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Hhip regulates zebrafish muscle development by both sequestering Hedgehog and modulating localization of Smoothened

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Abstract

Sharp borders between cells with different developmental fates are important for patterning of invertebrates, but are not well understood in vertebrates. Zebrafish slow muscle cells develop from adaxial cells, a one-cell-diameter-thick pseudo-epithelium immediately adjacent to the notochord. Hedgehog (Hh) signals from notochord specify adaxial cells to form slow muscle cells. Cells next to adaxial cells form fast muscle. This suggests that Hh signaling is locally regulated to produce a sharp border that separates slow and fast muscle precursors. To understand how Hh activity is locally regulated, we characterized the dynamic roles of Hhip, a protein that binds Hedgehog at the cell surface. Hhip is strongly expressed by adaxial cells and, together with Patched, the Hedgehog receptor, limits transduction of the Hedgehog signaling by Smoothened to adaxial cells. Hhip protein lacking its membrane associated domain still suppresses Hh activity but no longer acts synergistically with Patched. Hhip and Smoothened colocalize at the cell surface and, in response to Hedgehog, internalize together. Knockdown of Hhip blocks Smoothened internalization while increasing Hedgehog signaling and slow muscle formation. These data support a model in which Hhip regulates muscle development both by sequestering Hedgehog and by modulating localization of Smoothened.

Keywords: Clathrin; Endosomes; Hedgehog signaling; Patched; Skeletal muscle; Smoothened; Zebrafish

Introduction

During animal development, cells acquire particular fates in response to local cues. Some cues, such as the Hedgehog (Hh) family of secreted proteins, may act over a distance (Ingham and McMahon, 2001). Local regulation of this signaling is required to establish borders between cells with different fates. In zebrafish embryos, the border between skeletal muscle cell types is very sharp (Devoto et al., 1996) even though the fate decision is regulated by Hh secreted from neighboring cells (Blagden et al., 1997; Du et al., 1997; Ingham and Kim, 2005). The mechanisms that locally regulate this Hh activity are not well understood.

We previously showed that zebrafish myotomes are composed of superficial slow muscle cells, deeper fast muscle cells and a subset of slow muscle cells, muscle pioneers, located near the horizontal myoseptum that separates dorsal and ventral parts of the myotome (Devoto et al., 1996). Three of the zebrafish *hh* genes, *sonic hedgehog* (*shh*), *echidna hedgehog* (*ehh*) and *tiggy-winkle hedgehog* (*twhh*), are expressed in notochord and/or floor plate (Currie and Ingham, 1996; Ekker et al., 1995). Adaxial cells, a monolayer adjacent to the notochord, express the Hh receptors *patched1* (*ptc1*) and *patched2* (*ptc2*) (Lewis et al., 1999a,b) and in response to Hh signaling form slow muscle and muscle pioneers (Devoto et al., 1996; Hirsinger et al.,

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2004). A subset of adaxial cells subsequently migrates radially to form a superficial layer of mononucleate slow muscle cells, while muscle pioneer cells remain adjacent to the notochord. Later, cells of the fast lineage differentiate and fuse to form multinucleate fibers that comprise the bulk of the myotome (Devoto et al., 1996).

We originally proposed that different levels of Hh activity produce different cell types in the zebrafish myotome (Du et al., 1997), and subsequent studies have supported this view (Wolff et al., 2003; Nakano et al., 2004). High levels of Hh activity produce muscle pioneer cells, intermediate levels produce non-muscle pioneer slow muscle cells and low levels permit cells to become fast muscle. Several factors, including Ptc, Fused (Fu) and Suppressor of fused (Sufu), are thought to regulate the response of muscle to Hh (Wolff et al., 2003), although mechanisms that restrict Hh signaling to adaxial cells and that produce graded Hh levels to specify slow muscle and muscle pioneer fates are as yet poorly understood.

Initial steps in Hh signal transduction involve at least two proteins Ptc and Smoothened (Smo). Early studies indicated that Ptc negatively regulates Hh signaling by directly inhibiting Smo. Hh binding to Ptc relieves this inhibition and allows Smo to transduce the signal (Ingham and McMahon, 2001). Recent studies, however, suggest that the Ptc and Smo relationship may be nonstoichiometric and indirect rather than direct. In *Drosophila*, Hh treatment of cells results in removal of Ptc from the cell surface and subsequent accumulation of a phorylated form of Smo (Ingham et al., 2000; Denef et al., 2000; Zhu et al., 2003; Torroja et al., 2004; Gallet and Therond, 2005). This internalization of Hh and Ptc depends on dynamin and limits the Hh gradient in wing discs (Torroja et al., 2004). Studies in vertebrates suggest that Ptc and Smo colocalize prior to Hh exposure and enter the endosomal pathway after ligand binding. Subsequently, Smo segregates from the Hh–Ptc complex that is destined for degradation (Incardona et al., 2000, 2002). Consistent with this model, recent studies show that Smo internalizes via a Clathrin-dependent endocytic pathway in response to Hh activity (Chen et al., 2004) and that Rab23, a component of the vesicular transport machinery, is required for negative regulation of Hh signaling (Eggenschwiler et al., 2001). Although the exact mechanism by which Ptc interacts with Smo is still controversial, accumulation of Smo at the cell surface and endocytosis of the Hh–Ptc complex may be important mechanisms that regulate Hh signaling and formation of the morphogenetic gradient.

Recently, a new member of the Hh signaling pathway, Hedgehog interacting protein (Hhip), was identified as a type I membrane associated protein molecule that binds Hh (Chuang and McMahon, 1999). Genetic and biochemical analyses suggest that Hhip acts as a negative regulator of the Hh signaling pathway by binding Hh at the cell surface (Treier et al., 2001; Chuang et al., 2003; Kawahira et al., 2003) and by being released from cells where it can bind Hh extracellularly (Coulombe et al., 2004). Hence, the major reported function of Hhip is to titrate signaling by sequestering Hh protein.

We investigated the function of Hhip as a potential regulator of Hh signaling during zebrafish muscle development. We show that zebrafish *hhip* is expressed by adaxial cells and later is restricted to muscle pioneer cells and a subset of fast muscle cells. Experimentally induced gain and loss of Hhip function demonstrates that Hhip is required for restricted expression of *myod* in adaxial cells and subsequent slow muscle and muscle pioneer development. Epistatic analyses suggest that Hhip and Ptc synergistically suppress Hh activity in muscle cells and that Hhip suppresses the phenotype of *ptc*-MO-injected embryos. In contrast, Hhip lacking the membrane associated domain still suppresses Hh activity, but no longer suppresses the phenotype of *ptc*-MO-injected embryos, suggesting that the membrane anchoring domain is required for synergistic interaction with Ptc. This result was unexpected because previous studies suggested that Hhip simply binds Hh. In addition, we find that Hhip localizes with Smo but not Ptc at the cell surface. In response to Hh, Hhip and Smo internalize together associated with Clathrin-coated vesicles and endosomes. Knocking down Hhip activity suppresses Smo internalization and results in an increase in the number of slow muscle and muscle pioneer cells. These results suggest that Hhip regulates muscle development both by sequestering Hh and by modulating Smo localization.

Materials and methods

Animals

Wild-type (AB) and mutant zebrafish (*syu*^{t4}, *smu*^{b577}, *smu*^{b641}, *yot*^{ty119}, *uki*^{tc256d}) were provided by the University Oregon Zebrafish Facility. Embryos were maintained at 28.5°C and staged using standard morphological criteria (Kimmel et al., 1995).

Plasmids

We isolated a zebrafish *hhip* cDNA by screening a zebrafish presomitic stage cDNA library at low stringency using probes generated from mouse *Hhip* cDNA (Chuang and McMahon, 1999). Zebrafish genome informatics analysis (CSC GEnome Browser; Zebrafish Nov. 2003 Assembly) reveals that *hhip* lies on chromosome 1: 28,798,723–28,838,665 with a size of 43,268 basepairs (bp). For mRNA injections, PCR products of *hhip* were cloned into the *Eco*RV site of pTX (pTX *hhip*). To generate pTX *hhip*Δ C22, pTX *hhip*Δ 614–693 and pTX *hhip*Δ 415–693, we performed PCR using specific primers and inserted the products into *Spe*I or *Eco*RI sites of pTX. For antisense probes, PCR products of *hhip* were cloned into pCRII-TOPO (Invitrogen). To make a construct for myc-Hhip, PCR products of myc-tagged zebrafish Hhip were cloned into the *Not*I site of pcDNA3.1 (Invitrogen). Flag-tagged zebrafish Smo was cloned into the *Xho*I site of pcDNA3.1. To make Flag-tagged ShhN, amino acids 1–183 of zebrafish *shh* were amplified by PCR and cloned into the *Xho*I site of pcDNA3.1. pGEN/mSmoEAN (myc-his tagged) and pMT21-Ptc-HA were kindly provided by Philip Beachy (Taipale et al., 2002) and Henk Roelink (Incardona et al., 2000). We previously described pcDNA3.1-Hhip-YFP (Jeong and McMahon, 2005).

In vitro mRNA synthesis

Capped mRNAs were transcribed from PCR amplified DNA templates or linearized DNA using T7 and SP6 RNA polymerase in vitro transcription kits according to the manufacturer's instruction (mMESSAGE, mMACHINE; Ambion). The following plasmids were used: pS64TxB *ptc1* (Lewis et al., 1999b), pCS2dn*PKA* (Ungar and Moon, 1996).

In situ hybridization

The in situ labeling was performed as previously described (Westerfield, 2000). The following markers were used: *myod* (Weinberg et al., 1996), *ptc1* (Lewis et al., 1999b), *eng1a* (Ekker et al., 1992), *wnt11* (Makita et al., 1998) and *doublesex-related* (dmrt2) (Meng et al., 1999). *hhip* probe was synthesized from pCRII-TOPO *hhip* plasmid linearized with *Xba*I using SP6 RNA polymerase.

Microinjection and inhibition of endocytosis

mRNA was dissolved in double distilled H_2O to final concentrations of 10 ng/μl to 200 ng/μl. Phenol red was added to the solution. Approximately 1 nl of RNA or DNA was injected at the one-cell stage using published procedures (Westerfield, 2000). *hhip*-MOs were directed to the translation start and 5′ UTR of the respective RNAs (Gene Tools, LLC): MO-Zhip; AGAGCACAAATTTCAAATGCTTCAT, MO-Zhip UTR; AAAGCAACTACTCGCTAAATAGGTG. *ptc*-MOs were designed as previously described (Wolff et al., 2003); a combination of morpholinos targeted to both *ptc1* and *ptc2* (*ptc*-MO) were used. To inhibit endocytosis, 100 μM–500 μM Monodansylcadaverine, *N*-(5-Amino pentyl)-5-dimethyl amino naphthalene-1-sulfon amide (SIGMA) and 5 μg/ml Chlorpromazine (SIGMA) were added at 40% epiboly stage.

Cell culture and transfection

COS7 or NIH3T3 cells were cultured in 10 cm plastic plates in Dulbeco's modified Eagle's medium containing 10% fetal bovine serum (FBS) under 5% CO_2 at 37°C. Before each experiment, cells were treated with trypsin/EDTA and seeded in 24-well tissue culture plates. Plasmid DNA was prepared (QIAGEN) for transfection. Transient transfections of plasmid DNA were performed (Lipofectamine 2000, Invitrogen) according to the manufacturer's instructions. After 6 h of transfection, the medium was replaced with fresh 10% FBS. Cells were further incubated for 48 h.

Antibody labeling

Labeling with S58, F59, 4D9 and Prox1 was as previously described (Du et al., 1997). The primary antibodies were mAb 4D9 (anti-Eng) at a dilution of 1:20, mAb S58 (anti-MyHC) at 1:10, mAb F59 (anti-MyHC) at 1:10, rabbit anti-Prox1 (AngioBio Co.) at 1:500, mouse anti-c-myc (9E10; Santa Cruz Biotechnology) at 1:1000, rabbit anti-c-myc (A-14; Santa Cruz Biotechnology) at 1:1000, anti-FLAG M2 Monoclonal Antibody (SIGMA) at 1:1000, anti-HA clone 12CA5 (Roche) at 1:1000, anti-Calreticulin rabbit pAb (CALBIOCHEM) at 1:1000 and anti-mouse GM130 (BD Transduction Laboratories) at 1:1000. Secondary antibodies were Alexa Fluor 594 goat anti-mouse IgG at 1:1000 and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes) at 1:1000.

Microscopy

Embryos processed for whole-mount in situ hybridization and live embryos were photographed using Leica MZFGIII and Axiocam digital cameras on Zeiss Axioplan microscopes. For the colocalization of Hhip and Smo in COS7 cells and Prox1 and Eng in embryos, images were collected using a Bio-Rad Radiance 2100MP confocal system on a Nikon E600FN Upright microscope.

Results

Zebrafish Hhip is closely related to Hhip proteins in other vertebrates

We isolated a putative zebrafish Hhip cDNA. Sequence analysis demonstrates that the cDNA encodes a conceptual protein of 693 amino acids (Fig. 1A) that is very similar to Hhip proteins from *Takifugu* (76% identity), human (64% identity), mouse (64% identity) and *Xenopus* (64% identity). Like the other Hhip proteins (Chuang and McMahon, 1999), zebrafish Hhip contains one putative C-terminal membrane anchoring domain (Fig. 1A, blue) and a highly conserved EGF-like domain (Fig. 1A, red). The long sequence from position 25 to 600 (Fig. 1A, yellow) is novel, but highly conserved among Hhip proteins, suggesting that this region is functionally important. Assignment to the Hhip family is further supported by phylogenetic analysis (Fig. 1B) that shows zebrafish Hhip groups with the other known fish protein (Tfu) separate from tetrapod proteins. The high bootstrap values support the conclusion that zebrafish Hhip is an ortholog of mammalian Hhip.

Adaxial cells, muscle pioneers and slow muscle precursors express hhip mRNA

hhip transcripts are present in 1-cell stage embryos, as shown by mRNA in situ hybridization (Fig. 1C) and RT-PCR (not shown), indicating an abundant maternal supply of *hhip* message. From the 1-cell stage through gastrulation, *hhip* transcripts are widely distributed (Figs. 1D, E and not shown). We first detect localized *hhip* expression by bud stage in the anterior midline (Fig. 1F, arrow) and adaxial cells (Fig. 1F, arrowhead). During the segmentation period, cells distributed more laterally in the somites express *hhip* (Fig. 1G, arrow, I, upper panel) in addition to adaxial (Fig. 1H, arrow) and pronephric cells (Fig. 1H, arrowhead).

Previous studies in mouse showed that cells adjacent to *Hh* expressing cells express *Hhip* (Chuang and McMahon, 1999). Consistent with this, zebrafish cells located up to eight-cell diameters from the notochord and floor plate, sources of *shh* (Fig. 1I, lower panel), *ehh* (Krauss et al., 1993) and *twhh* (Ekker et al., 1995), express *hhip*. As development proceeds to late segmentation stages, *hhip* expression becomes restricted to muscle pioneer cells (Fig. 1K, left), a subpopulation of adjacent fast muscle cells (Fig. 1L, arrow) and to cells adjacent to the floor plate in the neural tube (Fig. 1L, arrowhead). To confirm that muscle pioneer cells express *hhip*, we double-labeled embryos for *hhip* mRNA and Eng protein that we previously showed is a marker for muscle pioneer cells (Hatta et al., 1991) and find colocalization (Fig. 1K). Previous reports showed that the Hh receptor Ptc is expressed by adaxial cells at early stages and is later restricted to cells next to the notochord, including muscle pioneer cells and adjacent fast muscle cells (Concordet et al., 1996; Barresi et al., 2000; Wolff et al., 2003). Thus, *hhip* and *ptc1* have very similar expression patterns in the paraxial mesoderm and developing somites.

By 48 h post-fertilization (hpf), *hhip* is expressed in other regions, including pectoral fin buds (Koudijs et al., 2005), tectum (Koudijs et al., 2005) and neural crest cells (Fig. 1M, bracket). In addition, *hhip* is detected in the branchial arch derived adductor mandibulae muscles (Koudijs et al., 2005) that also express slow myosin heavy chain (Hsiao et al., 2003).

Hhip and Patched sequester Hedgehog activity to adaxial cells

The expression pattern of *hhip* in zebrafish embryos suggests that Hhip may regulate Hh signaling during muscle

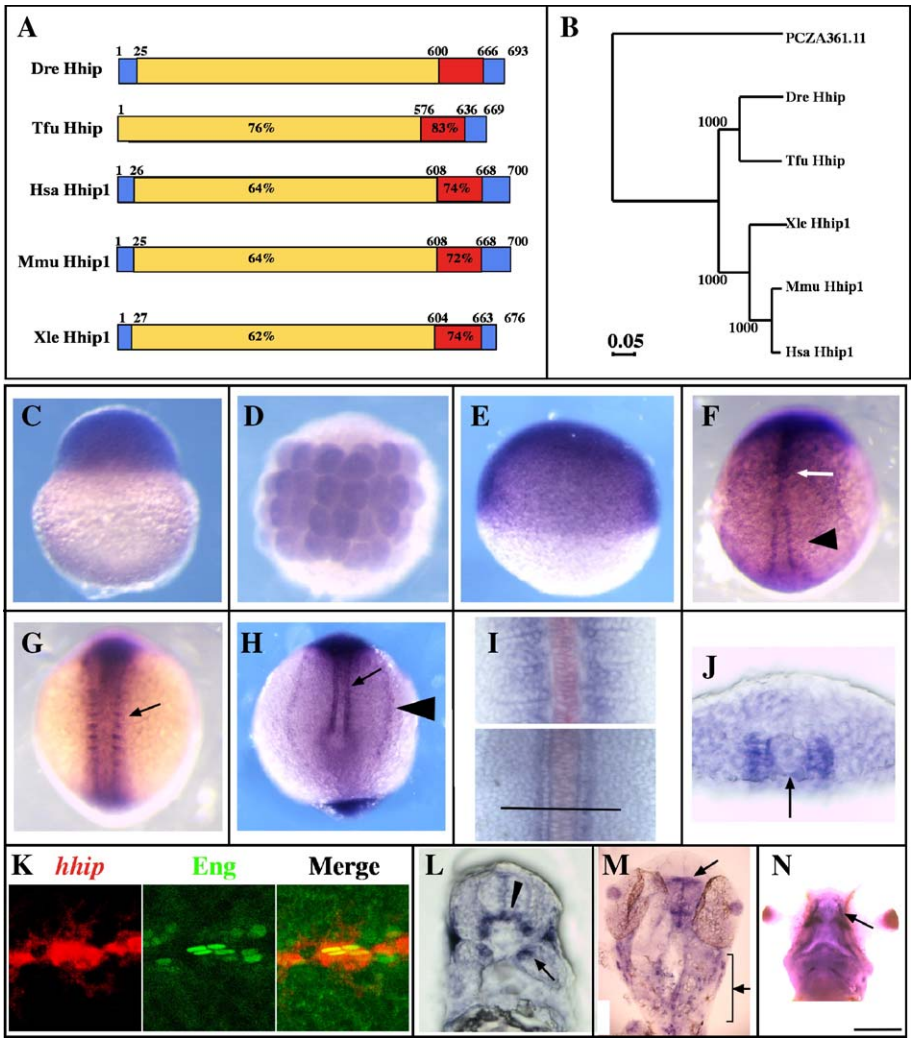

Fig. 1. Adaxial cells, muscle pioneer and slow muscle precursors express *hhip* mRNA. (A, B) Zebrafish Hhip protein is closely related to Hhip in other vertebrates. (A) The predicted amino acid sequences of *Danio rerio* (Dre, DQ177323), *Homo sapiens* (Hsa, NM_022475), *Mus musculus* (Mmu, AF116865), *Xenopus laevis* (Xle, BC046952) and *Takifugu rubripes* (Tfu, SINFRUT00000133748) Hhip protein. Red indicates the EGF-like domains, and blue indicates the hydrophobic stretches. Percentages indicate sequence identity of amino acids of each domain compared to the zebrafish sequence. The numbers indicate the locations of borders between domains. (B) Phylogenetic tree comparing zebrafish Hhip with other vertebrate Hhip proteins. The tree is based on the amino acid sequences of putative open reading frames of the proteins aligned with the Clustal method. PCZA361.11 (CAA11769) was used as an outgroup. Numbers indicate bootstrap support for the nodes. (C) Maternal *hhip* mRNA is present in the one-cell stage embryo. (D, E) *hhip* mRNA is present throughout the embryo at 32-cell (D) and 50% epiboly (E) stages. (F) *hhip* is expressed at higher levels in the midline (arrow) and adaxial cells (arrowhead) at bud stage. (G, H) *hhip* expression is apparent in the medial somite (G, arrow), adaxial cells (H, arrow) and pronephric tissue (H, arrowhead) at the 8-somite stage. (I) Comparison of *hhip* (blue) and *shh* (red) expression at the 12-somite stage. *hhip* mRNA is expressed adjacent to *shh* expressing cells in posterior, presomitic regions (bottom) and farther lateral in anterior, segmented regions (top). Bar indicates location of section shown in panel J. (J) *hhip* is expressed adjacent to the notochord (arrow, notochord). (K) Muscle pioneer cells express *hhip* mRNA (left panel) and Eng protein (middle panel). Double labeling with 4D9 (green) anti-Eng antibody and *hhip* (red) shows that *hhip* expressing cells contain Eng protein (right panel) at 24 hpf. (L) *hhip* mRNA is detected in a subset of fast muscle cells (arrow) at 24 hpf. (M) *hhip* is expressed in the tectum (arrow) and neural crest cells (bracket) at 24 hpf. (N) *hhip* expression in adductor mandibulae at 48 hpf (arrow). (C, E) Lateral views; (D, F, G, M) dorsal views; (H) posterior view of the tail bud; (J, L) transverse sections, dorsal towards the top; (K) lateral view; (N) ventral view. Scale bar: (C–H, K, M, N) 200 μm; (I, J, L) 50 μm; (K) 25 μm.

development. Previous studies in mouse (Chuang and McMahon, 1999) showed that Hhip binds to the N-terminal region of Shh and overexpression of Hhip mimics the phenotype of loss of function mutations in *Indian hedgehog* (*Ihh*), suggesting that Hhip acts as a negative regulator of Hh signaling by binding to Hh. Thus, zebrafish Hhip could act similarly to reduce the effectiveness of Hh on muscle. To test this hypothesis, we increased Hhip activity by injecting *hhip* mRNA into embryos and examined expression of *ptc1*, a downstream target of Hh signaling (Lewis et al., 1999a). In normal embryos, *ptc1* is expressed at high levels in adaxial cells and at lower levels in adjacent cells at bud stage (Lewis et al., 1999a). When we overexpress *hhip*, however, embryos exhibit reduced *ptc1* expression (Fig. 2B; 60%, $n = 28$), consistent with the idea that Hhip protein reduces Hh signaling. To learn whether *hhip* overexpression acts specifically on Hh signaling, we examined the expression of *wnt11*, a marker of notochord at bud stage (Makita et al., 1998), and *doublesex-related* (*dmrt2*) that is expressed in paraxial mesoderm and somites during segmentation stages (Meng et

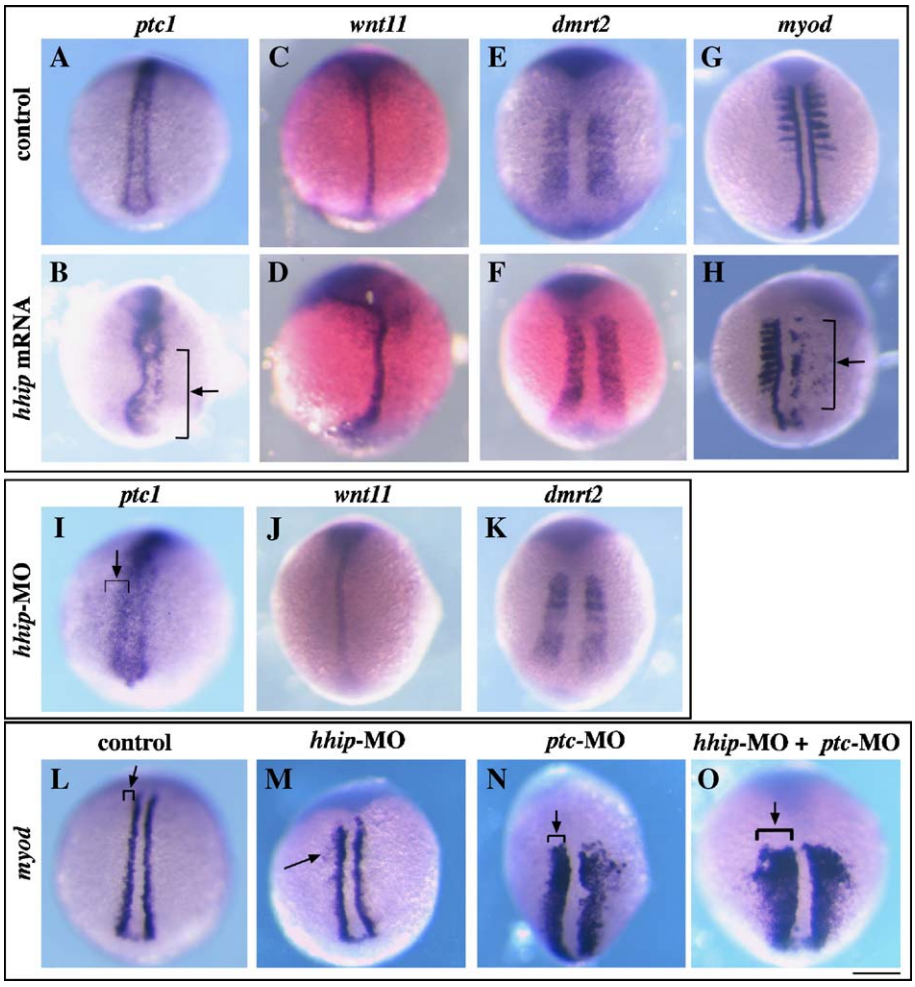

Fig. 2. Cooperative activity of Hhip and Patched is required for restricted expression of *myod* in adaxial cells. (A–B) *ptc1* is expressed in adaxial cells at bud stage in a control embryo (A) and is reduced by overexpression of *hhip* (B, arrow; 17/28 injected embryos). (C–F) In contrast, overexpression of *hhip* does not affect expression of *wnt11*, a marker of notochord, (C–D; 9/9 injected embryos) or *dmrt2*, a marker of paraxial mesoderm and somites (E–F, 13/14 injected embryos). (G–H) Overexpression of *hhip* inhibits *myod* expression. (G) *myod* is detected in adaxial cells and somites in a control embryo at the 8-somite stage. (H) *myod* expression is strongly reduced in a *hhip* mRNA-injected embryo (arrow, 25/46 injected embryos). (I–K) *ptc1* expression is increased in a *hhip*-MO-injected embryo (I, arrow; 7/9 injected embryos). *hhip*-MO does not affect expression of *wnt11* (J, 5/6 injected embryos) or *dmrt2* (K, 11/11 injected embryos). (L–O) Reduction of Hhip and Ptc results in spread of *myod* expression laterally in the paraxial mesoderm. (L) Expression of *myod* in a control embryo. (M) Expression of *myod* is slightly upregulated in cells adjacent to adaxial cells in a *hhip*-MO-injected embryo (arrow, 23/93 injected embryos). (N) Injection of *ptc*-MO has a stronger effect than injection of *hhip*-MO (arrow, 21/27 injected embryos). (O) Reduction of both Hhip and Ptc causes a significant increase in *myod* expression in paraxial mesoderm (arrow, 27/27 injected embryos). Dorsal views, bud stage (A–D, I–J, L–O) and 8-somite stage (E–H, K), anterior towards the top. Scale bar: 200 μm.

al., 1999). Neither expression pattern is affected by *hhip* overexpression (Figs. 2C–F). These data suggest that zebrafish Hhip specifically reduces Hh signaling, consistent with previous studies in mouse (Chuang and McMahon, 1999).

Expression of *myod*, a myogenic regulatory factor, is also affected by Hhip activity. In zebrafish, overexpression of Shh is sufficient to induce ectopic *myod* in the paraxial mesoderm (Weinberg et al., 1996; Coutelle et al., 2001), and *myod* expression in adaxial cells disappears in *smo* mutants that lack Hh signaling (Barresi et al., 2000; Chen et al., 2001). Injection of *hhip* mRNA reduces *myod* expression in adaxial cells and somites (Figs. 2G, H, arrow; 54%, $n = 46$). This result further supports our interpretation that Hhip functions in adaxial cell development by negatively regulating Hh signaling.

To understand how Hhip functions in adaxial cells, we used morpholino antisense oligonucleotides (MO) to reduce Hhip activity. The size of the *ptc1* expression domain increases in *hhip*-MO-injected embryos (Fig. 2I), whereas *wnt11* and *dmrt2* expression is unaffected (Figs. 2J, K). This result suggests that Hh signaling is upregulated in cells farther from the notochord in *hhip*-MO-injected embryos. Consistent with this interpretation, *hhip*-MO-injected embryos exhibit an increase in *myod* expression in cells lateral to adaxial cells (Fig. 2M, arrow, 25%, $n = 93$). These effects of *hhip*-MO injection are suppressed by *hhip* mRNA (Supplementary Fig. 1). The upregulation of *ptc1* by *hhip*-MO is reminiscent of the actions of *ptc1*-MO or *ptc2*-MO (Wolff et al., 2003). Ptc is thought to sequester Hh (Ingham and McMahon, 2001); thus as Ptc activity decreases, Hh signaling increases due to loss of sequestration (Wolff et al., 2003). To measure the sequestering ability of Hhip, we compared *hhip*-MO and *ptc*-MO-injected embryos. Injection of *ptc*-MO (a combination of *ptc1*-MO and *ptc2*-MO) results in

a pronounced lateral spread of *myod* expression in paraxial mesoderm (Fig. 2N; 78%, $n = 27$). Coinjection of *hhip*-MO and *ptc*-MO further enhances the lateral spread of *myod* expression (Fig. 2O; 100%, $n = 27$). Shh binding to Ptc relieves inhibition of Smo, leading to transcription of downstream genes including *ptc* itself (Chen and Struhl, 1996; Ingham and McMahon, 2001). The subsequent increase in Ptc protein is thought to buffer exogenous Shh, limiting its diffusion and signaling (Chen and Struhl, 1996; Ingham and McMahon, 2001). We find that *hhip* expression also depends upon Hh activity (Supplementary Fig. 1), suggesting that Hh signaling also increases expression of Hhip protein that subsequently buffers and limits Hh. Together, these results indicate that the combined activities of Hhip and Ptc are required for restricted *myod* expression in adaxial cells.

Hhip negatively regulates muscle pioneer cell development

The importance of Hhip in adaxial cell development predicts that it should also be required for proper formation of slow muscle and muscle pioneer cells because we previously showed that these cell types develop from adaxial cells (Devoto et al., 1996). Labeling with antibodies, S58, a marker of slow muscle (Devoto et al., 1996), and zm4, a marker of fast muscle (Barresi et al., 2000), reveals that overexpression of *hhip* suppresses formation of slow but not fast muscle cells (Fig. 3D). Labeling with 4D9 that recognizes Eng proteins in muscle pioneer cells (Hatta et al., 1991) and anti-Prox1, a slow muscle nuclear marker (Grunwald et al., 1988), shows that the numbers of muscle pioneer and slow muscle cells are reduced in embryos overexpressing *hhip* (Figs. 3F, M, Supplementary Fig. 2) compared to control embryos (Figs. 2, 3C, M). Conversely, *hhip*-MO increases the number of muscle pioneer cells (Figs. 3R–U and D′) and converts fast muscle cells to slow muscle cells, as indicated by S58 and F59 slow muscle markers (Fig. 3R, Supplementary Fig. 5). We observe a similar increase in the number of muscle pioneer cells in *hhip* mutant embryos (Supplementary Fig. 2). These results support the notion that Hhip acts as a negative regulator of Hh signaling in slow muscle and muscle pioneer cell development.

Hhip and Patched act synergistically to regulate slow muscle and muscle pioneer development

To understand the relationship between Hhip and Ptc functions, we examined genetic interactions between them. We find that fewer slow muscle and muscle pioneer cells form after coinjection of *hhip* and *ptc1* mRNAs than after injection of either mRNA alone (Figs. 3A–M), and knockdown of both Hhip and Ptc by MO injection produces a greater increase in slow muscle and muscle pioneer cells than injection of either MO alone (Figs. 3N–D′). *ptc*-MO-injected embryos have increased numbers of S58 and F59 labeled slow muscle cells in the region where fast muscle normally forms (Figs. 3V, W, D′ and Supplementary Fig. 5) and increased numbers of Prox1 positive slow muscle cells and 4D9 positive muscle pioneer cells (Figs. 3X, Y, D′). The numbers of slow muscle and muscle pioneer cells are unaffected or slightly increased by injection of either *hhip*-MO or *ptc*-MO alone (Fig. 3D′). In contrast, simultaneous injection of both *hhip*-MO and *ptc*-MO significantly enhances each other's effect (Figs. 3Z–D′) and produces numbers similar to injection of *shh* mRNA (Fig. 3D′). These results show that Hhip and Ptc act jointly to control the activity of Hh signaling during muscle development, consistent with previous work in mouse neural tube (Jeong and McMahon, 2005).

To position Hhip in the Hh pathway, we analyzed the epistatic relationships between Hhip and other Hh regulatory factors. *shh* mutants lack all myotomal *eng1a* expression (Fig. 4A and Schauerte et al., 1998). Injection of *hhip*-MO into *shh* mutants rescues *eng1a* expression (Figs. 4A, B). In contrast, the effect of *hhip*-MO is completely suppressed in *smo* (Figs. 4C, D) and *gli2* mutants (Figs. 4E, F). Protein Kinase A (PKA) is an intracellular transducer of Hh signaling, and a dominant negative form of Protein Kinase A (dn*PKA*) induces ectopic *eng1a* expression (Fig. 4H and Ungar and Moon, 1996; Du et al., 1997). Although injection of *hhip* mRNA decreases *eng1a* expression (Fig. 4I), this effect is suppressed by coinjection of dn*PKA* (Fig. 4J). These results suggest that Hhip functions downstream of Hh and upstream of Smo.

Injection of *hhip*-MO induces ectopic expression of *eng1a* in the myotome (Figs. 4K, L), and coinjection of *ptc1* mRNA rescues this effect of *hhip*-MO (Figs. 4M, W). Similarly, injection of *ptc*-MO results in U-shaped somites, ectopic *eng1a* expression and increased numbers of slow muscle cell and muscle pioneer cells (Figs. 4O, R, U, W, X) and (Wolff et al., 2003), and coinjection of *hhip* mRNA rescues these effects of *ptc*-MO (Figs. 4P, S, V, W, X). Because Ptc interacts with both Hh and Smo, the effect of changes in Hhip expression may reflect inhibition of either or both Hh and Smo. Together, these results indicate that Hhip acts synergistically with Ptc, genetically downstream of Hh and upstream of Smo.

The Hhip membrane anchoring domain is required for synergistic interaction with Patched but not for inhibition of Hedgehog activity

Previous studies showed that mouse Hhip attenuates Hh signaling by binding Hh on the cell surface (Chuang and McMahon, 1999) and as a secreted molecule (Coulombe et al., 2004). Thus, Hhip is thought to function by titrating extracellular Hh. We find that *hhip*-MO enhances the phenotype of *ptc*-MO-injected embryos (Figs. 3N–D′) and *hhip* mRNA rescues *ptc*-MO-injected embryo (Figs. 4N–X). If titration of extracellular Hh is the only function of Hhip, then it is unclear how knock down or overexpression of Hhip can affect the phenotype of embryos lacking Ptc. One possibility is that morpholinos may not completely suppress Ptc activity. This interpretation predicts that the Hh binding domain of Hhip alone should also rescue *ptc*-MO-injected embryos. To examine this possibility, we generated *hhip* deletion constructs (Fig. 5A) and evaluated expression of *myod*, Prox1 and Eng as indicators of Hh signaling.

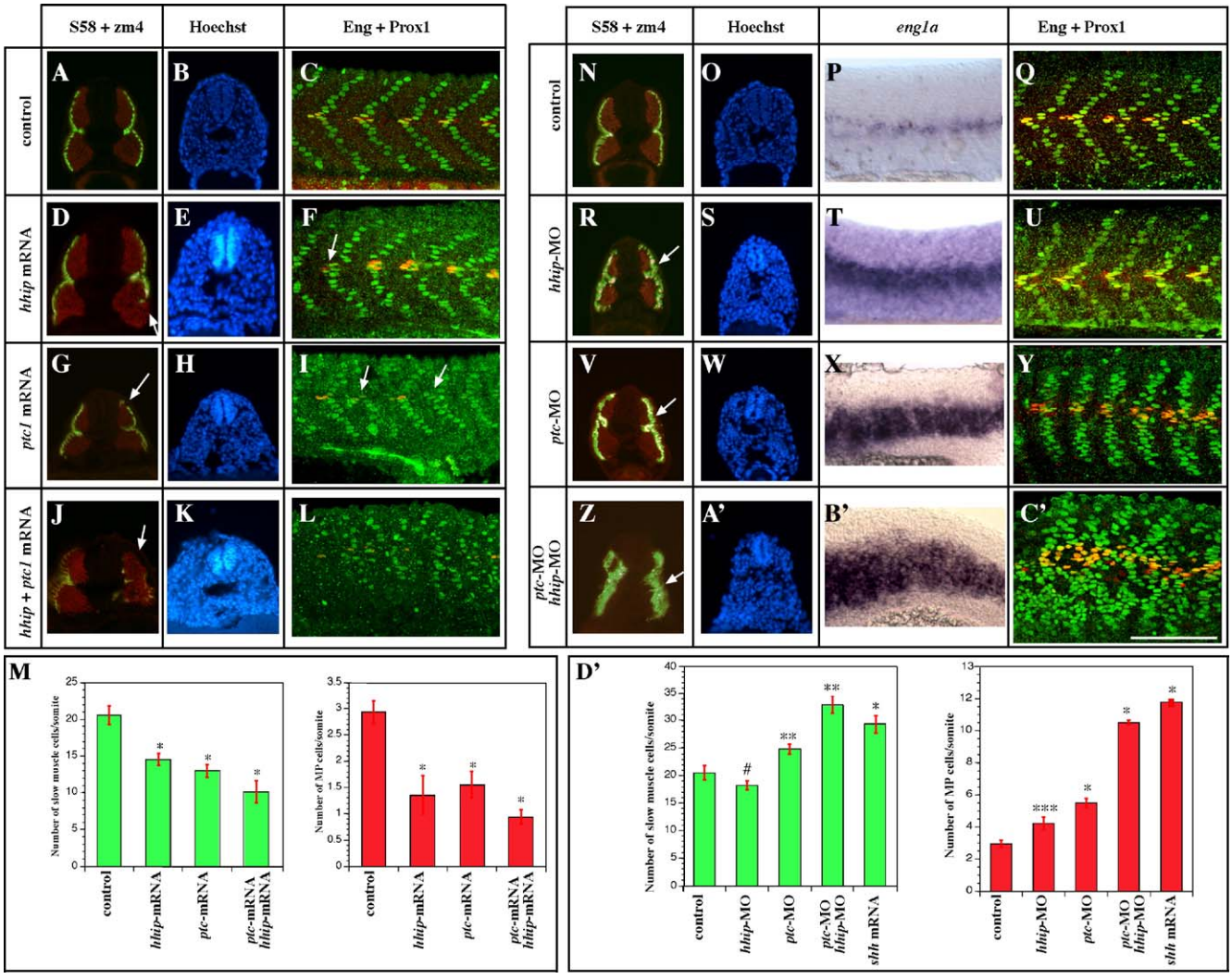

Fig. 3. Combined activities of Hhip and Patched are required for muscle pioneer and slow muscle cell development. (A–M) Overexpression of *hhip* mRNA inhibits muscle pioneer and slow muscle development. Control embryo (A–C), *hhip* mRNA-injected embryo (D–F), *ptc1* mRNA-injected embryo (G–I), *hhip* and *ptc1* mRNA-injected embryo (J–L). (A, B, D, E, G, H, J, K) Transverse sections of a 24 hpf embryo labeled with slow muscle marker S58 (green), fast muscle maker zm4 (red) (A, D, G, J) and Hoechst to mark nuclei (B, E, H, K). (C, F, I, L) Embryos labeled for nuclear slow muscle cell marker Prox1 (green) and the muscle pioneer marker 4D9 (red, Eng). Both *hhip* and *ptc1* inhibit slow muscle (D, G) and muscle pioneer development (arrows in F, I), but fast muscle cells are relatively unaffected. Simultaneous injection of *hhip* and *ptc1* mRNAs significantly enhances the phenotypes observed in the single injection experiments (J, L). (N–D′) Loss of Hhip or Ptc increases formation of muscle pioneer and slow muscle cells. (N–Q) Control embryo. (R–U) *hhip*-MO-injected embryo. (V–Y) *ptc*-MO-injected embryo. (Z–C′) *hhip*-MO and *ptc*-MO-injected embryo. (N, O, R, S, V, W, Z, A′) Transverse sections of 24 hpf embryo labeled with slow muscle marker S58 (N, R, S, V, Z) and stained with Hoechst to label nuclei (O, S, W, A′). (P, T, X, B′) Expression of *eng1a* in 24 hpf embryos. (Q, U, Y, C′) Embryos labeled with Prox1 and 4D9. An expansion of the slow muscle domain was observed in *hhip*-MO (arrow, R), *ptc*-MO (V, arrow) and *hhip*-MO + *ptc*-MO (Z, arrow)-injected embryos. In situ hybridization of *eng1a* and double labeling with Prox1 and 4D9 demonstrates that the number of muscle pioneer cells increases in *hhip*-MO-injected (T, U, D′) and *ptc*-MO-injected (X, Y, D′) embryos. Simultaneous injection of *hhip*-MO and *ptc*-MO enhances these effects (B′, C′ D′). This enhanced phenotype is similar to that observed in *shh* mRNA-injected embryos (D′). (M, D′) Numbers of slow muscle and muscle pioneer cells. Averages were calculated from the total number of cells labeled by the Prox1 antibody (slow muscle) and the total number of cells labeled by both the Prox1 and 4D9 antibodies (muscle pioneer cell) counted in four somites over the extended yolk per embryo at 24 hpf in 5–10 embryos. Data represent the average ± SEM. Significance: $*P < 0.01$, $**P < 0.05$, $***P < 0.075$, # no significant difference, Student's *t* test. Lateral views, anterior are towards the left (C, F, I, L, P, Q, T, U, X, Y, B′, C′). Scale bar (A, B, D, E, G, H, J, K, N, O, R, S, V, W, Z, A′): 150 μm (C, F, I, L, P, Q, T, U, X, Y, B′, C′), 100 μm.

Full-length Hhip blocks expression of *myod*, Prox1 and Eng (Figs. 5C, G, H; Supplementary Fig. 2). Hhip lacking the membrane anchoring domain (*hhip*ΔC22) is even more effective at blocking expression of *myod* (Figs. 5D, G, H). This inhibition indicates that extracellular Hh is very effectively titrated by HhipΔC22. Consistent with this interpretation, we confirmed that full-length Hhip protein accumulates on the surface of COS7 cells, whereas HhipΔC22 does not, even though the truncated form of the protein is just as stable (Supplementary Fig. 3). Hhip that lacks both the EGF-like domain and the membrane associated domain (*hhip*Δ614–693) also inhibits expression of *myod* (Figs. 5E, G, H), whereas Hhip lacking amino acid residues 415–693 (*hhip*Δ415–693) that include three

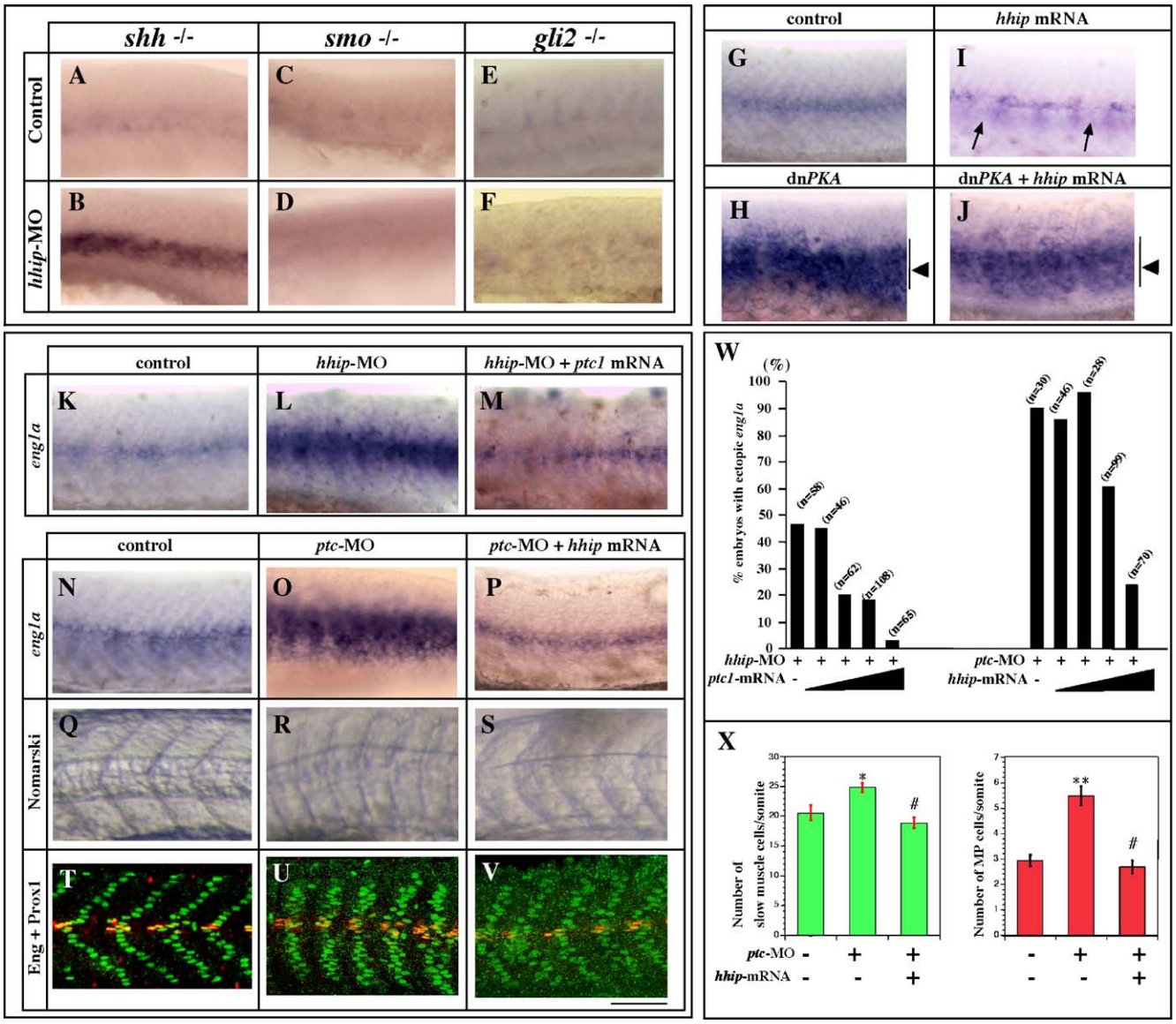

Fig. 4. Hhip and Patched act synergistically in muscle development. (A–J) *hhip* acts downstream of Hh and upstream of Smo. (A, B) Injection of *hhip*-MO rescues *eng1a* expression in *shh* mutants. Expression of *eng1a* is absent in myotomes of 25% of the siblings derived from crosses between $shh^{+/-}$ embryos (A, $n = 243$). In contrast, 90% of *shh* mutant embryos injected with *hhip*-MO exhibit increased *eng1a* expression in the myotome (B, $n = 84$). (C–F) The effect of *hhip*-MO is suppressed in *smo* and *gli2* mutant embryos. *smo* mutant embryos show no *eng1a* expression in the myotome (C, $n = 70$) and injection with *hhip*-MO fails to rescue *eng1a* expression (D, $n = 69$). *gli2* mutant embryos show no *eng1a* expression (E, $n = 216$) and *hhip*-MO injection fails to rescue *eng1a* expression (F, $n = 181$). (G–J) The effect of *hhip* mRNA injection is suppressed by dn*PKA*. (G) *eng1a* expression in control embryo. (H) Injection of dominant negative *PKA* induces ectopic expression of *eng1a* in the myotome (arrow, 23/34 injected embryos). (I) In contrast, *eng1a* expression is suppressed by injection of *hhip* mRNA (arrow). (J) No apparent difference can be detected between dn*PKA*-injected embryos and embryos coinjected with *hhip* mRNA + dn*PKA* (arrow, 26/36 injected embryos). (K–X) Hhip and Ptc can replace each other. (K–M, W) Ptc1 rescues the phenotype of *hhip*-MO-injected embryos. *eng1a* expression in control (K), 3.0 μg/μl *hhip*-MO-injected (L) and 3.0 μg/μl *hhip*-MO and 100 ng/μl *ptc1* mRNA-injected embryos (M). (N, Q, T) Control embryo, (O, R, U) 1.0 μg/μl *ptc*-MO-injected embryo, (P, S, V) 1.0 μg/μl *ptc*-MO and 100 ng/μl *hhip* mRNA-injected embryo. (N, O, W) 90% of *ptc*-MO-injected embryos show ectopic expression of *eng1a*. (P, W) The effects of *ptc*-MO injection are suppressed by overexpression of *hhip*. (Q–S) 59% of *ptc*-MO-injected embryos exhibit U-type somites (S). In contrast, only 36% of *ptc*-MO + *hhip* mRNA-injected embryos show U-type somites. (T–V, X) Double labeling of Prox1 and 4D9. The numbers of slow muscle and muscle pioneer cells increase in *ptc*-MO-injected embryos (U, X) compared to control embryos (T, X). The numbers of slow muscle and muscle pioneer cells are rescued by coinjection of *hhip* mRNA (V, X). (W) Percentage of embryos in which *eng1a* is ectopically induced in myotomes; *n*, number of embryos examined. Ramps indicate increasing concentrations of *ptc1* mRNA (left) or *hhip* mRNA (right), 10, 50, 100, 200 ng/μl *ptc* mRNA and *hhip* mRNA, respectively. (X) Average numbers of slow muscle and muscle pioneer cells per somite counted in 4 somites over the yolk extension in 5–10 embryos. The data represent the average ± SEM. Significance: $*P < 0.075$, $**P < 0.01$, # no significant difference, Student's *t* test. (A–V) Lateral views, anterior toward the left; scale bar: 100 μm.

putative N-linked glycosylation sites fails to inhibit *myod* expression (Figs. 5F, G, H). This result is consistent with analysis of the strongest *hhip* mutant allele (uki^{hu418b}) that has a stop codon at position 418 (Koudijs et al., 2005). These results demonstrate that Hhip lacking the EGF-like and membrane anchoring domain can inhibit Hh signaling, whereas amino acid residues 415–614 are necessary for Hh inhibition.

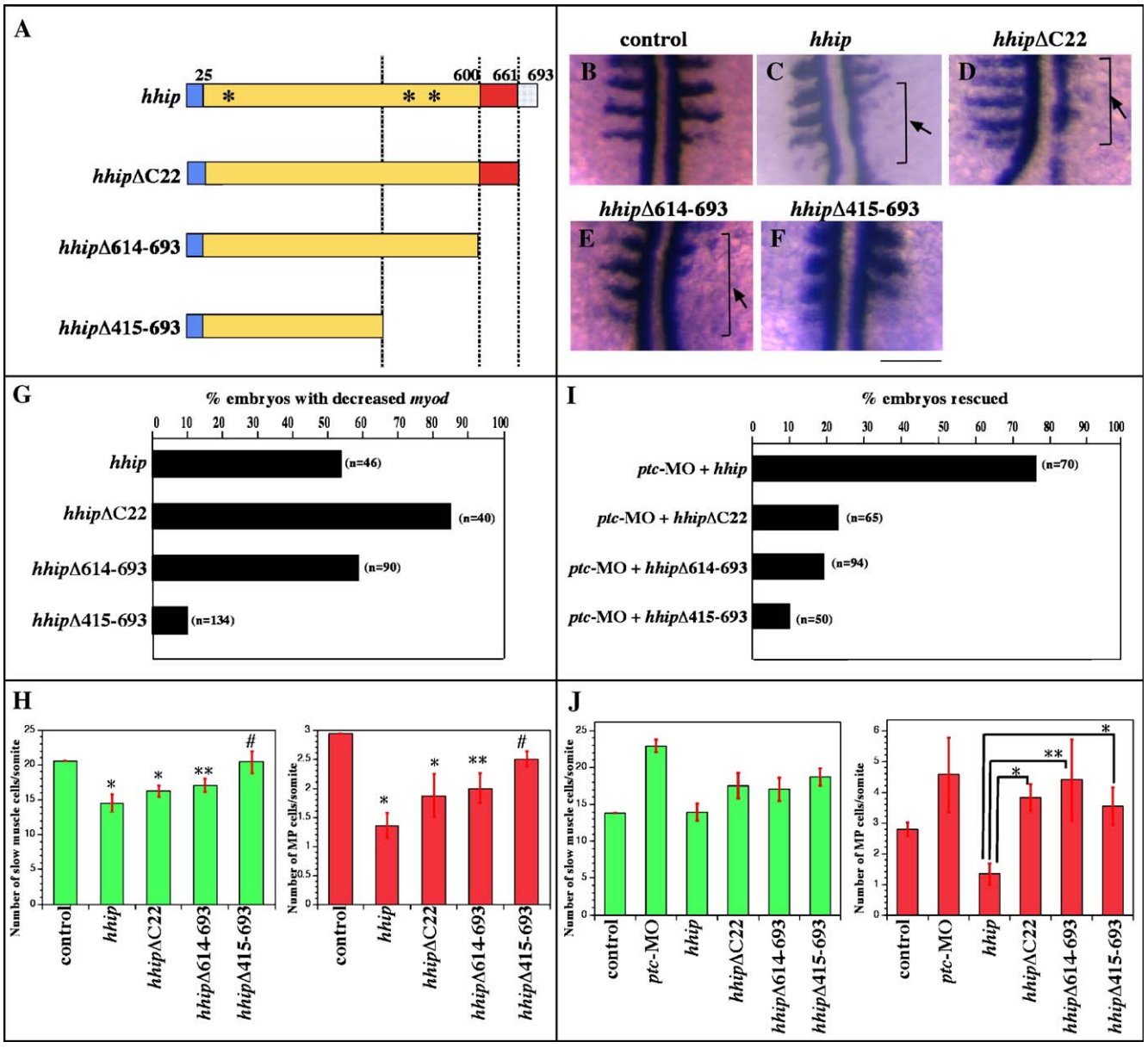

Fig. 5. The Hhip membrane anchoring domain is required for synergistic interaction with Patched but not for negative regulation of Hedgehog. (A) Schematic of Hhip deletion mutants. Blue indicates hydrophobic region, red EGF-like domain and speckled blue the membrane anchoring domain. Asterisks indicate potential N-linked glycosylation sites. (B–H) Hhip lacking the membrane anchoring domain (D) and the EGF-like domain (E, arrow) still inhibits *myod* expression, whereas Hhip lacking amino acids 415–693 fails to inhibit *myod* expression. We scored the percentage of embryos with decreased *myod* at segmentation stages (G, *n*, total number of embryos) and the total number of cells labeled by Prox1 or both Prox1 and 4D9 at 24 hpf (H, Supplementary Fig. 3). The data represent the average ± SEM. Significance: $*P < 0.01$, $**P < 0.05$, # no significant difference, Student's *t* test. (I, J) Hhip lacking the membrane anchoring domain fails to rescue *ptc*-MO-injected embryos. (I) *ptc*-MO induces ectopic *engl1a* expression in the myotome, and full-length *hhip* restores this expression (Supplementary Fig. 3). In contrast, *hhip*ΔC22 does not suppress ectopic expression *engl1a* in *ptc*-MO-injected embryos. (J) Full-length *hhip* but not *hhip*ΔC22 suppresses the number of muscle pioneer cells. The data represent the average ± SEM. Significance: $*P < 0.05$, $**P < 0.1$, Student's *t* test. (B–F) Dorsal views, anterior towards the top, scale bar, 50 μm.

Although the membrane anchoring domain is not required for Hhip to suppress Hh activity, surprisingly, membrane anchoring is required for Hhip to rescue the phenotype of *ptc*-MO-injected embryos. *ptc*-MO induces ectopic expression of *engl1a* in the myotome, and full-length Hhip rescues this phenotype (Figs. 5I, J; Supplementary Fig. 3). In contrast, Hhip that lacks the membrane anchoring domain fails to suppress ectopic *engl1a* expression in *ptc*-MO-injected embryos (Figs. 5I, J; Supplementary Fig. 3), and the numbers of 4D9 positive muscle pioneer cells and Prox1 positive slow muscle cells also are not rescued by injection of *hhip*ΔC22 (Fig. 5J). Hhip lacking the EGF-like domain (*hhip*Δ614–693) or amino acid residues 415–693 (*hhip*Δ415–693) also fails to suppress ectopic *engl1a* expression (Fig. 5I; Supplementary Fig. 2). Thus, even though loss of membrane anchoring increases the efficacy of Hhip to reduce Hh signaling (Fig. 5D), membrane anchoring, rather than titration of extracellular Hh, is required for Hhip to rescue *ptc*-MO-injected.

Hhip and Smoothened, but not Patched, colocalize on the cell surface and internalize together in response to Hedgehog

Because Hhip requires membrane anchoring to rescue *ptc*-MO-injected embryos, Hhip may interact with membrane-bound Ptc and/or Smo. To examine this possibility, we first compared the subcellular localization of Hhip, Smo and Ptc in COS7 cells. Hhip protein is localized at the cell surface and intracellularly (Fig. 6A). We used specific markers to confirm that intracellular Hhip is associated with the endoplasmic reticulum and Golgi (Supplementary Fig. 4). Comparison of Hhip, Smo and Ptc localization reveals that Hhip colocalizes with Smo at the cell surface but not with Ptc (Figs. 6A–F). We obtained similar results with unpermeabilized COS7 cells and with NIH3T3 cells. In contrast, Ptc localizes primarily in intracellular vesicles and only weak Ptc labeling is seen at the cell surface (data not shown). Together, these results suggest that Hhip may interact with Smo rather than Ptc and that Hhip and Ptc may have different modes of action.

Hhip and Smo translocate together in response to Hh. Previous studies showed that Smo internalizes in response to Hh activity via Clathrin-coated vesicles (Chen et al., 2004), and we confirmed this result in COS7 cells (Supplementary Fig. 4). COS7 cells express an endogenous Hhip-like protein as indicated by antibody labeling and Western blot analysis (data not shown). We find that Hhip colocalizes with Smo both at the cell surface in control cells (Figs. 6G–I) and in juxtanuclear and peripheral vesicular structures in cells exposed to Hh (Figs. 6J–L). The redistribution of Hhip and Smo is associated with Clathrin (Figs. 6M–R). The juxtanuclear Hhip also colocalizes with Transferrin–Alexa Fluor 594, a marker for early and recycling endosomes (Supplementary Fig. 4; Incardona et al., 2002; Cho et al., 2004). In contrast, Hhip does not colocalize with Ptc in cells exposed to Hh (Figs. 6S–X). These results suggest that Hhip and Smo internalize together in Clathrin-coated endocytotic vesicles and are sorted into recycling endosomes.

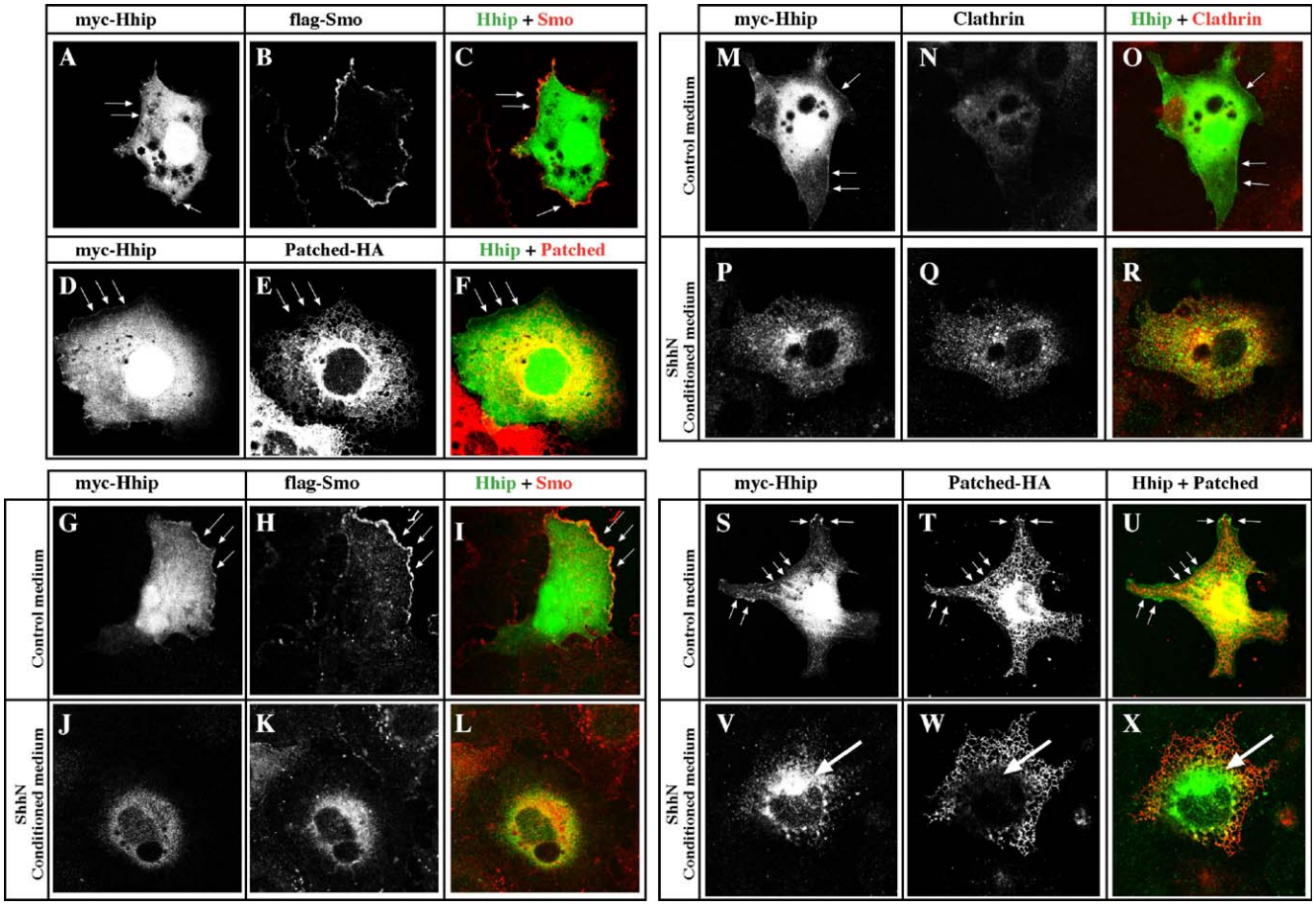

Fig. 6. Hhip and Smoothened colocalize at the cell surface and internalize together with Clathrin in response to Hh. (A–F) Hhip and Smo, but not Ptc, colocalize at the cell surface. COS7 cells transfected with myc-Hhip, flag-Smo or Ptc-HA. (A–C) Hhip is located on the cell surface (A, arrow) and intracellularly. Intracellular Hhip localizes with Calreticulin and GM130, markers of endoplasmic reticulum and Golgi, respectively (Supplementary Fig. 4). Myc-tagged Hhip (A), Flag-tagged Smo (B) and merged image (C). (E) Ptc is located predominantly intracellularly. Myc-tagged Hhip (D), HA-tagged Ptc (E) and merged image (F). (G–X) Hhip and Smo internalize together with Clathrin in response to Hh. COS7 cells transfected with myc-Hhip, flag-Smo or Ptc-HA and then incubated for 2 h with control medium (G–I, M–O, S–U) or ShhN conditioned medium (J–L, P–R, V–X). (G–R) Hhip and Smo distribute to juxtanuclear and peripheral vesicular structures. (M–R) Ptc does not colocalize with Hhip (R, arrow). (S–X) Hhip localizes with endogenous Clathrin in ShhN-treated cells. Arrows indicate membrane associated Hhip (S). Myc-tagged Hhip (S, V), Clathrin (T, W) and merged image (U, X). (A–X) Single confocal images.

Hhip regulates subcellular localization of Smoothened

The colocalization of Hhip and Smo suggests that Hhip may function with Smo during muscle development. We used a transiently expressed, myc-tagged form of Smo to localize Smo in embryos. In cells near the notochord, Smo protein is present both on the cell surface and intracellularly (Figs. 7A, B). We confirmed the membrane localization by colabeling with β-catenin, a plasma membrane marker (Fig. 7D). In contrast, Smo expressed in more lateral cells localizes primarily on the cell surface (Fig. 7C). To learn whether the intracellular localization of Smo in cells near the notochord requires endocytosis, we used monodansylcadaverine (MDC), an inhibitor of the membrane-bound transglutaminase, to block endocytosis. MDC is known to interfere with Clathrin-mediated receptor trafficking (Davies et al., 1980; Vabulas et al., 2002). Treatment with MDC significantly suppresses internalization of Smo (Figs. 7I, J, L, arrow), consistent with our interpretation that intracellular localization of Smo depends upon endocytosis. Disruption of Hhip activity by injection of *hhip*-MO produces a similar change in Smo localization in cells near the notochord (Figs. 7E, F, H). The internalization of Hhip in response to Hh application is also suppressed by MDC in COS7 cells (Supplementary Fig. 5). These results suggest that Hhip modulates the localization of Smo in zebrafish paraxial mesoderm.

Discussion

We previously showed the Hh signaling is both necessary and sufficient for formation of zebrafish slow muscle and muscle pioneer cells and suggested that different levels of Hh signal produce different fates (Du et al., 1997; Barresi et al., 2000;). Subsequent studies implicated Ptc, Fu and Sufu as regulators of Hh activity (Wolff et al., 2003). Ptc is thought to act by inhibiting Smo, thus repressing the Hh signaling pathway cell-autonomously. Consistent with this interpretation, Ptc overexpression decreases the number of slow muscle cells (Lewis et al., 1999b) and *ptc*-MO injection increases the number of slow muscle cells (Wolff et al., 2003). However, *ptc* mRNA injection fails to block formation of all slow muscle, and simultaneous injection of *ptc1*-MO and *ptc2*-MO is not sufficient to convert the entire myotome to the slow lineage (Wolff et al., 2003). These observations suggest that some other, previously unknown mechanisms regulate the relative proportions of slow and fast muscle. Our results suggest that Hhip is a likely candidate (Fig. 8). We find that (1) *hhip* is expressed by muscle precursor cells in response to Hh, (2) combined activity of Hhip and Ptc regulates restricted expression of *myod* in adaxial cells and subsequent slow muscle and muscle pioneer cell development, (3) Ptc and membrane associated Hhip act synergistically to regulate muscle cell fates and (4) Hhip modulates subcellular localization of Smo. Our results are consistent with the

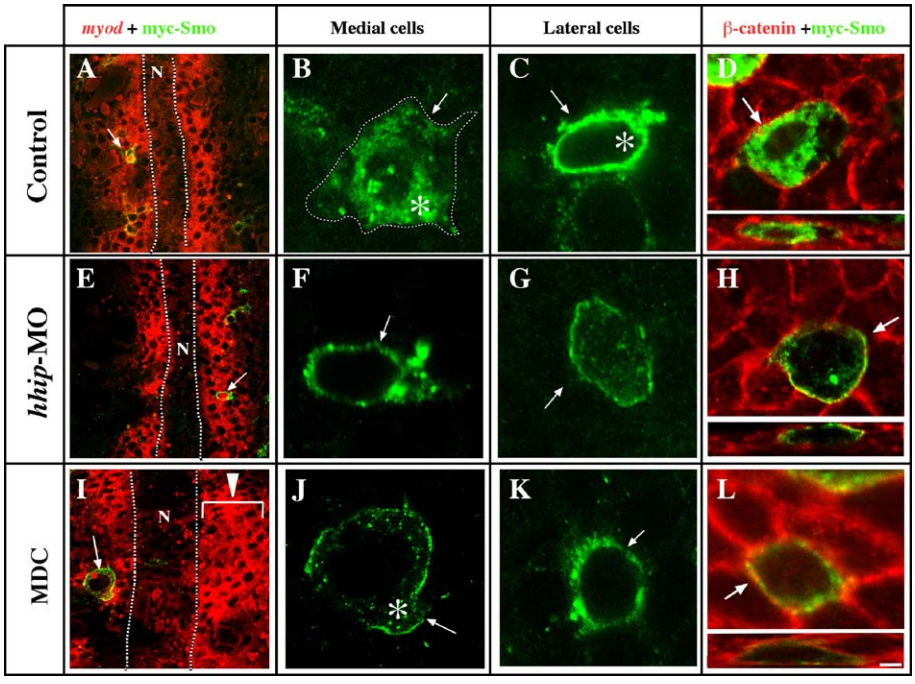

Fig. 7. Hhip regulates Smoothened localization. (A–D) Smo is detected intracellularly (B, asterisk) and at the cell surface (B, arrow and broken line) in cells near the notochord (A, B, D, 93%, $n = 16$) compared with lateral cells that contain primarily intracellular Smo (C, 50%, $n = 6$). (E, F, G, H) *hhip*-MO suppresses internalization of Smo in medial cells (83%, $n = 18$). (I, J, K, L) Inhibition of endocytosis suppresses internalization of Smo (100%, $n = 7$). 100 μM MDC added at the 40% epiboly stage and then incubated to the 8-somite stage. (A–L) Embryos were injected with myc-tagged *smo* plasmid at the 1-cell stage and incubated to the 8-somite stage. Single confocal images of myc-tagged Smo (A–G, I, J, K and D, H, L, upper panels). Embryos were labeled with anti-myc antibody and *myod* (A, E, I) or anti-β-catenin antibody (D, H, L), a marker of the membrane (Nagafuchi, 2001). (D, H, L, Lower panels) Confocal micrographs (transverse z series) through myc-tagged Smo expressing cells. (A, E, I) Arrows indicate the cells shown at higher magnification in right panels. (B, C, D, F, G, H, J, K) Asterisks and arrows indicate intracellular and surface localization of Smo, respectively. N, notochord, scale bar: (A, E, I) 10 μm, (B, C, D, F, G, H, J, K, L) 2.5 μm.

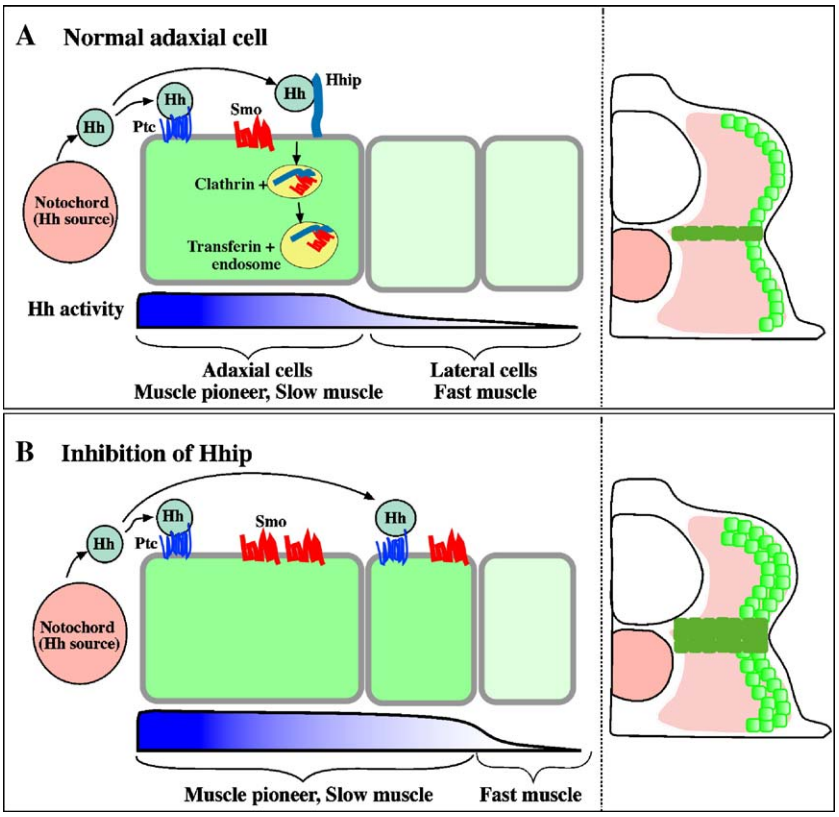

Fig. 8. Hhip regulates muscle development. (A) Hhip functions to sequester Hh and modulate localization of Smo. Hhip together with Ptc sequesters Hh and then Hhip internalizes together with Smo into endosomes. This function allows adaxial cells to accumulate high levels of Hh while preventing movement of Hh to more lateral cells. (B) Inhibition of Hhip allows Hh activity to spread to cells lateral to adaxial cells. Increased Hh activity allows lateral cells to express *myod* (green, left panel) and to development subsequently into extra muscle pioneer cells and slow muscle cells (green, right panel). Left panels; dark green, cells responding to Hh. Right panels; dark green, muscle pioneer cells; green, slow muscle cells; pink; fast muscle cells.

hypothesis that Hhip regulates zebrafish muscle development by sequestering Hh and modulating subcellular localization of Smo. Paraxial cells immediately adjacent to notochord, a source of Hh, express high levels of Ptc and Hhip that limit Hh activity. Thus, adaxial cells form a sharp border between slow and fast muscle precursors.

Hhip regulates muscle development

In the Hh pathway, Ptc acts by sequestering Hh ligands and inhibiting Smo. Therefore, inactivation of Ptc influences the distribution of the signaling activity and also causes a cell-autonomous derepression of the pathway (Wolff et al., 2003). Hhip was originally identified for its ability to bind ShhN, and overexpression of Hhip mimics the phenotype of *Ihh* knockout mice (Chuang and McMahon, 1999). Therefore, it was thought that Hhip acts as an attenuator of Hh signaling by titrating extracellular Hh. Consistent with this, we find that the expression of *myod* in Hhip-inactivated embryos is upregulated in an expanded domain near the midline, but not uniformly throughout the paraxial mesoderm. Subsequently, extra muscle pioneer cells form. These results suggest that Hhip helps restrict Hh signaling to adaxial cells. Similarly, *hhip* mRNA suppresses *myod* expression and subsequent muscle pioneer cell and slow muscle (but not fast muscle) development. In zebrafish, skeletal muscle cell fates are influenced by the level of Hh signaling; muscle pioneer cells require maximal levels of Hh activity, slow muscle cells require intermediate levels of Hh activity and fast muscle cells form in the absence of Hh activity (Blagden et al., 1997; Wolff et al., 2003). Our results indicate that Hhip participates in this process by fine tuning Hh activity.

Hhip and Patched act synergistically to regulate muscle cell fates

Because adaxial cells express both *ptc* (Lewis et al., 1999b) and *hhip*, we anticipated that Hhip would regulate slow muscle development together with Ptc. Ptc acts both by sequestering Hh and suppressing Smo, whereas Hhip was expected only to bind Hh. Although *ptc*-MO-injected embryos exhibit a slightly more severe phenotype than *hhip*-MO-injected embryos, simultaneous injection of both MOs dramatically affects muscle development, more than expected from an additive effect. One interpretation of this synergistic interaction is that titration of extracellular Hh is not the only function of Hhip in the Hh pathway. Our epistatic and deletion analyses support this interpretation. First, Hhip acts in the same part of the Hh signaling

pathway as Ptc, downstream of Hh and upstream of Smo. Second, Hhip lacking the membrane anchoring domain is unable to rescue *ptc*-MO-injected embryos even though it can still suppress Hh signaling. Hence, these results suggest that membrane-anchored Hhip has functions in addition to titration of Hh.

Hhip functions to localize smoothened

Studies in both vertebrates and *Drosophila* indicate that, when Hh protein binds to Ptc, the resulting complex is internalized and trafficked into endosomes where it is degraded (Denef et al., 2000; Incardona et al., 2002; Torroja et al., 2004). In *Drosophila*, treatment of cells with Hh results in removal of Ptc from the cell surface and subsequent accumulation of a phosphorylated form of Smo (Ingham et al., 2000; Denef et al., 2000; Zhu et al., 2003; Torroja et al., 2004; Gallet and Therond, 2005). Subsequently, Hh and Ptc internalize together (Torroja et al., 2004). Ptc protein also moves from the plasma membrane to the endocytic compartment in a ligand-independent manner. Although Ptc internalization does not apparently play a direct role in Hh signal transduction, this internalization regulates the Hh gradient (Torroja et al., 2004). In vertebrates, Smo internalizes in Clathrin-coated vesicles in a process dependent upon phosphorylation by GRK2 and interaction with β-Arrestin2 (Incardona et al., 2002; Chen et al., 2004). Although wild-type Smo localizes to the juxtanuclear region of KNRK cells, activated mutant SmoM2, isolated from human basal cell carcinomas, does not (Incardona et al., 2002). It is still unclear, however, whether internalization of Smo is essential for Hh signaling or formation of the Hh gradient.

Endocytosis is a generally important mechanism for modulating cell signaling. Mutation of fibroblast growth factor receptor 3 (FGFR3) slows receptor internalization and prolongs signaling (Monsonego-Ornan et al., 2000). In the Wnt signaling pathway, Kremen2, a type I transmembrane protein with a short cytoplasmic tail, forms a ternary complex with Dkk1 and the Wnt receptor LRP6 and induces rapid endocytosis and removal of LRP6 from the plasma membrane (Mao et al., 2002). Similarly, we find that Smo protein is concentrated at the cell surface in *hhip*-MO-injected embryos, whereas Smo localizes both at the surface and intracellularly in control embryos. In addition, because Hhip internalizes together with Smo and Clathrin in response to Hh activity, we suggest that Hhip may modulate Smo localization. In support of this interpretation, we also find that blocking endocytosis increases accumulation of Smo on the cell surface and expression of Hh target genes (Supplementary Fig. 5). Thus, membrane-anchored Hhip may be linked to Smo activity by modulating Smo localization.

We propose a novel explanation for how Hh signaling is regulated to produce a sharp border between cells with different development fates (Fig. 8). In zebrafish muscle precursors, Hh expression by notochord results in upregulation of Hhip and Ptc in adjacent adaxial cells. Initially, Hhip and Ptc sequester Hh protein to adaxial cells. Then, Hhip and Smo internalize by endocytosis and enter the endosomal pathway. Thus, Hhip may contribute to downstream processing of Smo in endosomes. Sequestering and internalization limit the spread of Hh to adaxial cells, allowing them to differentiate into slow muscle, whereas more lateral cells see only low levels of Hh activity and, thus, form fast muscle.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.05.001.

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