), who demonstrated that Hh acts at multiple times to regulate the expression of *engrailed*, first in embryonic slow fibers, and then in fast fibers.

Amniotes also have multiple, distinguishable waves of myogenic differentiation, but it is not yet clear whether Hh signaling is involved in one or more of them (Hadchouel et al., 2003). Myogenic regulatory factors are expressed prior to the establishment of a morphological dermomyotome (Pownall and Emerson, 1992). These cells are the likely precursors to early, post-mitotic slow muscle fibers with large nuclei (Cinnamon et al., 2006; Kahane et al., 1998b; Kahane and Kalcheim, 1998). This primary myotome then grows with the addition of new myotomal cells derived from the dermomyotome (Ordahl et al., 2000). Ectopic Shh applied to the dermomyotome leads to the up-regulation of *myoD* and down-regulation of *pax3*, which results in premature differentiation of myogenic precursors (Amthor et al., 1999). Ectopic Shh applied to myoblasts *in vitro* or to limb buds *in vivo* promotes myogenic differentiation, with a preferential effect on slow muscle differentiation and survival (Cann et al., 1999; Li et al., 2004). Loss of Hh leads to increased expression of *pax3* and reduced expression of *myf5* (Borycki et al., 1999). These effects of Hh in amniotes are thus more likely to be analogous to the second action of Hh in zebrafish, on *pax3/7* expressing dermomyotome cells. Whether there is also a separate, earlier effect of Hh on the embryonic myotome in amniotes has not been directly tested.

Hh acts directly on dermomyotome cells

Hh signaling regulates the differentiation of a number of different tissues in the trunk, including the dorsal aorta (Lawson and Weinstein, 2002), motor neurons (Lupo et al., 2006), and sclerotome (Dockter, 2000) in all studied vertebrates, and embryonic slow muscle fibers in zebrafish (Blagden et al., 1997; Du et al., 1997). Embryonic slow muscle fibers have been shown to regulate fast muscle fiber elongation in a Hh independent manner (Henry and Amacher, 2004). In amniotes, there is no unequivocal, direct evidence that Hh acts directly on the dermomyotome, and not via another tissue such as motor neurons or the dorsal aorta. The *myf5* promoter contains Gli binding sites that can respond to Hh signaling (Gustafsson et al., 2002), but it is not clear if this Hh response element is necessary for normal *myf5* regulation (Hadchouel et al., 2003). Hh-soaked beads in the embryo and soluble Hh in tissue culture can trigger myogenesis (Amthor et al., 1999; Cann et al., 1999), but this ectopic Hh but may be acting at non-physiological concentrations and/or may be exerting some of its effects via non-dermomyotome cells. Thus, it was important to determine if slow fibers or any of the other Hh dependent cell types were responsible for the effect of Hh signaling on dermomyotome *pax3/7* expression.

Embryonic slow muscle fibers are unlikely to play a major role in the regulation of the number of Pax7-positive cells. First, *yot(gli2)* mutants have a much smaller increase in the number of Pax7 cells than *smu(smo)* mutant embryos, even though both have a complete loss of slow muscle fibers. Second, blocking Hh signaling after slow muscle fibers were induced still triggered a large increase in the number of Pax7 cells. Third, we showed that wild-type cells were less likely than *smu(smo)* mutant cells to remain as Pax7-positive, undifferentiated cells when placed into a wild-type host somite (Fig. 6 and Table 1). These results strongly suggest that the regulation of $Pax7^+$ myogenic precursors is a direct effect of Hh and not mediated by other cell types. Transplantation of a few wild-type slow muscle fibers into an otherwise Hh unresponsive host somite was unable to rescue the Pax7 phenotype (Fig. 4). We did not test whether host muscle fiber elongation was rescued in these embryos. If elongation is rescued (Henry and Amacher, 2004), this would suggest that the excess $Pax7^+$ cells do not block elongation, and suggest that Hh has both direct and indirect effects on fast muscle differentiation.

Hedgehog as a regulator of proliferation and differentiation

The pax3 and pax7 genes are expressed in proliferative dermomyotome cells and then down-regulated as myogenic differentiation proceeds (Otto et al., 2006; Williams and Ordahl, 1994). The balance between proliferation and differentiation of dermomyotome cells may be regulated by Hh signaling (Amthor et al., 1999). Adaxial cells absolutely require Hh signaling to differentiate into embryonic slow muscle fibers (Barresi et al., 2000). However, we suspect that the $Pax7^+$ cells become muscles even without any Hh signaling, but do so less efficiently, because they might be delayed in exiting the cell cycle and/or in terminal differentiation. The increase in the number of $Pax7^+$ cells would then be a result of the prolonged expression in the dermomyotome, not the appearance of a novel population of $Pax7^+$ cells. Excess $Pax7^+$ cells do not undergo apoptosis later (data not shown), suggesting that they differentiate. The increase in the number of $Pax7^+$ cells also could be explained by the expression of Pax7 in a group of cells that normally do not express Pax7, such as the slow muscle precursors. We think this is unlikely because of the lack of correlation between the loss of slow muscle and the gain of $Pax7^+$ cells. Moreover, in the absence of Hh signaling, we have not observed Pax7 expression in the position where slow muscle fibers would normally develop, nor in the residual $Prox1^+$ nuclei remaining following late cyclopamine treatment (Fig. 6). There may be redundant mechanisms within every dermomyotome cell for myogenic differentiation, perhaps involving the appearance of other signals that can promote myogenic differentiation independently of Hh, or involving the disappearance of other signals that inhibit myogenic differentiation. Fgf8 was recently shown to promote fast muscle myogenesis (Groves et al., 2005); it will be important to learn whether Hh and Fgf8 are acting on the same or on different fast muscle precursors. We are currently testing whether other signaling pathways are also involved in the regulation of the dermomyotome in zebrafish. Lineage labeling of the zebrafish somite may indicate whether there are multiple, independent populations of myogenic precursors.

Acknowledgments

We thank Christina Hammond and Simon Hughes for sharing results prior to publication. We thank Anders Fjose for providing the *pax3* and *pax7* clones. We thank members of our laboratory, and Ann Burke, Laura Grabel, and Clarissa Henry

for valuable ideas during the course of this work, and for helpful comments on the manuscript. The MF20 hybridoma (Bader et al., 1982) and Pax7 hybridoma (Kawakami et al., 1997) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, The University of Iowa, Iowa City, IA 52242.Our work was supported by a Donaghue Investigator Award, and NIH grants R01 HD37509 and HD044929 to SHD.

References

Amthor, H., Christ, B., Patel, K., 1999. A molecular mechanism enabling continuous embryonic muscle growth—A balance between proliferation and differentiation. Development 126, 1041–1053.

Bader, D., Masaki, T., Fischman, D.A., 1982. Immunochemical analysis of myosin heavy-chain during avian myogenesis in vivo and in vitro. J. Cell Biol. 95, 763–770.

Barresi, M.J., Stickney, H.L., Devoto, S.H., 2000. The zebrafish slow-muscle-omitted gene product is required for Hedgehog signal transduction and the development of slow muscle identity. Development 127, 2189–2199.

Barresi, M.J., D'Angelo, J.A., Hernandez, L.P., Devoto, S.H., 2001. Distinct mechanisms regulate slow-muscle development. Curr. Biol. 11, 1432–1438.

Blaess, S., Corrales, J.D., Joyner, A.L., 2006. Sonic hedgehog regulates Gli activator and repressor functions with spatial and temporal precision in the mid/hindbrain region. Development 133, 1799–1809.

Blagden, C.S., Currie, P.D., Ingham, P.W., Hughes, S.M., 1997. Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog. Genes Dev. 11, 2163–2175.

Borycki, A.G., Mendham, L., Emerson Jr., C.P., 1998. Control of somite patterning by Sonic hedgehog and its downstream signal response genes. Development 125, 777–790.

Borycki, A.G., Brunk, B., Tajbakhsh, S., Buckingham, M., Chiang, C., Emerson Jr., C.P., 1999. Sonic hedgehog controls epaxial muscle determination through Myf5 activation. Development 126, 4053–4063.

Borycki, A., Brown, A.M., Emerson, C.P., 2000. Shh and Wnt signaling pathways converge to control Gli gene activation in avian somites. Development 127, 2075–2087.

Bren-Mattison, Y., Olwin, B.B., 2002. Sonic hedgehog inhibits the terminal differentiation of limb myoblasts committed to the slow muscle lineage. Dev. Biol. 242, 130–148.

Brent, A.E., Schweitzer, R., Tabin, C.J., 2003. A somitic compartment of tendon progenitors. Cell 113, 235–248.

Briscoe, J., Chen, Y., Jessell, T.M., Struhl, G., 2001. A hedgehog-insensitive form of patched provides evidence for direct long-range morphogen activity of sonic hedgehog in the neural tube. Mol. Cell 7, 1279–1291.

Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Montarras, D., Rocancourt, D., Relaix, F., 2003. The formation of skeletal muscle: from somite to limb. J. Anat. 202, 59–68.

Cann, G.M., Lee, J.W., Stockdale, F.E., 1999. Sonic hedgehog enhances somite cell viability and formation of primary slow muscle fibers in avian segmented mesoderm. Anat. Embryol. (Berl.) 200, 239–252.

Cayuso, J., Ulloa, F., Cox, B., Briscoe, J., Marti, E., 2006. The Sonic hedgehog pathway independently controls the patterning, proliferation and survival of neuroepithelial cells by regulating Gli activity. Development 133, 517–528.

Chen, J.K., Taipale, J., Cooper, M.K., Beachy, P.A., 2002. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev. 16, 2743–2748.

Christ, B., Ordahl, C.P., 1995. Early stages of chick somite development. Anat. Embryol. (Berl.) 191, 381–396.

Cinnamon, Y., Ben-Yair, R., Kalcheim, C., 2006. Differential effects of N-cadherin-mediated adhesion on the development of myotomal waves. Development 133, 1101–1112.

Cortes, F., Daggett, D., Bryson-Richardson, R.J., Neyt, C., Maule, J., Gautier, P., Hollway, G.E., Keenan, D., Currie, P.D., 2003. Cadherin-mediated differential cell adhesion controls slow muscle cell migration in the developing zebrafish myotome. Dev. Cell 5, 865–876.

Denetclaw Jr., W.F., Christ, B., Ordahl, C.P., 1997. Location and growth of epaxial myotome precursor cells. Development 124, 1601–1610.

Devoto, S.H., Barnstable, C.J., 1989. Expression of the growth cone specific epitope CDA 1 and the synaptic vesicle protein SVP38 in the developing mammalian cerebral cortex. J. Comp. Neurol. 290, 154–168.

Devoto, S.H., Melancon, E., Eisen, J.S., Westerfield, M., 1996. Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. Development 122, 3371–3380.

Devoto, S.H., Stoiber, W., Hammond, C.L., Steinbacher, P., Haslett, J.R., Barresi, M.J., Patterson, S.E., Adiarte, E.G., Hughes, S.M., 2006. Generality of vertebrate developmental patterns: evidence for a dermomyotome in fish. Evol. Dev. 8, 101–110.

Dockter, J.L., 2000. Sclerotome induction and differentiation. Curr. Top. Dev. Biol. 48, 77–127.

Du, S.J., Devoto, S.H., Westerfield, M., Moon, R.T., 1997. Positive and negative regulation of muscle cell identity by members of the hedgehog and TGF-beta gene families. J. Cell Biol. 139, 145–156.

Duprez, D., Fournier-Thibault, C., Le Douarin, N., 1998. Sonic Hedgehog induces proliferation of committed skeletal muscle cells in the chick limb. Development 125, 495–505.

Frank-Kamenetsky, M., Zhang, X.M., Bottega, S., Guicherit, O., Wichterle, H., Dudek, H., Bumcrot, D., Wang, F.Y., Jones, S., Shulok, J., Rubin, L.L., Porter, J.A., 2002. Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. J. Biol. 1, 10.

Goulding, M., Lumsden, A., Paquette, A.J., 1994. Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. Development 120, 957–971.

Grimaldi, A., Tettamanti, G., Martin, B.L., Gaffield, W., Pownall, M.E., Hughes, S.M., 2004. Hedgehog regulation of superficial slow muscle fibres in *Xenopus* and the evolution of tetrapod trunk myogenesis. Development 131, 3249–3262.

Gritli-Linde, A., Lewis, P., McMahon, A.P., Linde, A., 2001. The whereabouts of a morphogen: direct evidence for short- and graded long-range activity of hedgehog signaling peptides. Dev. Biol. 236, 364–386.

Gros, J., Scaal, M., Marcelle, C., 2004. A two-step mechanism for myotome formation in chick. Dev. Cell 6, 875–882.

Groves, J.A., Hammond, C.L., Hughes, S.M., 2005. Fgf8 drives myogenic progression of a novel lateral fast muscle fibre population in zebrafish. Development 132, 4211–4222.

Gustafsson, M.K., Pan, H., Pinney, D.F., Liu, Y., Lewandowski, A., Epstein, D.J., Emerson Jr., C.P., 2002. Myf5 is a direct target of long-range Shh signaling and Gli regulation for muscle specification. Genes Dev. 16, 114–126.

Hadchouel, J., Carvajal, J.J., Daubas, P., Bajard, L., Chang, T., Rocancourt, D., Cox, D., Summerbell, D., Tajbakhsh, S., Rigby, P.W., Buckingham, M., 2003. Analysis of a key regulatory region upstream of the Myf5 gene reveals multiple phases of myogenesis, orchestrated at each site by a combination of elements dispersed throughout the locus. Development 130, 3415–3426.

Henry, C.A., Amacher, S.L., 2004. Zebrafish slow muscle cell migration induces a wave of fast muscle morphogenesis. Dev. Cell 7, 917–923.

Hernandez, L.P., Patterson, S.E., Devoto, S.H., 2005. The development of muscle fiber type identity in zebrafish cranial muscles. Anat. Embryol. (Berl.) 209, 323–334.

Hirsinger, E., Stellabotte, F., Devoto, S.H., Westerfield, M., 2004. Hedgehog signaling is required for commitment but not initial induction of slow muscle precursors. Dev. Biol. 275, 143–157.

Huang, R., Christ, B., 2000. Origin of the epaxial and hypaxial myotome in avian embryos. Anat. Embryol. (Berl.) 202, 369–374.

Ingham, P.W., McMahon, A.P., 2001. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 15, 3059–3087.

Johnson, R.L., Laufer, E., Riddle, R.D., Tabin, C., 1994. Ectopic expression of Sonic hedgehog alters dorsal–ventral patterning of somites. Cell 79, 1165–1173.

Jowett, T., 1997. Tissue In Situ Hybridization: Methods in Animal Development. John Wiley and Sons, Inc, New York.

Kahane, N., Kalcheim, C., 1998. Identification of early postmitotic cells in distinct embryonic sites and their possible roles in morphogenesis. Cell Tissue Res. 294, 297–307.

Kahane, N., Cinnamon, Y., Kalcheim, C., 1998a. The cellular mechanism by which the dermomyotome contributes to the second wave of myotome development. Development 125, 4259–4271.

Kahane, N., Cinnamon, Y., Kalcheim, C., 1998b. The origin and fate of pioneer myotomal cells in the avian embryo. Mech. Dev. 74, 59–73.

Kalcheim, C., Cinnamon, Y., Kahane, N., 1999. Myotome formation: a multistage process. Cell Tissue Res. 296, 161–173.

Karlstrom, R.O., Tyurina, O.V., Kawakami, A., Nishioka, N., Talbot, W.S., Sasaki, H., Schier, A.F., 2003. Genetic analysis of zebrafish gli1 and gli2 reveals divergent requirements for gli genes in vertebrate development. Development 130, 1549–1564.

Kawakami, A., Kimura-Kawakami, M., Nomura, T., Fujisawa, H., 1997. Distributions of PAX6 and PAX7 proteins suggest their involvement in both early and late phases of chick brain development. Mech. Dev. 66, 119–130.

Kimmel, C.B., Warga, R.M., 1987. Cell lineages generating axial muscle in the zebrafish embryo. Nature 327, 234–237.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.

Kruger, M., Mennerich, D., Fees, S., Schafer, R., Mundlos, S., Braun, T., 2001. Sonic hedgehog is a survival factor for hypaxial muscles during mouse development. Development 128, 743–752.

Lawson, N.D., Weinstein, B.M., 2002. Arteries and veins: making a difference with zebrafish. Nat. Rev., Genet. 3, 674–682.

Lewis, K.E., Concordet, J.P., Ingham, P.W., 1999. Characterisation of a second patched gene in the zebrafish *Danio rerio* and the differential response of patched genes to hedgehog signalling. Dev. Biol. 208, 14–29.

Li, X., Blagden, C.S., Bildsoe, H., Bonnin, M.A., Duprez, D., Hughes, S.M., 2004. Hedgehog can drive terminal differentiation of amniote slow skeletal muscle. BMC Dev. Biol. 4, 9.

Lum, L., Beachy, P.A., 2004. The Hedgehog response network: sensors, switches, and routers. Science 304, 1755–1759.

Luo, R., An, M., Arduini, B.L., Henion, P.D., 2001. Specific pan-neural crest expression of zebrafish Crestin throughout embryonic development. Dev. Dyn. 220, 169–174.

Lupo, G., Harris, W.A., Lewis, K.E., 2006. Mechanisms of ventral patterning in the vertebrate nervous system. Nat. Rev., Neurosci. 7, 103–114.

Lyons, K.M., Hogan, B.L., Robertson, E.J., 1995. Colocalization of BMP 7 and BMP 2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development. Mech. Dev. 50, 71–83.

Marcelle, C., Ahlgren, S., Bronner-Fraser, M., 1999. In vivo regulation of somite differentiation and proliferation by sonic hedgehog. Dev. Biol. 214, 277–287.

McDermott, A., Gustafsson, M., Elsam, T., Hui, C.C., Emerson Jr., C.P., Borycki, A.G., 2005. Gli2 and Gli3 have redundant and context-dependent function in skeletal muscle formation. Development 132, 345–357.

McMahon, J.A., Takada, S., Zimmerman, L.B., Fan, C.M., Harland, R.M., McMahon, A.P., 1998. Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes Dev. 12, 1438–1452.

Munsterberg, A.E., Kitajewski, J., Bumcrot, D.A., McMahon, A.P., Lassar, A.B., 1995. Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. Genes Dev. 9, 2911–2922.

Ordahl, C.P., Williams, B.A., Denetclaw, W., 2000. Determination and morphogenesis in myogenic progenitor cells: an experimental embryological approach. Curr. Top. Dev. Biol. 48, 319–367.

Otto, A., Schmidt, C., Patel, K., 2006. Pax3 and Pax7 expression and regulation in the avian embryo. Anat. Embryol. (Berl.) 211, 293–310.

Pownall, M.E., Emerson Jr., C.P., 1992. Sequential activation of three myogenic regulatory genes during somite morphogenesis in quail embryos. Dev. Biol. 151, 67–79.

Relaix, F., Rocancourt, D., Mansouri, A., Buckingham, M., 2005. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. Nature 435, 948–953.

Rowlerson, A., Veggetti, A., 2001. Cellular mechanisms of post-embryonic muscle growth in aquaculture species. In: Johnston, I.A. (Ed.), Muscle Development and Growth, vol. 18. Academic Press, San Diego, pp. 103–140.

Roy, S., Wolff, C., Ingham, P.W., 2001. The u-boot mutation identifies a Hedgehog-regulated myogenic switch for fiber-type diversification in the zebrafish embryo. Genes Dev. 15, 1563–1576.

Seo, H.C., Saetre, B.O., Havik, B., Ellingsen, S., Fjose, A., 1998. The zebrafish Pax3 and Pax7 homologues are highly conserved, encode multiple isoforms and show dynamic segment-like expression in the developing brain. Mech. Dev. 70, 49–63.

Stickney, H.L., Barresi, M.J., Devoto, S.H., 2000. Somite development in zebrafish. Dev. Dyn. 219, 287–303.

Tajbakhsh, S., 2003. Stem cells to tissue: molecular, cellular and anatomical heterogeneity in skeletal muscle. Curr. Opin. Genet. Dev. 13, 413–422.

Tajbakhsh, S., Rocancourt, D., Cossu, G., Buckingham, M., 1997. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell 89, 127–138.

Te Kronnie, G., Reggiani, C., 2002. Skeletal muscle fibre type specification during embryonic development. J. Muscle Res. Cell Motil. 23, 65–69.

Tyurina, O.V., Guner, B., Popova, E., Feng, J., Schier, A.F., Kohtz, J.D., Karlstrom, R.O., 2005. Zebrafish Gli3 functions as both an activator and a repressor in Hedgehog signaling. Dev. Biol. 277, 537–556.

van Eeden, F.J., Granato, M., Schach, U., Brand, M., Furutani Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh, R.N., Mullins, M.C., Odenthal, J., Warga, R.M., Allende, M.L., Weinberg, E.S., Nusslein Volhard, C., 1996. Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*. Development 123, 153–164.

Vanderlaan, G., Tyurina, O.V., Karlstrom, R.O., Chandrasekhar, A., 2005. Gli function is essential for motor neuron induction in zebrafish. Dev. Biol. 282, 550–570.

Varga, Z.M., Amores, A., Lewis, K.E., Yan, Y.L., Postlethwait, J.H., Eisen, J.S., Westerfield, M., 2001. Zebrafish smoothened functions in ventral neural tube specification and axon tract formation. Development 128, 3497–3509.

Waterman, R.E., 1969. Development of the lateral musculature in the teleost, *Brachydanio rerio*: a fine structural study. Am. J. Anat. 125, 457–493.

Weinberg, E.S., Allende, M.L., Kelly, C.S., Abdelhamid, A., Andermann, P., Doerre, G., Grunwald, D.J., Riggleman, B., 1996. Developmental regulation of zebrafish *MyoD* in wild-type, *no tail*, and *spadetail* embryos. Development 122, 271–280.

Westerfield, M., 1995. The Zebrafish Book. University of Oregon Press, Eugene, OR.

Williams, B.A., Ordahl, C.P., 1994. Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. Development 120, 785–796.

Wolff, C., Roy, S., Ingham, P.W., 2003. Multiple muscle cell identities induced by distinct levels and timing of hedgehog activity in the zebrafish embryo. Curr. Biol. 13, 1169–1181.