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The zebrafish *slow-muscle-omitted* gene product is required for Hedgehog signal transduction and the development of slow muscle identity

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SUMMARY

Hedgehog proteins mediate many of the inductive interactions that determine cell fate during embryonic development. Hedgehog signaling has been shown to regulate slow muscle fiber type development. We report here that mutations in the zebrafish *slow-muscle-omitted* (*smu*) gene disrupt many developmental processes involving Hedgehog signaling. *smu*$^{-/-}$ embryos have a 99% reduction in the number of slow muscle fibers and a complete loss of Engrailed-expressing muscle pioneers. In addition, mutant embryos have partial cyclopia, and defects in jaw cartilage, circulation and fin growth. The *smu*$^{-/-}$ phenotype is phenocopied by treatment of wild-type embryos with forskolin, which inhibits the response of cells to Hedgehog signaling by indirect activation of cAMP-dependent protein kinase (PKA). Overexpression of Sonic hedgehog (Shh) or dominant negative PKA (dnPKA) in wild-type embryos causes all somitic cells to develop into slow muscle fibers. Overexpression of Shh does not rescue slow muscle fiber development in *smu*$^{-/-}$ embryos, whereas overexpression of dnPKA does. Cell transplantation experiments confirm that *smu* function is required cell-autonomously within the muscle precursors: wild-type muscle cells rescue slow muscle fiber development in *smu*$^{-/-}$ embryos, whereas mutant muscle cells cannot develop into slow muscle fibers in wild-type embryos. Slow muscle fiber development in *smu* mutant embryos is also rescued by expression of rat Smoothened. Therefore, Hedgehog signaling through Slow-muscle-omitted is necessary for slow muscle fiber type development. We propose that *smu* encodes a vital component in the Hedgehog response pathway.

Key words: Slow muscle, Muscle fiber type, Zebrafish, Muscle pioneer, Axial muscle, Hedgehog signaling, Slow-muscle-omitted, Smoothened, Patched, Protein kinase A, Forskolin.

INTRODUCTION

Vertebrate skeletal muscle fibers can be subdivided into multiple fiber types based on contraction speeds, innervation, metabolism, morphology and the expression of specific contractile proteins. Muscle cells become committed to specific fiber type identities very early in development (for reviews, see Hughes and Salinas, 1999; Stockdale, 1992). However, the signals that regulate embryonic fiber type development in amniotes are unknown. Recent work in zebrafish suggests that some of the positive and negative signals from surrounding tissues that are known to influence myogenesis may also influence the development of muscle fiber type identity (Blagden et al., 1997; Currie and Ingham, 1996; Du et al., 1997; for a recent review, see Currie and Ingham, 1998).

Zebrafish have three distinct embryonic muscle fiber types: muscle pioneer slow muscle fibers, non-pioneer slow muscle fibers and fast muscle fibers. Slow muscle fibers develop from adaxial cells, which are adjacent to the notochord in the segmental plate. These cells are the first to express myogenic transcription factors such as MyoD (Weinberg et al., 1996) and the first to differentiate into muscle fibers (Devoto et al., 1996; van Raamsdonk et al., 1978). Adaxial cells begin to elongate while adjacent to the notochord and then migrate radially to the surface of the somite, forming a superficial monolayer of embryonic slow twitch muscle fibers (Devoto et al., 1996). A small subset of these cells express Engrailed proteins and are known as muscle pioneer slow muscle cells (Felsenfeld et al., 1991; Hatta et al., 1991). Muscle pioneers span the somite from its medial to its lateral surface at the future position of the horizontal myoseptum, which establishes a separation between the dorsal and ventral myotome (Waterman, 1969). The position of slow muscle precursors adjacent to the notochord suggests that notochord signaling may play a role in their development. In support of this, mutants with disrupted notochord development have a loss of muscle pioneers, and muscle pioneer development can be rescued by transplanting wild-type notochord cells into mutant embryos (Halpern et al., 1993). Non-pioneer slow muscle cells are also dependent on notochord signaling (Blagden et al., 1997). After adaxial cell migration to the surface of the myotome, fast muscle fibers develop from cells that were initially lateral to adaxial cells in the segmental plate. Their development does not depend on notochord signaling (Blagden et al., 1997).

Sonic hedgehog (Shh) is a secreted protein that underlies

many of the notochord signaling properties. Hedgehog (Hh) gene family members have been proposed to play critical roles in many diverse biological processes, ranging from segmentation in insects to cancer in humans (reviewed by Hammerschmidt et al., 1997). In every system that has been analyzed, components of the Hh signaling pathway are conserved. The Hh receptor Patched (Ptc) is a 12-pass transmembrane protein that forms a heterodimer with Smoothened, a 7-pass transmembrane protein with homology to G-protein coupled receptors (Chen and Struhl, 1998; Murone et al., 1999; Stone et al., 1996). When Hh binds to Ptc, it inhibits Ptc's repression of Smoothened, enabling Smoothened to signal to downstream Hh signaling components such as Cubitus interruptus (Ci) in *Drosophila* and its homologs Gli1, Gli2 and Gli3 in vertebrates (for recent reviews of the mechanism of Hh signal transduction, see Ingham, 1998; Johnston and Scott, 1998; McMahon, 2000). Hh signaling can be modulated by the activity of cAMP-dependent protein kinase (PKA), an antagonist to Hh signaling in vertebrates and in *Drosophila* (Hammerschmidt et al., 1997; Perrimon, 1995).

Ectopic overexpression of Hh in zebrafish is sufficient to transform the entire myotome into slow muscle (Blagden et al., 1997; Du et al., 1997). Inhibition of PKA signaling is also sufficient to induce slow muscle fibers, while hyperactivation of PKA blocks slow muscle development (Du et al., 1997; Hammerschmidt et al., 1996). From these data, we and others have proposed that Hh signaling specifies fiber type identity in the early zebrafish embryo (Blagden et al., 1997; Currie and Ingham, 1996; Du et al., 1997).

In this paper, we have used genetic and pharmacological approaches to further characterize the role of Hh in slow muscle development. We introduce a gene, *slow-muscle-omitted* (*smu*), that is necessary for slow muscle fiber type development. *smu* function is required for the response of muscle precursors to Hh, but not for their response to the inhibition of PKA or the overexpression of Smoothened. We discuss the implications of these data with respect to the Hh signaling pathway and the development of vertebrate muscle fiber type identity.

MATERIALS AND METHODS

Animals and mutagenesis

Wild-type embryos were obtained from the Oregon AB line, which was maintained in the Wesleyan University zebrafish colony (details of animal husbandry are available on request). Embryos were staged by hours (h) or days (d) post-fertilization at 28.5°C (Kimmel et al., 1995; available on the World Wide Web: http://zfish.uoregon.edu/).

Mutations were induced with N-ethyl-N-nitrosourea (ENU), following published procedures (Riley and Grunwald, 1995). Embryos were screened for morphological defects through the ongoing screen at the University of Oregon.

Two independent alleles of ***s**low-**mu**scle-omitted* (*smu*) were identified, b577 and b641. Both alleles have been maintained on the AB background, with at least three outcrosses from the original stock of mutants. Both alleles appear to have identical phenotypes and all experiments were done at least once on each allele. Both mutations are recessive lethals inherited in simple Mendelian ratios, and all phenotypes are fully penetrant. Each phenotype that we have labeled as $smu^{-/-}$ was present in 1/4 of the embryos from a cross of two heterozygous carriers.

By complementation analysis, *smu* is not allelic to *helix, you-too, iguana* or *you*; no mutant embryos were seen in a cross of an identified *smu* mutant carrier and carriers of mutant alleles of these genes. The only other mutants that mildly resemble *smu* mutants, *uboot* and *sonic you*, are rescued by *shh* mRNA injections (Schauerte et al., 1998), indicating that these genes are distinct from *smu* (see Figs 3, 4, below).

Plasmids, in vitro mRNA synthesis and microinjection

Zebrafish *shh* and mouse *dnPKA* RNAs were transcribed from DNA plasmids T7TS*shh* and CS2+*dnPKA-bGFP*, respectively (Ekker et al., 1995a; Ungar and Moon, 1996). Capped mRNAs were transcribed from linearized DNA template with a T7 (*shh*) and SP6 (*dnPKA*) RNA polymerase in vitro transcription kit (mMESSAGE mMACHINE T7 or SP6, Ambion, Inc., Austin, TX, USA) according to the manufacturer's instructions. Rat *smoothened* cDNA under the control of the CMV promoter (pRK5.rsmoflag) was obtained from Donna Stone at Genentech (Stone et al., 1996).

For microinjections, mRNA and DNA were dissolved in double distilled H_2O to final concentrations of 150-200 μg/ml and 17 μg/ml, respectively. Phenol Red was added to the solution at a final concentration of 0.1% to facilitate visualization during microinjections. Approximately 2 nl of RNA or DNA was microinjected into the yolk of zebrafish embryos at the one- or two-cell stage using published procedures (Westerfield, 1995).

Forskolin treatment of wild-type embryos

Wild-type embryos at specific stages were treated with 0.3 mM forskolin dissolved in 4% DMSO in embryo medium. To examine the effect of forskolin on eye separation, slow muscle fibers, and *myoD*, *ptc1* and *shh* expression in the notochord, wild-type embryos were dechorionated and soaked in forskolin solution from 5.5h to the desired stage. For *shh* expression in the limb buds, forskolin treatment began at 8h, whereas for cartilage staining and pectoral fin outgrowth, treatment began at 24h. 100% of the treated animals exhibited the phenotype shown. Wild-type controls consisted of corresponding treatments with embryo medium alone and 4% DMSO in embryo medium. There were no differences between controls in the phenotypes assessed.

Transplantations

We created genetic mosaics between wild-type and mutant embryos essentially as described (Ho and Kane, 1990). Donor embryos were injected at the 1- to 4-cell stage with lysinated rhodamine dextran (10,000 kDa, Molecular Probes). Between 3h and 5h, 10-50 cells were transplanted from these embryos into similarly staged embryos. Transplant pipettes were made on a sanding disk constructed from a discarded hard drive coated with diamond lapping film. Transplantations were done using an Olympus SZX12 dissecting microscope. At 24h, the $smu^{-/-}$ embryos were identified on the basis of partial cyclopia and the U-shape of their somites. Embryos were fixed and sectioned on a cryostat; sections were then labeled with F59 to identify muscle fiber type (Devoto et al., 1996). Slow and fast muscle fibers derived from donor cells were counted in every third section in all cases (Table 1).

Antibodies

F59 is an IgG1 monoclonal antibody raised against chicken myosin (Crow and Stockdale, 1986) that labels slow muscle strongly and fast muscle faintly in zebrafish (Devoto et al., 1996). S58 is an IgA monoclonal antibody specific for slow isotypes of myosin heavy chain in chicken (Crow and Stockdale, 1986) and slow muscle fibers in zebrafish (Devoto et al., 1996). Tissue culture supernatants of F59 and S58 were generously provided by Frank Stockdale at Stanford University and used at a dilution of 1:10. The IgG monoclonal antibody zm4 is specific for fast muscle fibers in zebrafish; supernatants were generously provided by Monte Westerfield at the University of Oregon and used at a dilution of 1:5. 4D9 is an IgG1

monoclonal antibody, generated against the *Drosophila invected* homeodomain (Patel et al., 1989), which recognizes Engrailed proteins in zebrafish (Ekker et al., 1992; Hatta et al., 1991); 4D9 supernatant was obtained from the Developmental Studies Hybridoma Bank and diluted 1:3. Secondary antibodies from Sigma were used as follows: horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG at a dilution of 1:200, HRP-conjugated goat anti-mouse IgA at 1:100, TRITC-conjugated goat anti-mouse IgG at 1:200, and FITC-conjugated goat anti-mouse IgA at 1:100.

Immunocytochemistry, in situ hybridization and histology

Antibody labeling with F59 and 4D9 was carried out as previously described (Du et al., 1997; Hatta et al., 1991), with a few minor modifications. Briefly, embryos were fixed in 4% paraformaldehyde in PB (0.05 M phosphate buffer, pH 7.0) for 3 hours at room temperature (RT), or overnight at 4°C. Embryos were washed in 0.1 M PB (2× 5 minutes), rinsed in 50% methanol and soaked in 100% methanol at –20°C for at least 20 minutes. Embryos were rehydrated with 50% methanol (1× 5 minutes) followed by PBS-Tw (0.1% Tween20 in PBS, 1× 5 minutes) and incubated with 0.5% Triton X-100 (45 minutes, RT). Subsequent steps of antibody labeling were as described (Du et al., 1997).

Antibody labeling with S58 and zm4 was performed on embryos fixed with Carnoys fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid). Embryos were rehydrated through 95, 85, 70, 50 and 30% ethanol (each for 10 minutes, RT) into distilled H_2O or PB. Whole-mount S58 labeling was performed as described for F59 above, except that a goat anti-mouse IgA secondary antibody was used (Du et al., 1997). Labeling on sections was carried out as previously described (Devoto et al., 1996).

To count slow muscle fibers for the general description of *smu*$^{-/-}$ and forskolin-treated embryos, 24h-26h embryos were labeled with F59 or S58 in whole mount. Slow muscle fibers were identified on the basis of position (superficial in the myotome) and intensity of antibody labeling. For the counts shown in Figs 4 and 7, only S58 was used, and any labeling was counted as a complete muscle fiber. In these cases, individual embryos were separated and coded; the number of fibers in each embryo was counted ‘blind’, i.e. without knowing whether the embryo had been injected or not.

In situ hybridization of zebrafish embryos was performed using published procedures (Jowett, 1997). Vertebral and jaw cartilages of 3.5d embryos were visualized with Alcian Blue as described (Schilling et al., 1996).

Imaging

Whole-mount embryos were viewed and photographed using either Nomarski (DIC) optics on a Zeiss Axioskop compound microscope or with an Olympus SZX12 stereo microscope. Sections were photographed at 40× magnification using a fluorescence microscope. Rhodamine, fluorescein and DAPI or Hoechst images were overlaid using Adobe Photoshop. For comparisons between wild-type, mutant and treated embryos, photography and image manipulations were done identically and on the entire image.

RESULTS

In all species and systems that have been tested, activation of PKA inhibits Hh signaling, while inhibition of PKA mimics Hh signaling (Ingham, 1998; Perrimon, 1995). PKA can be activated pharmacologically using agents such as forskolin that increase cellular cAMP levels by direct stimulation of adenylyl cyclase (Seamon and Daly, 1981; Tesmer and Sprang, 1998). We reasoned that if mutations in *slow-muscle-omitted* (*smu*) disrupted all Hh signaling, the phenotype should resemble that of forskolin-treated embryos. We have therefore compared these two phenotypes in detail, focusing on muscle fiber type development.

Phenotypes in *slow-muscle-omitted* mutants resemble those resulting from a deficiency in Hedgehog signaling

24h wild-type embryos had straight tails and chevron-shaped somites (Fig. 1A). In contrast, *smu*$^{-/-}$ and forskolin-treated embryos had ventrally curved tails and U-shaped somites (Fig. 1B,C). Morphologically distinct muscle pioneers were absent in both *smu*$^{-/-}$ and forskolin-treated embryos (Fig. 1A-C; data not shown). Both *smu*$^{-/-}$ and forskolin-treated embryos had varying degrees of ventral cyclopia (Fig. 1D,F,H) and a loss of head cartilage as compared to wild-type embryos (Fig. 1E,G,I). They also both had circulation defects, leading to cardiac edema and death by 5d (data not shown).

Shh is expressed in the posterior limb bud region known as the zone of polarizing activity (Roelink et al., 1994) and is necessary for limb bud outgrowth and anterior-posterior polarity in the limb (Neumann et al., 1999; for a review, see Tickle, 1995). *shh* was expressed appropriately in the fin buds of wild-type, *smu*$^{-/-}$ and forskolin-treated embryos (Fig. 1J,L,N). At about 3d, however, *smu*$^{-/-}$ and forskolin-treated pectoral fins were severely reduced in size as compared to wild-type fins (Fig. 1K,M,O).

In zebrafish, as in other vertebrates, *shh* is expressed in the axial mesoderm just after the beginning of gastrulation (Krauss et al., 1993). As the axial mesoderm differentiates into the notochord during the segmentation period, it continues to express *shh*. *smu*$^{-/-}$ and forskolin-treated embryos expressed *shh* in the notochord at levels comparable to wild-type (Fig. 1P,R,T). *tiggy winkle hedgehog* and *echidna hedgehog* were also expressed normally in mutant and treated embryos (data not shown). The development of the hypochord and floor plate was apparently normal in mutant embryos (data not shown, see also Schauerte et al., 1998).

The types of defects in *smu* mutant embryos and their similarity to defects in forskolin-treated embryos suggested that *smu* mutations are disrupting Hh signaling. An early response to Hh signaling in zebrafish is the transcriptional activation of *patched1* (*ptc1*) (Concordet et al., 1996; Lewis et al., 1999a). In wild-type embryos, *ptc1* was expressed at high levels in mesodermal cells immediately adjacent to the notochord (Fig. 1P,Q; Concordet et al., 1996). *smu* mutant and forskolin-treated embryos did not express detectable *ptc1* mRNA in the paraxial mesoderm (Fig. 1R-U).

slow-muscle-omitted$^{-/-}$ and forskolin-treated embryos have defects in fiber type development

Three muscle fiber types develop during the segmentation period of zebrafish embryogenesis: fast muscle, slow muscle and muscle pioneer slow muscle cells. These three fiber types can be unambiguously identified using monoclonal antibodies. The S58 antibody exclusively labels muscle pioneer and non-pioneer slow muscle cells. The F59 antibody preferentially labels both types of slow muscle fibers, but also weakly labels fast muscle cells (Devoto et al., 1996). Using these two antibodies to label slow muscle fibers at 24-26h for counting, we found that all wild-type embryos had over 1000 slow muscle fibers per embryo (approximately 20 fibers per somite), whereas *smu* mutants had on average 11.3±0.5 (n=498) and

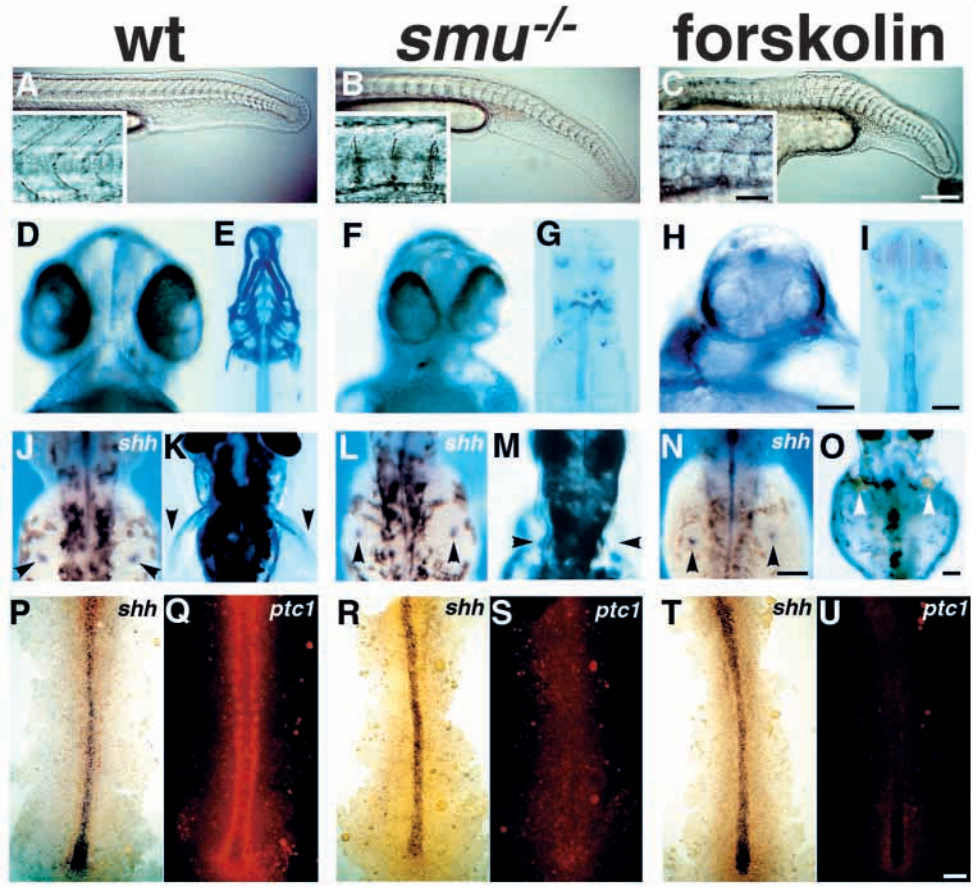

Fig. 1. *smu* mutant phenotypes resemble those seen in forskolin-treated embryos. (A-C) Side views of 24h live embryos; insets show higher magnification views of somite morphology. Wild-type embryos (A) have typical chevron-shaped somites while *smu*$^{-/-}$ (B) and forskolin-treated (C) embryos have blocky, U-shaped somites and ventrally curled tails. (D,F,H) Ventral views of live 48h embryos. The eyes in wild-type embryos (D) are separated, whereas *smu*$^{-/-}$ (F) and forskolin-treated (H) embryos have partial cyclopia. (E,G,I) Ventral views of Alcian Blue-stained 3.5d embryos. Jaw, head, fin and trunk cartilage is well developed in wild-type embryos (E), while *smu*$^{-/-}$ (G) and forskolin-treated (I) embryos have a nearly complete loss of jaw and head cartilage. Trunk cartilage is similar in wild-type, *smu*$^{-/-}$ and forskolin-treated embryos. (J,L,N) Dorsal views of 36h embryos labeled by in situ hybridization for *shh*. The fin buds (arrowheads) of wild-type (J), *smu*$^{-/-}$ (L), and forskolin-treated (N) embryos all express *shh* in a similar pattern. (K,M,O) Dorsal views of live 3d embryos. Pectoral fins (arrowheads) are well developed in wild-type embryos (K), whereas pectoral fins are severely reduced in *smu*$^{-/-}$ (M) and forskolin-treated (O) embryos. (P-U) Dorsal views of *shh* (blue) and *ptc1* (red) expression in 12h embryos. A white light (P,R,T) and a fluorescence (Q,S,U) image of each embryo is shown. Wild-type (P), *smu*$^{-/-}$ (R), and forskolin-treated (T) embryos express *shh* similarly in the notochord. Wild-type (P,Q) embryos express high levels of *ptc1* in paraxial mesoderm cells adjacent to the notochord. *smu*$^{-/-}$ (R,S) and forskolin-treated (T,U) embryos do not express detectable *ptc1* in the paraxial mesoderm. All images are oriented such that anterior is to the left (side views) or to the top (dorsal and ventral views). Bars: 200 μm (A-C); 50 μm (A-C insets); 50 μm (D,F,H); 100 μm (E,G,I); 100 μm (J,L,N); 100 μm (K,M,O); 50 μm (P-U).

forskolin-treated wild-type embryos had on average 10.8±4.6 (*n*=23) fibers per embryo (Fig. 2A-C) (values are means ± s.e.m.). Engrailed proteins were expressed in muscle pioneer slow muscle fibers, but not in other slow muscle fibers, in 24h wild-type embryos (Fig. 2D; Hatta et al., 1991). No Engrailed expression was detectable in the trunk of *smu*$^{-/-}$ or forskolin-treated embryos (Fig. 2E,F), though Engrailed was expressed appropriately at the mid-hindbrain junction in these animals (data not shown).

Slow muscle fibers develop from adaxial cells adjacent to the notochord (Devoto et al., 1996). To determine if adaxial cells are initially present in *smu*$^{-/-}$ embryos, we examined *myoD* mRNA levels in wild-type and *smu*$^{-/-}$ embryos. At the 6-somite stage, *myoD* was expressed in the adaxial cells still adjacent to the notochord in the segmental plate (Fig. 2G; Weinberg et al., 1996). Mutant embryos lacked all adaxial cell staining except for very faint labeling in the posteriormost region of the segmental plate (Fig. 2H), whereas somitic labeling was apparently normal. Forskolin-treated embryos had a similar loss of adaxial *myoD* expression (Fig. 2I), although somitic staining was somewhat more disrupted in forskolin-treated embryos than in *smu*$^{-/-}$ embryos.

We used slow and fast muscle cell-specific antibodies to examine the differentiation of fast muscle fibers and to further characterize slow fibers. S58 is specific for slow muscle in zebrafish (Devoto et al., 1996), while zm4 is specific for fast muscle in zebrafish (Fig. 2J; M. Westerfield, personal communication). In sections of wild-type embryos approximately 20 slow muscle fibers in each somite formed a superficial monolayer bordering the zm4 staining of fast muscle fibers (Fig. 2J). Sections of *smu*$^{-/-}$ and forskolin-treated embryos were almost entirely devoid of S58-labeled slow muscle fibers (Fig. 2K,L). In contrast, zm4-labeled fast muscles were still present, suggesting that fast muscle development is not as dependent on *smu* function as is slow muscle. However, there was a variable reduction in the amount of fast muscle in both *smu*$^{-/-}$ and forskolin-treated embryos (compare Figs 2K,L, 3E,F, 6E,F); further experiments will be necessary to determine if this is due to an effect on cell proliferation or cell growth, leading to a smaller number or a smaller size of fast muscle fibers.

Shh overexpression does not rescue slow muscle in *slow-muscle-omitted*$^{-/-}$ or forskolin-treated embryos

Ectopic overexpression of Shh in wild-type embryos is sufficient to induce ectopic slow muscle cells in the paraxial

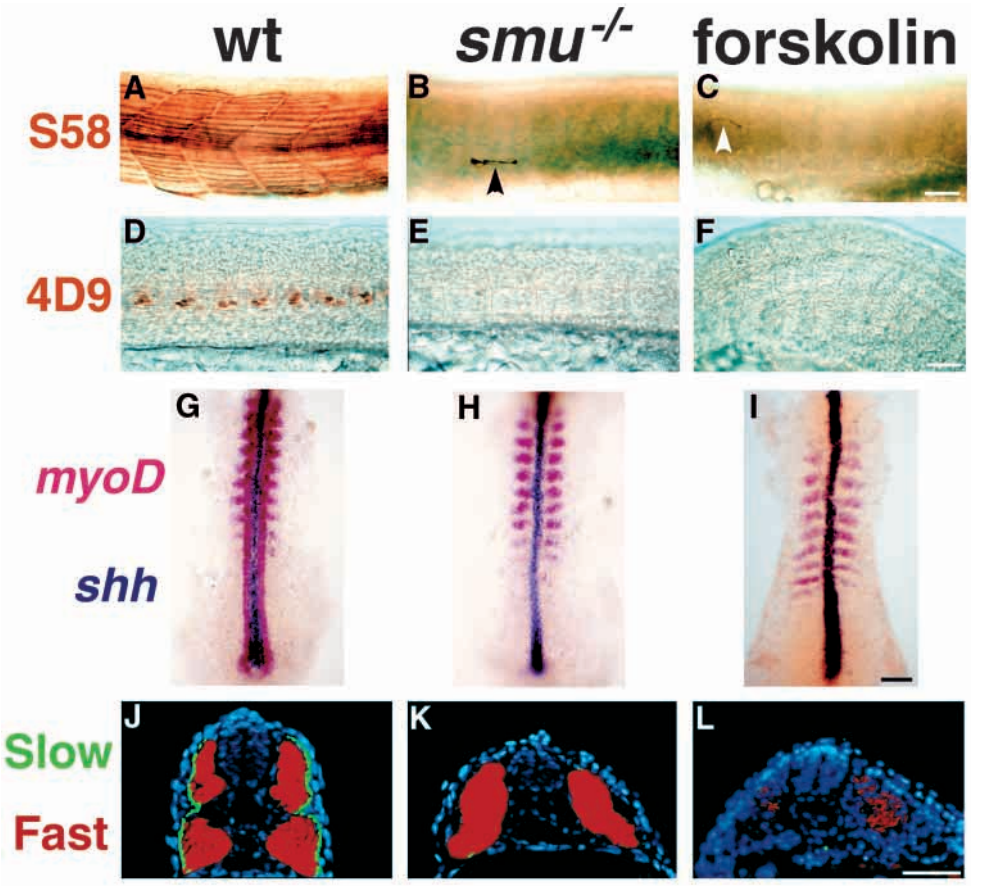

Fig. 2. *smu*$^{-/-}$ and forskolin-treated embryos have defects in slow muscle fiber type development. (A-C) Side views of S58 antibody labeled 24h embryos. S58 specifically labels slow muscle fibers. In wild-type embryos (A) approximately 20 slow muscle fibers span each chevron-shaped somite very clearly. In *smu*$^{-/-}$ (B) and forskolin-treated (C) embryos, slow muscle is markedly reduced; individual slow muscle fibers are marked with arrowheads. (D-F) Side views of 4D9 Engrailed antibody labeling of muscle pioneer slow muscle fibers at 24h. 4D9 labels 2-5 muscle pioneer nuclei per somite in wild-type (D), while *smu*$^{-/-}$ (E) and forskolin-treated (F) embryos have a complete loss of muscle pioneer slow muscle cells. (G-I) Dorsal view of 12h-14h embryos hybridized to show *myoD* (red) and *shh* (dark blue) expression. *MyoD* is expressed in wild-type embryos (G) in the somites and in the adaxial cells of the presomitic mesoderm. *smu*$^{-/-}$ (H) and forskolin-treated (I) embryos express *myoD* in the somites, but do not have any expression in cells adjacent to the notochord. (J-L) Transverse sections through the trunk of 30h embryos labeled with S58 (green) and zm4 (red) monoclonal antibodies to identify slow and fast muscle fibers, respectively. In sections of wild-type embryos (J), slow muscle fibers form a monolayer around the deeper fast muscle fibers. *smu*$^{-/-}$ (K) and forskolin-treated (L) embryos have a nearly complete loss of S58 labeling throughout the entire trunk, whereas zm4 staining is still present. Bars, 50 μm (A-F); 50 μm (G-I); 100 μm (J-L).

mesoderm (Blagden et al., 1997; Du et al., 1997). To determine if *smu*$^{-/-}$ muscle precursors are able to respond to Hh, we overexpressed Shh by microinjection of mRNA at the 1- to 4-cell stage. We also microinjected *shh* into embryos that were subsequently treated with forskolin at 5.5h. Embryos were labeled by whole-mount RNA in situ hybridization for *myoD* at 12h, and at 30h sections were labeled with S58 and zm4 monoclonal antibodies to identify slow and fast muscle fibers, respectively. Overexpression of Shh did not have any effect on *smu* mutant embryos. In a cross of two heterozygous carriers, 3/4 of the embryos had both normal adaxial *myoD* labeling and ectopic labeling in the lateral presomitic cells induced by the ectopic *shh* (Fig. 3A), whereas the remaining 1/4 of the embryos had no *myoD* labeling in adaxial cells and no expansion of myoD labeling into the lateral presomitic cells (Fig. 3B). Labeling of slow and fast muscle fibers at 30h showed the same patterns: *smu* mutant embryos did not respond to ectopic overexpression of Shh (Fig. 3D,E). We also

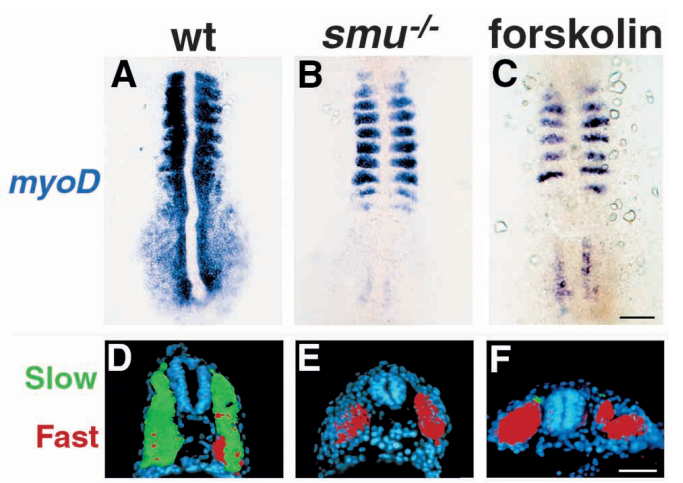

Fig. 3. Shh overexpression in *smu*$^{-/-}$ and forskolin-treated embryos does not rescue slow muscle development. (A-C) *shh* mRNA injected embryos were fixed at 12h and labeled by in situ hybridization for *myoD*; dorsal views are shown. In 3/4 of the embryos from a cross of 2 *smu* heterozygotes (60/86), *myoD* expression is expanded into the segmental plate as a result of Shh overexpression (A). In 1/4 of the embryos (26/86), *myoD* expression is not affected by Shh overexpression (B). 100% of the forskolin-treated embryos failed to respond to Shh overexpression (C). (D-F) *shh* mRNA injected embryos were fixed at 30h and transverse sections were labeled for S58 (green) and zm4 (red). In wild-type embryos (D), most of the zm4-labeled fast muscle (red) is replaced by S58-labeled slow muscle (green), compare to Fig. 2J. Slow muscle is not rescued by Shh overexpression in *smu*$^{-/-}$ (E) or in forskolin-treated (F) embryos; compare to Fig. 2K,L. See also Fig. 4. Bars, 100 μm (A-C); 50 μm (D-F).

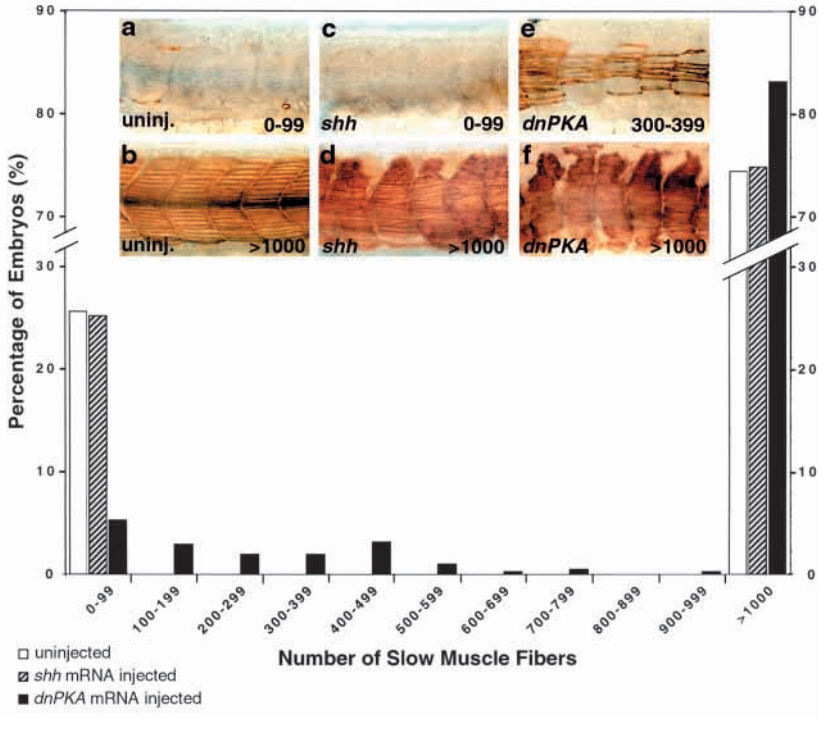

Fig. 4. Quantitative analysis of slow muscle fiber numbers in uninjected, *shh* injected and *dnPKA* injected embryos from a cross of two $smu^{+/-}$ animals. S58-labeled slow muscle fibers in uninjected and injected embryos were counted blind, and fiber tallies binned in sets of 100 for clarity of presentation. Uninjected siblings of *shh* injected, *dnPKA* injected and *rsmo* injected embryos (Fig. 7) were pooled. *shh* injection (n=453) has no effect on the expected Mendelian proportion of mutant embryos; as in uninjected embryos (n=1219), approximately 25% had <100 fibers, and approximately 75% had >1000 fibers. Conversely, only 5% of the embryos injected with *dnPKA* exhibited <100 fibers (black bars, n=421). Numbers of dead and malformed embryos (in which we did not count fibers) for injected embryos and their siblings were very similar (*shh* injected: 58 of 511, siblings 34 of 607; *dnPKA* injected 10 of 431, siblings 29 of 651). Although we saw variation between clutches of uninjected embryos in the number of slow muscle fibers per embryo, among embryos with <1000 slow muscle fibers *shh* injection did not affect the number of fibers within clutches, whereas *dnPKA* injection consistently increased the mean number of slow muscle fibers. *shh*-injected embryos had an average of 3.9±0.14 slow muscle fibers per fish (n=114), and their uninjected siblings had an average of 3.2±0.3 (n=154). This difference is not significant (t-test, P≤0.10). *dnPKA* injected embryos had an average of 259.0±25.6 slow muscle fibers per fish (n=71), while their uninjected siblings had an average of 8.3±0.6 (n=162). This difference is significant (t-test, P≤0.0005). Values are means ± s.e.m. Insets depict representative embryos for uninjected controls with 0-99 (a) and >1000 (b) fibers, *shh* injected embryos with 0-99 (c) and >1000 (d) fibers, and *dnPKA* injected embryos with 300-399 (e) and >1000 (f) fibers.

counted whole-mount, S58-labeled slow muscle fibers in *shh*-injected embryos to test for any quantitative rescue by Shh overexpression. 25% of both uninjected and *shh*-injected embryos had <100 fibers, while 75% had >1000 (Fig. 4). We conclude that slow muscle in mutant embryos is unaffected by *shh* overexpression.

Shh overexpression in forskolin-treated embryos also had no effect on *myoD* expression at 12h (Fig. 3C), or on slow muscle differentiation at 30h (Fig. 3F). The observation that treatment with forskolin at 5.5h blocked the effect of very early overexpression of Hh resulting from mRNA injections at the 1-2 cell stage suggests that cells are unable to respond to Hh overexpression until after 5.5h, when endogenous Hh is normally expressed. Neither of the likely Hh receptors, Ptc1 or Ptc2, is expressed before 8h, supporting this conclusion (Concordet et al., 1996; Lewis et al., 1999a).

Table 1. *smu* is required cell-autonomously

	Donor-derived muscle cells	
Donor→Host	Slow	Fast‡
wt*→wt* (n=31)	450	1136
wt→$smu^{-/-}$ (n=10)	96	201
$smu^{-/-}$→wt (n=17)	0	663

The fiber type identity of every donor-derived muscle cell was determined by labeling with F59. The number of individual slow and fast fibers are shown. n, the number of host embryos examined.

*wt, wild type: includes both $smu^{+/-}$ and $smu^{+/+}$ embryos.

‡This is a conservative estimate of the number of fast muscle fibers; individual fast fibers were sometimes difficult to distinguish from each other.

slow-muscle-omitted function is required in slow muscle precursors

The above results suggest that *smu* function is required for muscle precursor response to Shh. To determine if the *smu* gene product is necessary in muscle cells, we transplanted 10-50 cells from wild-type into mutant embryos, from mutant into wild-type embryos and from wild-type into wild-type embryos. We then assayed the fiber type identity of donor-derived muscle cells by F59 antibody labeling. We expected that donor cells that developed adjacent to the notochord would develop into slow muscle fibers, whereas donor cells positioned more laterally in the segmental plate would develop into fast muscle cells. As displayed in Table 1, we found that about 1/3 of transplanted wild-type muscle precursor cells developed into slow muscle fibers when placed into wild-type hosts. Roughly the same proportion of wild-type donor muscle precursors developed into slow muscle fibers when placed into $smu^{-/-}$ hosts. In striking contrast, none of the muscle precursors derived from a $smu^{-/-}$ donor developed into slow muscle fibers in wild-type hosts. The rescue of slow muscle by wild-type cells in mutant hosts occurred irrespective of whether other, non-muscle, donor cells were present. In some cases, wild-type donor cells differentiated only into slow muscle fibers in a *smu* mutant host embryo (Fig. 5A-C), whereas in other cases both slow and fast muscle fibers developed from wild-type cells in the mutant host (Fig. 5D-F). No rescue of slow muscle was observed when wild-type cells differentiated into notochord or floor plate cells (data not shown), consistent with the observation that mutant cells are unable to differentiate into

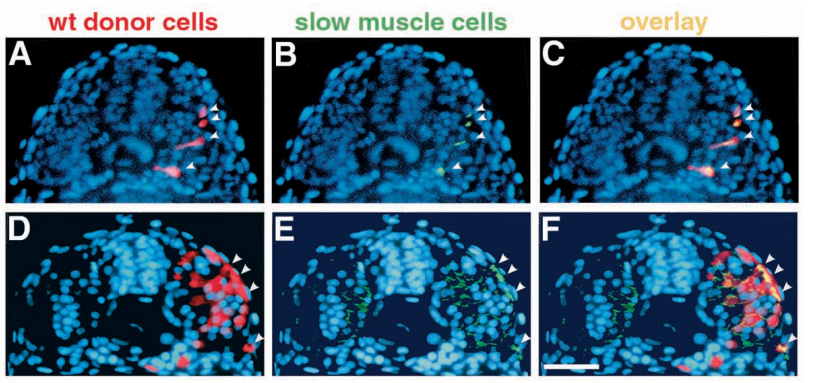

Fig. 5. Transplanted wild-type muscle cells rescue slow muscle development in *smu*$^{-/-}$. Transverse sections from two different *smu*$^{-/-}$ embryos show donor-derived, wild-type muscle cells. Sections were labeled with the F59 antibody, detected with a fluorescein-conjugated secondary antibody, and counterstained with Hoechst 33258 (blue). (A,D) Rhodamine-labeled wild-type cells (red, arrowheads). (B,E) F59-labeled slow muscle fibers (green, arrowheads). (C,F) merged micrographs. In A-C, four transplanted wild-type cells have developed into slow muscle fibers in an approx. 22h *smu*$^{-/-}$ host (C, yellow, arrowheads). Cells are still migrating through the somite. In D-F, transplanted wild-type cells have differentiated into both slow (4 cells; F, yellow, arrowheads) and fast (approx. 10 cells) muscle fibers in a 24h *smu*$^{-/-}$ host. Slow fibers can be distinguished from fast on the basis of the intensity of F59 labeling. Bar, 50 μm.

slow muscle in wild-type hosts (Table 1). We conclude that *smu* gene function is required only in muscle precursors in order for slow muscle development to occur.

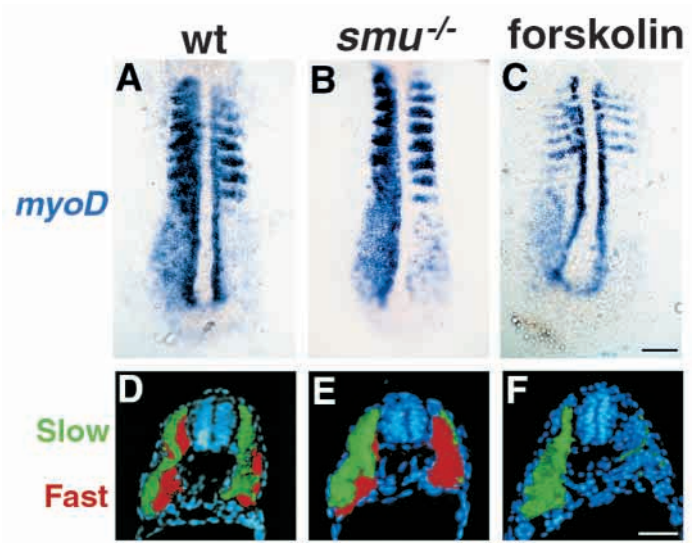

Fig. 6. *dnPKA* overexpression in *smu*$^{-/-}$ and forskolin-treated embryos rescues slow muscle development. *dnPKA* mRNA was injected into forskolin-treated embryos and embryos derived from a cross of two animals heterozygous for *smu* mutations. (A-C) Dorsal view of embryos fixed at 12h and labeled by in situ hybridization for *myoD*. (A) In the majority of embryos (187/194), *myoD* expression is expanded into the segmental plate as a result of *dnPKA* overexpression. (B) In some of these embryos, *myoD* labeling on one side of the embryo shows the same pattern as injected wild-type embryos, whereas the labeling on the other side resembles uninjected *smu* mutant embryos. We interpret these as mutant embryos in which the *dnPKA* mRNA was effective only on one side of the embryo. (C) Forskolin-treated embryos also respond to *dnPKA* mRNA. (D-F) *dnPKA* mRNA injected embryos fixed at 30h and labeled for S58 (green) and zm4 (red). (D) In wild-type embryos most of the zm4-labeled fast muscle (red) is replaced by S58-labeled slow muscle (green), compare to Fig. 2J (see also Du et al., 1997). (E) Slow muscle is on occasion rescued by *dnPKA* overexpression only on one side (compare to Fig. 2K); we interpret these as *smu*$^{-/-}$ embryos. (F) *dnPKA* overexpression rescues forskolin-treated embryos; compare to Fig. 2L. See also Fig. 4. Bars, 100 μm (A-C); 50 μm (D-F).

dnPKA overexpression rescues slow muscle in *slow-muscle-omitted*$^{-/-}$ and forskolin-treated embryos

Overexpression of a dominant negative form of PKA (dnPKA) leads to the induction of ectopic muscle pioneer and non-pioneer slow muscle cells (Du et al., 1997; Hammerschmidt et al., 1996). If the Smu protein acts upstream of the PKA target, then microinjecting *dnPKA* should rescue slow muscle in mutant embryos. Forskolin-treated embryos should also respond to dnPKA, as dnPKA does not respond to elevated cAMP levels (Clegg et al., 1987). *smu* mutant embryos did respond to dnPKA. In a cross of two heterozygous carriers, greater than 90% of the embryos injected with dnPKA had normal *myoD* labeling in adaxial cells and/or the expansion of *myoD* labeling into the lateral presomitic cells (Fig. 6A,B). Similarly, dnPKA overexpression reversed the effects of forskolin on adaxial *myoD* expression at 12h (Fig. 6C). Labeling of slow and fast muscle fibers at 30h showed the same patterns: wild-type, forskolin-treated and *smu* mutant embryos all responded to ectopic overexpression of dnPKA (Fig. 6D-F). Some of these embryos have rescue of *myoD* and slow muscle on only one side of the trunk; we suspect that these are partially rescued *smu*$^{-/-}$ embryos (Fig. 6B,E), a phenotype never observed following *shh* mRNA injections.

Unlike *shh* overexpression, *dnPKA* overexpression led to a large reduction in the proportion of embryos with less than 100 fibers, and an increase in the proportion with greater than 100 fibers (Fig. 4). We conclude that *dnPKA* rescued the mutant embryos (see also Fig. 4 insets).

Rat smoothened overexpression rescues slow muscle in *slow-muscle-omitted*$^{-/-}$ embryos

Since *smu* mutant cells can respond to dnPKA overexpression, we next focused on Smoothened, a component in the Hh signaling pathway upstream of the action of PKA. Because the zebrafish homologue(s) of the *smoothened* gene has not yet been identified and members of the Hh signaling pathway are widely conserved, we tested rat *smoothened* cDNA for its ability to rescue slow muscle development in *smu* mutant embryos. We injected 1-2 cell embryos with rat *smoothened* cDNA under the control of the CMV promoter (Stone et al., 1996), allowed the embryos to develop to 24h, and then labeled for slow muscle fibers (S58). Like *dnPKA* overexpression, overexpression of *smoothened* led to a large reduction in the

proportion of embryos with less than 50 fibers and an increase in the proportion with greater than 50 fibers (Fig. 7). We conclude that *smoothened* rescued the mutant embryos (see also Fig. 7 insets).

DISCUSSION

Mutations in the *slow-muscle-omitted* gene lead to the loss of hedgehog signaling and the consequent loss of slow muscle fibers. *smu* functions cell-autonomously in the responding cells, and *smu* mutant cells do not respond to overexpression of Shh. Slow muscle development is rescued in *smu* mutant embryos by inhibition of PKA, as well as by expression of rat Smoothened. Below, we discuss these results in the context of what is known about Hh signaling.

The role of *slow-muscle-omitted* in Hedgehog signaling

The first step in Hedgehog signal transduction is fairly well-established: Hh binds to its receptor Patched and inhibits Patched's repression of Smoothened (for reviews, see Ingham, 1998; McMahon, 2000). Less is known about transduction of the Hh signal from the Patched-Smoothened complex to Hh target genes. The homology of Smoothened to G-protein linked receptors suggests that Smoothened could have an as-yet-unidentified extracellular ligand. Recent work in *Drosophila* has demonstrated that cells homozygous for *smoothened* null mutations are unable to respond to Hh signaling and that inactivation of PKA yields a Hh response in *smoothened* null cells (Chen and Struhl, 1998). These results suggest that all Hh signaling in *Drosophila* depends on Smoothened and that at least some functions of Smoothened can be replaced by the repression of PKA activity. Recently, evidence has emerged that Ci is directly phosphorylated by PKA (Chen et al., 1999b, 1998; Wang et al., 1999) and that Hh signaling may depend on the activity of a protein phosphatase (Chen et al., 1999a; Krishnan et al., 1997). Vertebrate Gli proteins appear to function similarly to Ci (Aza-Blanc and Kornberg, 1999; Ruiz i Altaba, 1999; von Mering and Basler, 1999).

Our data eliminate some potential candidates for the *smu* gene. The lack of rescue by Hh and the cell-autonomy results indicate that *smu* is not required for Hh synthesis, processing or presentation to muscle precursor cells. Rescue by *dnPKA* makes it also very unlikely that *smu* function is required downstream of the target of PKA action. Thus, *smu* is probably not one of the *gli* homologues, as the products of these genes act downstream of PKA (Aza-Blanc and Kornberg, 1999). Moreover, mutations in *yot*, the zebrafish homologue of *gli2* (Karlstrom et al., 1999), are not rescued by inhibition of PKA with dnPKA (Schauerte et al., 1998).

We favor a model in which *smu* encodes a component directly within the Hedgehog pathway, acting upstream or at the same point as Smoothened. The Hedgehog receptor Patched (Concordet et al., 1996; Hooper and Scott, 1989; Nakano et al., 1989; Stone et al., 1996), and the Hh binding protein Hip (Chuang and McMahon, 1999) are both inhibitory components of the Hh signaling cascade. Thus, unless both alleles of *smu* are gain of function mutations, *smu* is unlikely to encode these components. Our data do not exclude the possibility that *smu* encodes a ligand or other cofactor required for Smoothened function. However, Smu protein must be required cell-autonomously (Fig. 5), and must not be required in the context of dnPKA or rat Smoothened overexpression (Figs 6, 7). As in the *Drosophila smoothened* mutant (Chen and Struhl, 1998), the simplest model that accounts for the rescue of *smu* mutant embryos by *smoothened* and the lack of rescue by *shh* is that *smu* encodes a zebrafish homologue of the *smoothened* gene.

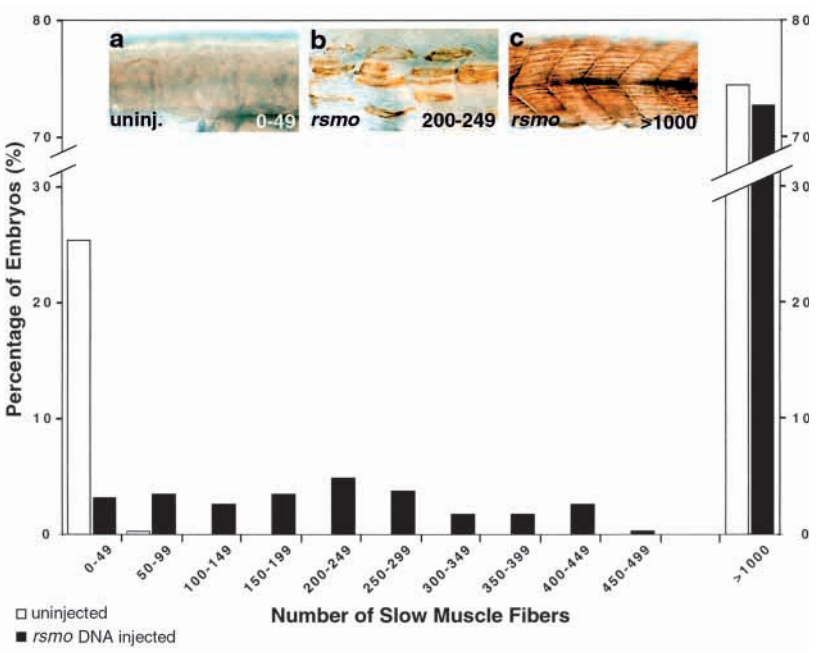

Fig. 7. Smoothened overexpression rescues slow muscle development in *smu* mutants. A plasmid encoding rat *smoothened* cDNA under the control of the CMV promoter was injected into embryos derived from a cross of two animals heterozygous for *smu* mutations. S58-labeled slow muscle fibers in uninjected and injected embryos were counted blind, and fiber tallies binned in sets of 50 for clarity of presentation. Uninjected siblings of *shh* injected and *dnPKA* injected embryos (Fig. 4) and *rsmo* injected embryos were pooled. Uninjected embryos (white bars, n=1219) displayed the expected Mendelian proportions: approx. 25% had <50 fibers, approx. 75% had >1000 fibers. *rsmo* overexpression resulted in a dramatic decrease in the percentage of embryos with <50 fibers and a consequent increase in the percentage of embryos with >50 fibers (black bars, n=351). Numbers of dead and malformed embryos (in which we did not count fibers) for injected embryos and their siblings were very similar (*rsmo* injected: 71 of 422, siblings: 80 of 492). Therefore we conclude that *rsmo* is not increasing the number of dead or deformed embryos. Among the embryos with <1000 slow muscle fibers, *rsmo* injected embryos had an average of 210.0±12.7 slow muscle fibers (n=96) and their uninjected siblings had an average of 22.0±1.3 (n=98). This difference is significant (t-test, $P \leq 0.0005$). Values are means ± s.e.m. Insets depict representative embryos for uninjected controls with 0-49 (a) fibers and *rsmo* injections with 200-249 (b) and >1000 (c) fibers.

Other possibilities for *smu* function can be envisaged. If both alleles of *smu* are partial loss of function mutations, it is possible that Smu functions downstream of Smoothened. In this case, overexpression of Smoothened compensates for the partial loss of Smu function. If the outcome of Hh signaling depends on a quantitative balance between the activity of Smoothened and the activity of PKA, it is also possible that *smu* encodes an inhibitor of PKA activity. In this case, *smu* mutations would lead to an increase in PKA activity, which could be overcome by a greater increase of Smoothened signaling following *smoothened* overexpression. Although we cannot formally exclude these possibilities, we believe that they are less likely than the model we propose above. Conclusive evidence of the nature of the *smu* gene product will require the cloning of the *smu* gene.

Is slow-muscle-omitted required for all Hedgehog signal transduction?

Vertebrates have several homologues of each of the genes involved in Hedgehog signaling and many of them have overlapping expression patterns. For example, in zebrafish, *tiggy winkle hedgehog*, *echidna hedgehog* and *sonic hedgehog* are all expressed in the midline (Currie and Ingham, 1996; Ekker et al., 1995b; Krauss et al., 1993), and *ptc1* and *ptc2* are both expressed in the paraxial mesoderm (Concordet et al., 1996; Lewis et al., 1999a). The extent of functional overlap, crosstalk and redundancy between the different homologues is not clear.

A variety of mutants exhibit a disruption in the development of tissues dependent on midline signaling. These include mutations in *sonic you* (*syu*), which encodes *shh*, *you-too* (*yot*), which encodes *gli2*, *you*, *chameleon* (*con*) and *uboot* (*ubo*), which have all been proposed to be part of the Hh signaling pathway (Karlstrom et al., 1999; Schauerte et al., 1998; van Eeden et al., 1996). These mutants show a set of common phenotypes, many of which are also seen in mutants with notochord defects. Phenotypes include loss of muscle pioneers, adaxial cells and dorsal aorta, partial cyclopia, ectopic lens in the ventral head midline, and pectoral fin and jaw cartilage defects (Beattie et al., 1997; Blagden et al., 1997; Halpern et al., 1993; Karlstrom et al., 1999; Lewis, 1999b; Schauerte et al., 1998; Talbot et al., 1995; van Eeden et al., 1996). The severity of each phenotype varies considerably between the different mutants, with some phenotypes absent in some mutants. $smu^{-/-}$ embryos show all of the phenotypes seen in these mutants, and in every case the phenotype is as strong or stronger than that seen in other mutants. Moreover, the phenotypes seen in *smu* mutants are analogous to many of the phenotypes seen in chick and mice embryos and in humans that are deficient in Hh signaling, including defects in the development of limbs, head cartilage, eye spacing and somite patterning (Ahlgren and Bronner-Fraser, 1999; Chiang et al., 1996; Hu and Helms, 1999; Marcelle et al., 1999; Ming and Muenke, 1998). Furthermore, the $smu^{-/-}$ phenotypes closely resemble the extreme Hh-deficient phenotypes seen in forskolin-treated embryos (Figs 1, 2). These observations together support the hypothesis that *smu* encodes a necessary component for all Hh signaling, which acts downstream of the three different Hh proteins and the two different Ptc proteins. If this is true, *slow-muscle-omitted* mutant embryos provide a very useful genetic tool for dissecting both the multifaceted role of Hh signaling in vertebrate embryogenesis and the complex nature of Hh signal transduction.

The role of Hedgehog signaling in vertebrate slow muscle development

Hh is a critical component in the development of vertebrate muscle (for a recent review, see Hughes et al., 1998). We and others have suggested that it also regulates the development of fiber type identity (Blagden et al., 1997; Cann et al., 1999; Currie and Ingham, 1996; Du et al., 1997; Lewis et al., 1999b). In zebrafish, overexpression of Hh is sufficient to induce slow muscle cells in the paraxial mesoderm, and mutations in *yot*, the zebrafish homolog of *gli2*, show a deficiency in slow muscle development (Du et al., 1997; Blagden et al., 1997; Lewis et al., 1999b). Moreover, slow muscle development can be blocked by either the activation of PKA, which is known to disrupt Hh signal transduction, or overexpression of *ptc1*, which represses Hh signaling (this paper; Du et al., 1997; Lewis et al., 1999b). If *smu* mutants have an almost complete loss of slow muscle, as we have shown, and if *smu* is required for all Hh signaling, as we propose, then this confirms that Hh signaling is necessary for slow muscle development in zebrafish.

Two important issues are raised by our results. First, we do not know the fate of $smu^{-/-}$ cells adjacent to the notochord that receive but cannot transduce the Hh signal. They may differentiate into fast muscle or sclerotome, remain undifferentiated, or die. Second, a small number of slow muscle fibers remain in $smu^{-/-}$ embryos. These fibers may develop from adaxial cells if the *smu* mutants that we have characterized do not lead to a complete loss of Hh signaling. Alternatively, they may develop from a population of slow muscle precursors that is distinct from the adaxial population. If a small number of slow fibers are also present in other mutants with a disruption in Hh signaling, then this would suggest that there is a Hh-independent population of slow muscle precursors. However, our results clearly show that the vast majority of embryonic slow muscle fibers are dependent on Smu mediated Hh signaling.

The development of muscle fiber type has not been examined in mice or chicken that have deficits in Hh signaling due to mutations or experimental perturbations. The molecular identification of the *smu* gene and the identification of *smu* homologues in mouse and chicken will help to indicate whether slow muscle development in other vertebrates also depends on Slow-muscle-omitted mediated Hh signaling.

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