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Scube activity is necessary for Hedgehog signal transduction *in vivo*

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ABSTRACT

The Hedgehog (HH) signaling pathway is a central regulator of embryonic development, controlling the pattern and proliferation of a wide variety of organs. Previous studies have implicated the secreted protein, Scube2, in HH signal transduction in the zebrafish embryo (Hollway et al., 2006; Kawakami et al., 2005; Woods and Talbot, 2005) although the nature of the molecular function of Scube2 in this process has remained undefined. This analysis has been compounded by the fact that removal of Scube2 activity in the zebrafish embryo leads to only subtle defects in HH signal transduction *in vivo* (Barresi et al., 2000; Hollway et al., 2006; Ochi and Westerfield, 2007; van Eeden et al., 1996; Wolff et al., 2003). Here we present the discovery of two additional *scube* genes in zebrafish, *scube1* and *scube3*, and demonstrate their roles in facilitating HH signal transduction. Knocking down the function of all three *scube* genes simultaneously phenocopies a complete loss of HH signal transduction in the embryo, revealing that Scube signaling is essential for HH signal transduction *in vivo*. We further define the molecular role of *scube2* in HH signaling.

Introduction

HH signaling plays significant roles in normal embryonic development and erroneous HH signaling leads to human disease (reviewed in Bale, 2002; Cohen, 2003; Mullor et al., 2002; Nieuwenhuis and Hui, 2005; Taipale and Beachy, 2001; Wicking and McGlinn, 2001). Mutations in components of the HH signal transduction pathway have been directly implicated as causative in a number of developmental abnormalities, such as craniofacial and polydactyly conditions (Hui and Joyner, 1993; Kang et al., 1997; Radhakrishna et al., 1997; Roessler et al., 1996; Vortkamp et al., 1991). In addition, mutation and mis-regulation of HH signaling components are present in a wide variety of malignant tissues (reviewed in Evangelista et al., 2006). Many of the genes that regulate Hedgehog signal transduction have been identified and the majority of the molecular components are conserved from *Drosophila melanogaster* through to vertebrates (Hooper and Scott, 2005; Ingham and McMahon, 2001). In its most basic form the pathway involves reception of the secreted HH glyco-protein signal by the transmembrane receptor Patched, which in the absence of ligand inhibits the activity a second transmembrane receptor, Smoothened. When HH ligand is present, Smoothened inhibition is released, leading to activation of the Gli transcription factor and transcription of HH target genes.

In the developing zebrafish embryo, HH signaling specifies two different muscle fiber types, slow twitch and fast twitch muscle (Blagden et al., 1997, Du et al., 1997). Varying levels of HH signaling are required to specify the different cell types found within the myotome; high levels of HH are required to induce the Engrailed (En) positive muscle pioneer slow cells (MPs) and the En positive medial fast fibers, while lower concentrations of HH specify the non-pioneer slow twitch muscle fibers, which arise from the adaxial cells adjacent to the notochord and migrate laterally to form a superficial layer of slow muscle (Blagden et al., 1997; Currie and Ingham, 1996; Wolff et al., 2003). Fast muscle fibers differentiate after slow muscle migration has begun and are thought to receive the lowest amount of HH signal (Ochi and Westerfield, 2007; Wolff et al., 2003). The *you*-type group of zebrafish mutants have "U"-shaped rather than chevron-shaped somites and disrupt the formation of cell fates that are known to

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be controlled by HH signaling such as MPs and slow twitch muscle cells (van Eeden et al., 1996) and many of the *you*-type mutants possess mutations in genes encoding HH signaling pathway components (Barresi et al., 2000; Hollway et al., 2006; Karlstrom et al., 1999; Kawakami et al., 2005; Woods and Talbot, 2005).

Mutations in the coding region of the gene *scube2* were identified in multiple alleles of a canonical member of the *you*-type mutant class, termed *you* (Hollway et al., 2006; Kawakami et al., 2005; Woods and Talbot, 2005). *you* mutants display the weakest phenotype of the class, with a reduction in MPs and a variable reduction of the slow twitch muscle cell population (Lewis et al., 1999; Hollway et al., 2006; Kawakami et al., 2005; Woods and Talbot, 2005). *you* mutants demonstrate an anterior to posterior progression in their phenotype whereby the loss of HH sensitive fates becomes more severe in the posterior of the embryo (Hollway et al., 2006). Expression of the HH receptor *ptc* and the muscle specific transcription factor *myod,* both sensitive to HH signaling, are reduced in the posterior adaxial cells of *you* homozygous mutant embryos (Hollway et al., 2006; Kawakami et al., 2005; Lewis et al., 1999; Woods and Talbot, 2005) but remain relatively unaffected in anterior somites.

Scube2, a member of the signal peptide-CUB domain-EGF-related (*scube*) gene family, was first identified in mouse as a novel gene encoding an epidermal growth factor (EGF)-related protein containing a CUB domain (Grimmond et al., 2000). Three distinct *scube* gene family members have been identified and called *scube1–3*, based on their order of discovery in mammals (Grimmond et al., 2000; Grimmond et al., 2001; Wu et al., 2004; Yang et al., 2002). Scube family proteins contain a conserved domain organization, consisting of an N-terminal domain containing nine EGF-like repeats of the calcium binding class, and a single CUB (Complement subcomponents C1r/C1s, EGF related sea urchin protein, bone morphogenetic protein-1) domain near the C-terminus. The EGF-like repeats and CUB domain are linked by a spacer region and are thought to be involved in protein-protein interactions and ligand binding. Additionally the Scube proteins are known to be secreted and cell-surface associated (Grimmond et al., 2000; Wu et al., 2004; Yang et al., 2002). Homodimerization has been demonstrated for all three human SCUBE proteins, while hSCUBE1 has been shown to heterodimerize with both hSCUBE2 and hSCUBE3 (Wu et al., 2004; Yang et al., 2002).

Despite previous studies (Hollway et al., 2006; Kawakami et al., 2005; Woods and Talbot, 2005) the exact nature of Scube2's role in Hedgehog signaling remains elusive. We and others have previously shown, using transplants between *you* mutant and wildtype embryos, that *scube2* is required non-cell autonomously for correct specification of HH target cells, (Hollway et al., 2006; Woods and Talbot, 2005). These and other analyses suggest that Scube2 may be required for the transport or stability of the HH signal (Hollway et al., 2006; Woods and Talbot, 2005). The notion that Scube2 may act upstream or adjacent to reception of the HH signal is reinforced by studies that show components of the HH signaling pathway downstream of Shh are functional in Scube2 mutants (Hollway et al., 2006).

Given the lack of a universally accepted mode of action for Scube2, we sought to further characterize the role of the Scube gene family in HH signaling. The you^{ty97} mutant phenotype is relatively weak compared to other *you*-type mutants, and thus its central importance to HH signal transduction has remained unclear. The existence of three *scube* genes in both mouse and humans suggested there might also be additional *scube* genes functioning in HH signaling in zebrafish, and that the original models of Scube2 function in HH signal transduction may have been confounded by redundant action of the Scube protein class. In this study, we have determined there are three *scube* genes in zebrafish and that all three genes play a role in HH signaling and slow muscle development in the zebrafish embryo, and that combined knockdown of function for all three *scube* genes completely abrogates HH signaling in the zebrafish embryo. We also further show that despite being required for normal HH signal transduction *in vivo* the necessity for collective scube activity can be overcome with application of sufficient HH ligand.

Results

Identification and mRNA expression of scube1 and 3 genes in zebrafish

Mouse, human and chicken *scube1* and *scube3* sequences were used to identify partial, orthologous sequences within the integrated Whole Genome Shotgun (WGS) assembly of the zebrafish genome available at Ensembl (www.ensembl.org/Danio_rerio/Info/Index). Partial sequences of two zebrafish *scube* genes, additional to *scube2*, were identified and assembled using Genewise (http://www.ebi.ac.uk/Tools/Wise2/index.html). Following RACE, full-length sequences corresponding to the predicted partial sequences were isolated from a zebrafish cDNA library (GenBank accession numbers JF900367 (*scube1*) and JF900368 (*scube3*)). Alignment of the corresponding protein sequences for zebrafish Scube1 and Scube3 with mouse and human Scube orthologues, showed high homology across these species for both Scube1 and Scube3 proteins (Fig. S1 and data not shown). No additional *scube* sequences were identified in the zebrafish genome. Analysis of the zebrafish Scube1 and Scube3 protein sequences determined these Scube family members also have the signal peptide, EGF repeats, CUB domains and spacer regions found in Scube2 and all known Scube family members. Zebrafish Scube1 and Scube3 proteins cluster with their mouse and human orthologues in a phylogenetic tree (Fig. S1). Sequence regions of low similarity between the three zebrafish *scube* genes were used to design *in situ* hybridization probes for each gene. This analysis revealed that *scube1* is maternally expressed (Fig. 1A) and at ~90% epiboly is expressed in the developing notochord (Fig. 1B). From 1–2 somites, embryos exhibit diffuse neural and hindbrain staining, weak expression in the pre-somitic mesoderm (psm) and notochord which persists at the 15 somite stage (Fig. 1C). At later somite stages embryos exhibit stronger *scube1* expression in the somitic mesoderm and notochord, as well as in the forebrain, midbrain, hindbrain and rhombomeres (Fig. 1D, E). Analysis of histological sections confirmed expression within specific regions of the neural tube and brain, with strong expression in the floorplate and lateral ventral regions of the neural tube, areas known to be patterned by HH signal transduction (Fig. 1F, G, I). Expression within the somitic mesoderm was also confirmed via sectioning (Fig. 1H). Maternal expression of *scube3* was also found (Fig. 1J), early somite stages exhibited expression in the notochord, developing somites as well as diffuse neural expression. The neural, notochord, somitic mesoderm and presomitic mesoderm staining persisted throughout somitogenesis (Fig. 1K). Strong floorplate expression and weak notochord expression is evident at the 26 somite stage, which shows an anterior (high) to posterior (low) gradient of expression (Fig. 1L–P). Weak somitic mesoderm expression of *scube3* is also evident at this stage. In summary *scube1* and *scube3* are expressed within tissues that are known to secrete HH or are patterned by HH, although clear exceptions to this can be seen, that hint at a broader role for Scube proteins than modulation of HH signaling alone.

Morpholino knockdown of scube1 and scube3 expression enhances the phenotype of the scube2 mutant, you^{ty97}

The weak phenotype of you^{ty97} mutant embryos, compared with other members of the *you*-type class (Hollway et al., 2006; van Eeden et al., 1996), together with the overlapping nature of

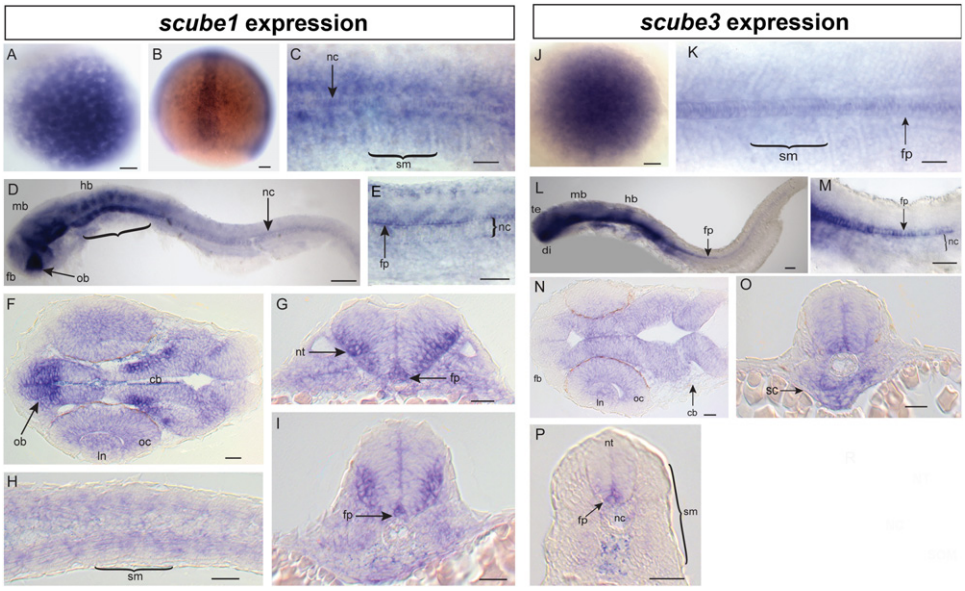

Fig. 1. mRNA expression patterns of *scube1* and *scube3*. (A–I) *scube1* mRNA expression. (A) Maternal expression of *scube1* in 256 cell embryo, (B) *scube1* is expressed in a stripe of cells corresponding to the developing notochord (nc) at ~90% epiboly. (C) At 15 somites, *scube1* is expressed in the somitic mesoderm (sm), and notochord. (D, E) At 26 somites *scube1* expression is in the forebrain (fb), olfactory bulb (ob), midbrain (mb), hindbrain (hb), rhombomeres (bracket), with diffuse staining present in notochord and floorplate (fp) of neural tube [E closeup of axial somites in embryo equivalent to D]. (F–I) Sections of 24 hpf embryos. (F) *scube1* expression in forebrain, cerebellar neurons (cb), lens (ln), optic cup (oc) with strong expression in the olfactory (ol) forebrain region. (G, I) *scube1* is expressed throughout the neural tube (nt) with strong floorplate and lateral nt expression. (H, I) *scube1* somitic mesoderm expression in tail sections. (J–P) *scube3* mRNA expression. (J) Maternal expression of *scube3* in 256 cell embryo. (K) *scube3* expression in the floorplate, notochord, somitic mesoderm, and PSM at 15 somites. (L, M) Extensive neural expression (telencephalon te, diencephalon, di, midbraim, mb, and hindbrain, hb), expression in the floorplate, somites and diffusely in the notochord at 26 somites. (M) Magnification of tail region in (L) showing strong ventral floorplate staining and weak notochord staining. N–P sections of 24 hpf embryos. (N) *scube3* is expressed in the cerebellum, the lens of the eye and the optic cup and weakly in the forebrain. (O) *scube3* expression is strong in sclerotome ventral to the notochord. (P) *scube3* expression throughout neural tube, strong expression in floorplate and weak notochord expression. Scale Bars: 100 μm. (C, K) Dorsal view, (D, E, L, M) lateral view (F, H, N) longitudinal sections, (G, I, O, P) transverse sections.

the expression of the *scube* genes, suggested the possibility that the three *scube* genes may be functioning redundantly in HH signaling and slow muscle development. Alternatively, it remained formally possible that the allele of *scube2* mostly utilised for analyses, *you*^{ty97}, which is predicted to encode a truncated form of Scube2 missing the C-terminal 443 amino acids, is not a complete null allele. In order to distinguish between these hypotheses, 41.1 ηg of *scube2*MO was injected into homozygous *you*^{ty97} mutant embryos. Consistent with *you*^{ty97} being a null allele of *scube2*, injection of the *scube2*MO did not appear to alter the *you*^{ty97} phenotype ($n=15$) (data not shown) and the loss of slow muscle fibers in the posterior of homozygous *you*^{ty97} embryos injected with *scube2*MO, does not appear to be more severe than *you*^{ty97} alone.

To investigate the possibility of functional redundancy acting between the *scube* encoding genes, translation-blocking morpholinos to *scube1* and *scube3* were injected, individually and in combination, into wildtype and *you*^{ty97} embryos. In homozygous *you*^{ty97} mutant embryos, in addition to U-shaped somites, there is a loss of slow twitch muscle fibers in the posterior tail and the ordered array of fibers becomes patchy, slow myosin heavy chain (MyHC) positive muscle fibers are often absent from the tip of the tail (Fig. 2B). Injection of *scube1* MO or *scube3* MO individually into wildtype embryos did not alter the ordered slow muscle fiber array ($n=40$ for each morpholino) (Fig. 2C, E), although *scube1* morphants appear to have a shorter body axis indicative of a mild axis extension defect (Fig. S2). Furthermore co-injection of *scube1*MO and *scube3*MO into wildtype embryos, also failed to cause an effect on the slow MyHC staining pattern ($n=50$), and the gross phenotype resembled that of embryos injected with *scube1*MO alone (Fig. 2C, G, Fig. S2). In contrast to the injections into wildtype embryos, injection of *scube1*MO or *scube3*MO into homozygous *you*^{ty97} embryos elicited a dramatic fiber loss phenotype, enhancing the phenotype seen in homozygous *you*^{ty97} mutant embryos. Homozygous *you*^{ty97} embryos injected with *scube*1MO have very little or no slow MyHC staining in the first 10–12 somites (Fig. 2D) ($n=44$). Posterior to this level, slow muscle formation occurs normally within yolk extension level somites until the posterior tail at which point the staining pattern reflects the slow muscle loss normally seen in homozygous *you*^{ty97} embryos (Fig. 2B, D). In addition to the enhanced fiber loss, *you*^{ty97}+*scube1*MO injected embryos possessed somites more rounded in appearance than uninjected homozygous *you*^{ty97} embryos (data not shown). Intriguingly, homozygous *you*^{ty97} embryos injected with *scube3*MO display a distinct phenotype, where normal slow MyHC staining is evident in the most anterior ~9 somites, after which the somites completely lack slow MyHC posterior of this point (Fig. 2F) indicating that distinct *scube* genes act at different anterior–posterior regions of the embryo ($n=30$).

Notably, morpholino knockdown of *scube1* and *scube3* in homozygous *you*^{ty97} mutant embryos resulted in an almost complete absence of slow MyHC fibers at all axial levels (Fig. 2H), in addition to a total loss of the wildtype chevron somite shape, resulting in block-like somites (Fig. 3C, F) ($n=25$). To confirm the phenotypic enhancement of *scube1MO* or *scube3MO* occurred only in homozygous *you*^{ty97} mutant embryos, injected embryos were genotyped. All *scube1*MO into *you*^{ty97} embryos displaying the enhanced fiber loss phenotype were found to be homozygous for the *you*^{ty97} mutation ($n=44$), similar results were found for *scube3*MO injections ($n=30$) and *scube1*MO+*scube3*MO injections ($n=25$). Genotyping of ten embryos unaffected by each morpholino injection showed they were either wildtype or *you*^{ty97} heterozygote embryos. Importantly, morpholino knockdown of all three *scube* genes simultaneously, *scube1*MO+*scube2*MO+*scube3*MO, hereafter referred to as *scube* triple MO embryos, in wildtype embryos produced a phenotype equivalent to *you*^{ty97}+*scube1*MO+ *scube3*MO (Figs. 2H, I, 3C) ($n=34$).

Homozygous *you*^{ty97} mutant embryos have reduced expression of the HH target genes *myod*, *patched1* (*ptc1*) and *engrailed* (*eng*) (Fig. 3J–L) (Hollway et al., 2006; Kawakami et al., 2005;

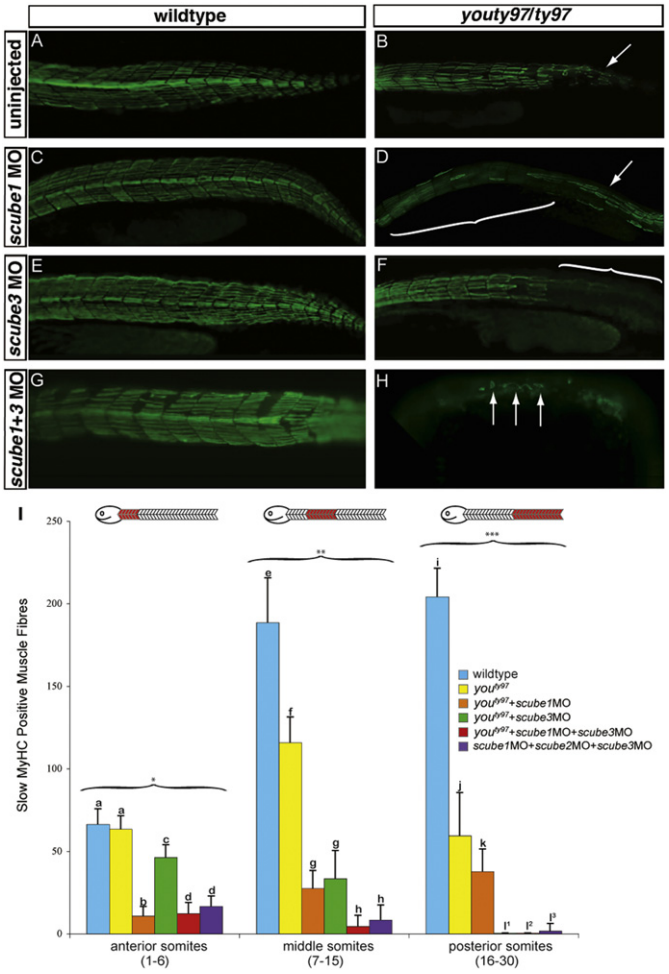

Fig. 2. *scube1* and *scube3* morpholinos enhance the phenotype of *you*^{ty97} mutant embryos. The wildtype pattern of slow MyHC (A) is disrupted in the posterior tail region of *you*^{ty97} homozygotes (B) (arrow). The pattern of slow MyHC is not visibly affected when *scube1*MO (C) *scube3*MO (E) or both *scube1*MO+*scube3*MO (G) are injected into wildtype embryos. (D) *scube1*MO injected into *you*^{ty97} homozygotes enhanced the *you*^{ty97} phenotype. Slow MyHC expression is disrupted after ~somite 6, however slow MyHC expression returns in the posterior somites reflecting the pattern seen in *you*^{ty97} homozygotes in this region (compare B to D). Bracket highlights the region in *you*^{ty97}+*scube1*MO embryos lacking slow MyHC fibers. An arrow indicates the somitic region where slow MyHC expression resembles *you*^{ty97} mutants. (F) *scube3*MO injected into *you*^{ty97} homozygotes also enhanced the *you*^{ty97} mutant phenotype. Slow MyHC is disrupted after ~8–10 somites, all remaining somites completely lack slow MyHC expression. Bracket highlights the somitic region in *you*^{ty97}+*scube3*MO embryos devoid of slow MyHC staining. (H) Injection of *scube1*-MO+*scube3*MO together into *you*^{ty97} mutant embryos essentially eliminates slow MyHC staining. Arrows highlight the few remaining slow MyHC positive fibers remaining in *you*^{ty97}+*scube1*MO+*scube3*MO embryos (H). *Scube* triple MO injection into wildtype embryos faithfully reproduces the phenotype seen in *you*^{ty97}+*scube1*MO+*scube3*MO (H and data not shown). All embryos ~30 hpf. F59 monoclonal antibody labels slow MyHC positive muscle fibers, Alexa 488-conjugated donkey anti-mouse secondary. (I) Slow MyHC positive muscle fibers were counted in anterior (1–6 somites), middle (7–15 somites) and posterior (16–30) somites of wildtype, *you*^{ty97} homozygous mutants, *you*^{ty97}+*scube1*MO embryos, *you*^{ty97}+*scube3*MO embryos, *you*^{ty97}+*scube1*MO+ *scube3*MO embryos, and *scube* triple MO embryos (*scube1*-MO+*scube2*MO+*scube3*MO). Statistical analysis demonstrated the phenotypic enhancement of the *you*^{ty97} mutant phenotype seen following MO injection is significant. The dramatic loss of slow MyHC positive muscle fibers seen following *scube1*MO+*scube3*MO injection into *you*^{ty97} homozygotes was equivalent to the loss of slow MyHC positive muscle fibers seen following *scube* triple MO injection into wildtype embryos (I1-3). *ANOVA $F(5,137)=200.11$, $p<0.001$. **ANOVA $F(5,137)=266.10$, $p<0.001$. ***ANOVA $F(5,137)=391.35$, $p<0.001$. Within each somite region, bars with different letters are statistically significant from one another.

Lewis et al., 1999; Woods and Talbot, 2005), as compared to expression in wildtype embryos (Fig. 3G–I). However, this *you*^{ty97} phenotype is considerably weaker than the reduction of HH target gene expression evident in other mutations disrupting the HH signaling pathway in zebrafish (Hollway et al., 2006; Lewis et al., 1999; Woods and Talbot, 2005) and is restricted to the posterior somitic region, correlating with the loss of slow myhc expressing muscle cells evident within this domain (Figs. 2B, 3J–L). The loss of slow muscle fibers at all axial levels in *scube* triple MO embryos (Fig. 2H) suggested that Scube mediated HH signaling may be required for specification of HH target cells in zebrafish somites. This possibility was investigated by examining HH target gene expression (*myod*, *eng*, *ptc1*) within *scube* triple MO embryos. As seen for Slow MyHC expression (Fig. 2H), HH target gene expression was reduced to barely detectable levels at all axial levels in *scube* triple MO embryos (Fig. 3M–O) (*myod* $n=36$, *ptc* $n=38$, *eng* $n=56$). A similar decrease in HH target gene expression occurred in embryos treated with cyclopamine, a small molecule inhibitor of *smo* signaling (Fig. 3P–R), which were stained in parallel. Similar results were obtained for gene expression analyses examining the role of *scube* gene activities in HH dependent signaling events in neural tissues (Fig. 3S–X″). *ptc1* expression is prominent in the ventral neural tube of the trunk (Fig. 3S–S″) where its expression is known to be induced by HH signaling from the midline. In the brain, *ptc1* is highly expressed within the diencephalon (Fig. 3S″), an expression domain induced by HH signaling from the underlying zona limitans intrathalamica (Scholpp et al., 2006). In both *scube* triple MO embryos (Fig. 3T–T″) and in cyclopamine treated embryos (Fig. 3U–U″) expression of *ptc1* in both these tissue is severely reduced or absent. A second ventral neural marker *foxa2* is also severely reduced in expression in *scube* triple MO embryos (Fig. 3V–X″). In wildtype embryos at 26 somites, *foxa2* is expressed in floor plate (Fig. 3V, V′ both medial and lateral cells) and in two wings of expression in the ventral diencephalon (Strähle et al., 1996) (arrow, Fig. 3V″). HH signaling is known to induce formation of the lateral floor plate cells and is also required for the maintenance of *foxa2* expression in medial floor plate cells (Odenthal et al., 2000). In homozygous *you*^{ty97} mutants both these *foxa2* expression domains are reduced (Fig. 3W–W″), and in *scube* triple MO embryos this reduction of *foxa2* expression is more severe (Fig. 3X–X″). Collectively, these results suggest that Scube proteins are required for normal levels of HH signaling *in vivo*, and act redundantly to facilitate HH signal transduction. Furthermore, the anterior–posterior restriction of the *scube1* and *scube3* loss of function phenotypes, in the absence of Scube2 activity suggests a regional requirement for obligate heterodimerization with Scube2 mediating HH signal transduction.

Molecular basis of scube function in the HH signal transduction pathway

Previously, we proposed, based on the secreted nature and non-cell autonomous action of Scube2, that one possible function of Scube2 may be at the level of signal reception in the HH signaling pathway. To further examine the requirement for Scube activity in HH signal transduction we undertook a series of molecular epistasis experiments to examine this question in the *scube* triple MO context. Ectopic HH pathway activation has been shown to induce ectopic slow muscle at the expense of fast muscle (Blagden et al., 1997), as well as inducing ectopic muscle pioneer cells (Currie and Ingham, 1996; Du et al., 1997; Hammerschmidt et al., 1996), resulting in excess slow twitch muscle and decreased fast twitch muscle. One regulatory component of the HH pathway, involved in the modulation of the Gli transcription factor processing is protein kinase A (PKA). Injection of RNA encoding a dominant negative form of PKA (*dnPKA*) into wildtype embryos resulted in activation of the HH pathway and ectopic slow myhc expression ($n=100$) (Fig. 4C). If Scube proteins act at the level of HH signal reception

to endocytose the HH signal, activation of the pathway downstream of signal reception, by *dnPKA* RNA injection, should be unaffected in *scube* triple MO embryos. Over-expression of *dnPKA* in *scube* triple MO embryos rescued the loss of slow muscle fibers seen in *scube* triple MO embryos, resulting in a slow MyHC staining pattern equivalent to the pattern seen in *dnPKA* RNA-injected wildtype embryos ($n=72$) (Fig. 4C, D). This result is consistent with *dnPKA* functioning downstream of the Scube proteins in the HH pathway.

We hypothesized that injection of *shh* RNA should not be able to induce formation of slow muscle in *scube* triple MO embryos if Scube proteins act in the reception of the HH signal. Surprisingly however, *shh* RNA injection into *scube* triple MO embryos was also able to partially rescue the loss of slow MyHC expressing muscle cells ($n=30$) (Fig. 4E, F). In order to examine this question in more detail we injected increasing amounts of *shh* mRNA into *scube* triple MO embryos and compared the ability of these injections to induce ectopic expression of the *shh* target gene *myod*. We determined that 4 times the amount of *shh* mRNA is required to induce ectopic *myod* expression in *scube* triple MO embryos compared to the amount required for wildtype embryos (Fig. 4K, J, O). These results suggest that although Scube activity is clearly required for normal levels of HH signaling *in vivo*, this need can be bypassed if sufficient ligand is provided.

One explanation for the ability of injected *shh* mRNA to activate target gene expression in *scube* triple MO embryos could be that global over expression of *shh* mRNA in every cell of the developing embryo may induce signaling directly within the cells that receive it, therefore bypassing the need for Scube activity in the reception of secreted HH ligand. To test this possibility we generated small mosaic clones of cells that express SHH fused to GFP under the control of the *pax3a* promoter. The *pax3a* gene is expressed throughout the psm and the dorsal neural tube at the stages these cells are competent to respond to activation of HH signaling. Injection of this construct into wildtype embryos generated ectopic slow muscle both cell autonomously and non-cell autonomously ($n=73$) (Fig. 4Q–S″) and this ability was not altered within *scube* triple MO embryos ($n=30$) (Fig. 4T–V″). Thus

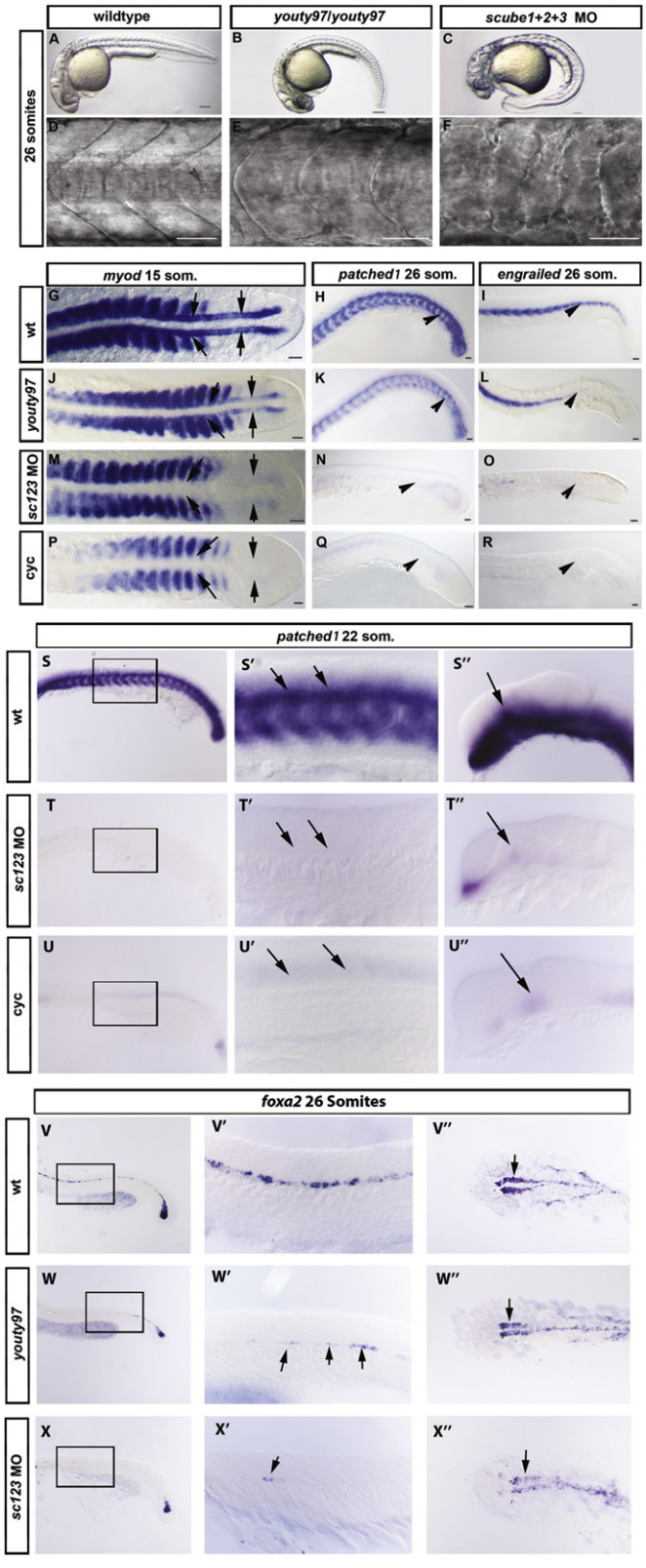

Fig. 3. *scube1* and *scube3* morpholinos enhance the phenotype of *you*^{ty97} mutant embryos. (A–F). Somite shape in live 30 hpf embryos, lateral view anterior to left. (A, D) The characteristic chevron shape of the wildtype zebrafish somite became rounded or 'U' shaped in the *you*^{ty97} mutant (B, E). (C, F) Morpholino knockdown of all 3 *scube* genes enhanced the *you*^{ty97} mutant phenotype, a severe loss of the chevron shape resulted in 'blocky' shaped somites. Scale Bars: A–C 150 μm, D–F 100 μm. (G–R). *In situ* hybridization revealed Hh target gene expression (*myod*, *patched1* (*ptc1*), and *engrailed* (*eng*)), decreased dramatically in *scube* triple MO embryos. (G) *myod* is expressed in somites and adaxial cells of wildtype embryos, (J) adaxial expression of *myod* is reduced in *you*^{ty97} homozygous mutants, (M) adaxial *myod* expression is absent from *scube* triple MO embryo. (P) Adaxial *myod* expression is also absent from cyclopamine treated embryos. (G, J, M, P) Arrows indicate location of adaxial cells. (H) *ptc* expression in ventral neural tube and somites in wildtype embryos. (K) Reduced *ptc* expression in posterior somites of *you*^{ty97} embryo, (N) somitic expression of *ptc* is absent from *scube* triple MO and (Q) cyclopamine treated embryos. (H, K, N, Q) Arrowheads highlight regions of altered gene expression. (I) In wildtype embryos *eng* is expressed in the muscle pioneer cells and a subset of fast muscle fibers, (L) *eng* expression is reduced in the posterior of *you*^{ty97} mutant embryos, (O) in *scube* triple MO embryos *eng* expression is severely reduced with only small patches of *eng* positive cells visible. (R) Cyclopamine treated embryos display the most severe loss of *eng* staining. (I, L, O, R) Arrowheads highlight regions of altered gene expression. (G, J, M, P) 15 somite stage, dorsal view, (H, K, N, Q) 22 somite stage, lateral view anterior to left, (I, L, O, R) 26 somite stage, lateral view, anterior to left. Scale bars: 50 μm. (S–U″) *ptc1* expression is reduced in neural tissue of *scube* triple MO embryos. In wildtype, uninjected 22 somite embryos (S–S″), *ptc1* expression is prominent in the ventral neural tube of the trunk (S and arrows in S′) as well as the somites, where its expression is known to be induced by HH signaling from the midline. In the brain, *ptc1* is highly expressed within the diencephalon (S″, arrow), an expression domain known to be induced by HH signaling from the underlying zona limitans intrathalamica. In both *scube* triple MO embryos (T–T″) and in cyclopamine treated embryos (U–U″) expression of *ptc1* in both these tissues is severely reduced or absent. (V–X″) Expression of the ventral neural marker *foxa2* is severely reduced in *scube* triple MO embryos. In wildtype embryos at 26 somites *foxa2* is expressed in floor plate (V, V′ both medial and lateral cells) and in two wings of expression in the ventral diencephalon (arrow, V′). In homozygous *you*^{ty97} mutants both these regions of expression are reduced (Fig. 3W–W″), and in *scube* triple MO embryos this reduced expression is greatly enhanced (X–X″). (S–X″) Within each row, the same letter indicates a different region of the same embryo.′ Add –(S–X″) anterior to left, (S–U″) 22 somites, lateral view (V,V′, W, W′, X, X′) 26 somites, lateral view, (V″, W″, X″) dorsal view.

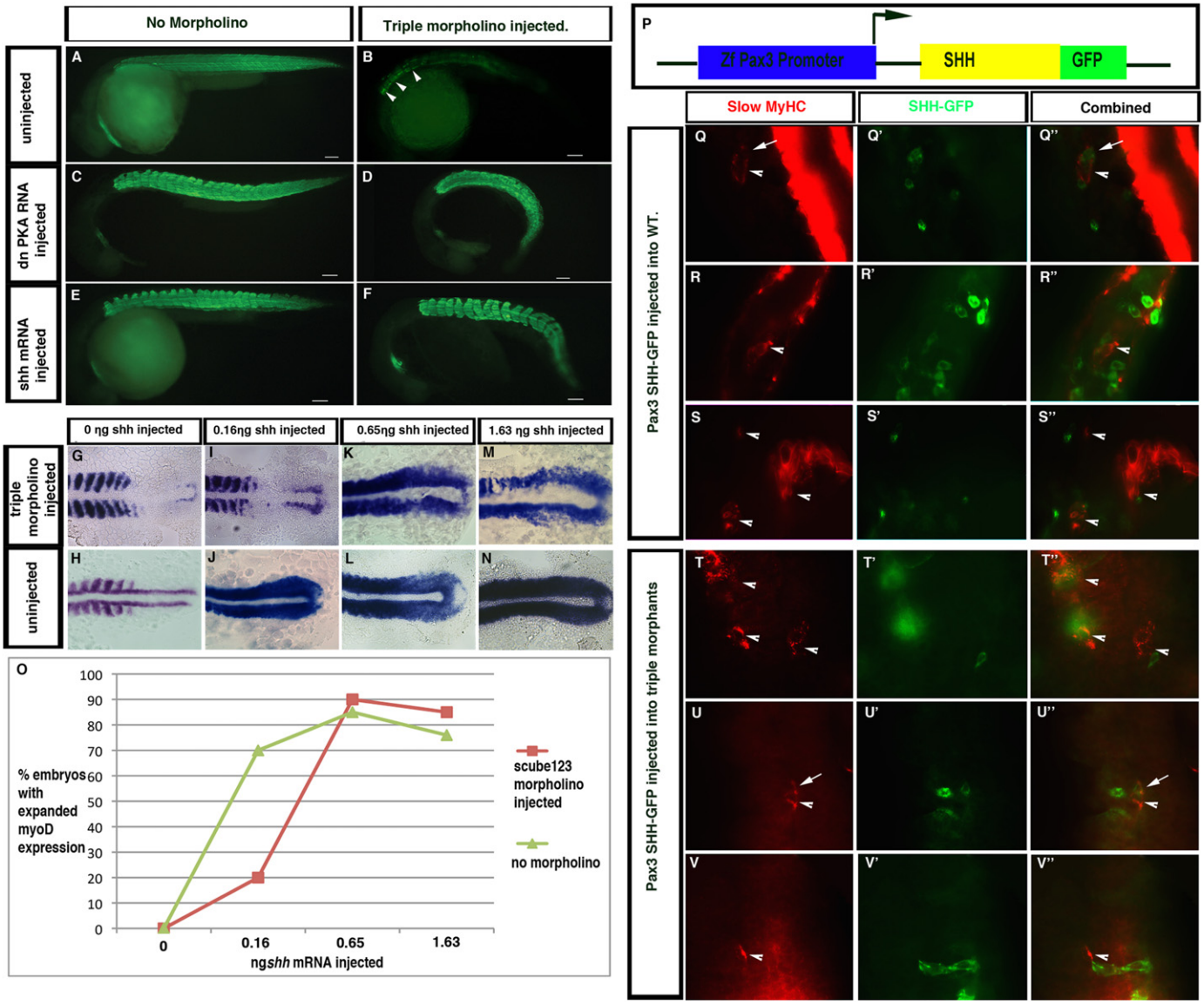

Fig. 4. Epistatic analysis of *scube* function in the Hedgehog pathway. Hedgehog pathway overexpression induces ectopic slow MyHC expression in zebrafish embryos. (A–F). Slow MyHC expression in treated and untreated embryos. (A) Slow MyHC localization in wildtype embryos. (B) *scube* triple MO embryos show severe reductions in slow MyHC expression. Representative embryo (B) has 15 slow MyHC positive fibers (arrowheads). (C) Overexpression of *dnPKA* RNA turns all muscle in the embryo into slow muscle. (D) Overexpression of *dnPKA* in *scube* triple MO embryos rescued the loss of slow muscle seen in *scube* triple MO embryos. (E) *shh* RNA overexpression induced ectopic slow MyHC expression in wildtype embryos. (F) *shh* RNA overexpression in *scube* triple MO embryos rescued the loss of slow muscle in *scube* triple MO embryos. Lateral views of ~30 hpf embryos. F59 monoclonal antibody labels slow MyHC positive muscle fibers, Alexa 488 conjugated-donkey anti-mouse secondary antibody, 0.65 ηg of *dnPKA* RNA and *shh* RNA was injected at 100 μg/mL. Scale Bars: 100 μm. (G–O) Titration of *shh* RNA in *scube* triple MO embryos (G, I, K, M) and uninjected 10 somite embryos (H, J, L, N). Expansion of myod expression is seen at 0.16 ng in embryos not injected with Scube morpholinos (J) compared to 0.65 ng in scube triple MO embryos (K). (O) Graph showing percentage of embryos with expanded *myod* expression. (P–V″) Mosaic analysis of cells expressing SHH fused to GFP under the control of the *pax3a* promoter. Mosaic expression of *pax3*a:Shh-GFP generated ectopic slow muscle both cell autonomously (arrows) and non-cell autonomously (arrowheads) in both wildtype (Q–S″) and *scube* triple MO embryos (T–V″). (Q, R, S, T, U, V) F59 monoclonal antibody, (Q′, R′, S′, T′, U′, V′) anti-GFP antibody, (Q″, R″, S″, T″, U″, V″) merged images.

we conclude that although Scube activity is essential for signaling via endogenous HH, downstream signaling can still be activated when sufficient ectopic HH ligand is provided. These results reinforce the idea that Scube activity regulates the bioavailability or uptake of the HH ligand in target cells.

Zebrafish Scube2 can bind SHH and does not affect formation of primary cilia

Currently, zebrafish is the only model system in which Scube activity has been implicated in the control of HH signal transduction *in vivo*, and mutations in *you* mutants remain the only loss of function models for *scube* function (Hollway et al., 2006; Kawakami et al., 2005; Woods and Talbot, 2005). Previous studies have shown that human Scube2 protein can bind to SHH *in vitro* (Tsai et al., 2009), therefore it was important to determine whether zebrafish Scube2 can similarly bind SHH. In order to examine this question, we expressed a version of Scube2 that is Myc tagged at its N-terminus in culture cells cotransfected with SHH. Previously we had demonstrated that this version of Scube2 is highly expressed and effectively secreted in cultured cells (Hollway et al., 2006). Our results demonstrate that zebrafish Scube2 binds strongly to SHH *in vitro* in co-immunoprecipitation assays (Fig. 5), thus confirming that zebrafish Scube2 is also a SHH binding protein.

We also examined whether *scube2* played any role in the morphogenesis of the primary cilia as modulation of HH signal

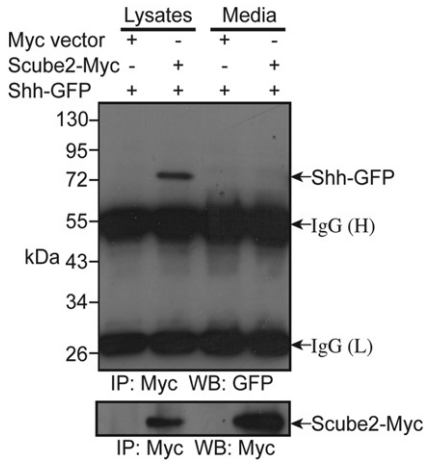

Fig. 5. Zebrafish Scube2 binds SHH when co-expressed in COS-1 cells. Binding of Scube2-Myc to SHH-GFP occurs only in the membrane fraction. COS-1 cells were transiently transfected with Scube2-Myc and Shh-GFP. As a control, an empty Myc vector was co-transfected with SHH-GFP. Immunoprecipitation (IP) was performed on lysates and media fractions using anti-Myc antibody. Western blotting (WB) of immunoprecipitated proteins was performed using anti-GFP (upper panel) or anti-Myc (lower panel) antibodies.

transduction in vertebrates has recently been shown to depend on primary cilia formation (Aanstad et al., 2009; Corbit et al., 2005; Haycraft et al., 2005; Huangfu and Anderson, 2006; Rohatgi et al., 2007). Aspects of the *scube* triple MO phenotype could be explained by a requirement for Scube activity in formation of primary cilia, although the non-cell autonomous requirement for Scube activity in HH target tissues suggests that this would have to act via an indirect mechanism. However, an evaluation of a subset of primary cilia in the presomitic mesodern, somites and neural tube of *scube* triple MO embryos ($n=40$) reveals no difference between wildtype and triple morphant embryos at either early somite stages, where HH signaling is known to control specification of the primary myotome or later stages after specification of the myotome is complete (Hirsinger et al., 2004) (Fig. 6A–M).

Discussion

In this study we describe the partially redundant function of the zebrafish *scube* gene family during HH signal transduction within the myotome. Scube2 has previously been implicated in HH signaling during zebrafish myotome development (Hollway et al., 2006; Kawakami et al., 2005; Woods and Talbot, 2005). We

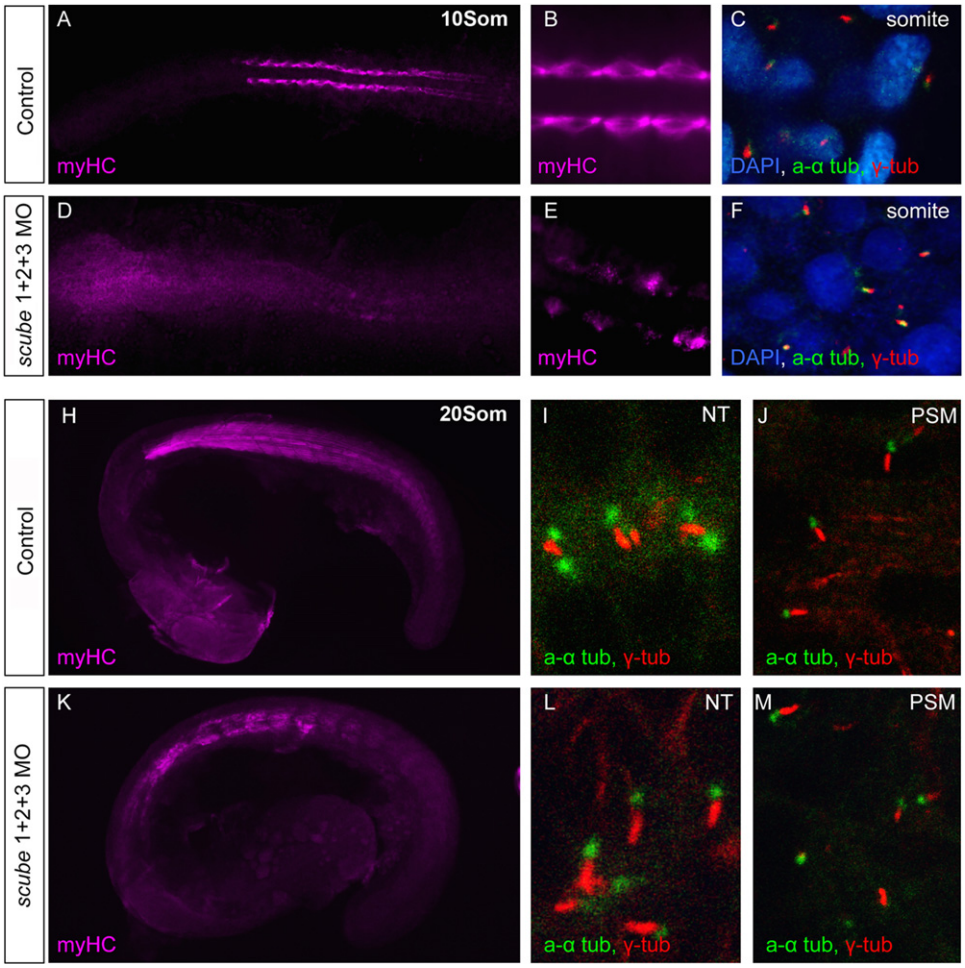

Fig. 6. Scube activity is not required for primary cilia formation. (A–M) Scube triple MO injections do not affect formation of primary cilia. (A–C). Water injected control 10 somite stage embryos display normal slow muscle development visualized by the slow myHC specific antibody F59. (A, B) Primary cilia are present in the somites, marked by acetylated alpha (a α)-tubulin (green) and gamma (γ)-tubulin (red) (Nuclei marked with DAPI) (C). (D–F). 10 somite stage embryos injected with *scube* triple MO have disrupted slow muscle myogenesis (D, E), yet appear to possess normal primary cilia (F). (H–J). In control 20 somite embryos (H) slow myHC staining shows a well-organised myotome with chevron shaped somites. (I) In wildtype embryos, cilia are visible in the neural tube (NT) and presomitic mesoderm (PSM) (J). (K–M). In *scube* triple MO embryos at 20 somites, myogenesis is disrupted, with somites displaying a U-shaped morphology (K). Cilia visible in the neural tube (L) and paraxial mesoderm (M) appear normal in *scube* triple MO embryos.

have presented evidence that zebrafish, like humans and mice; possess three *scube* genes, orthologous to their mammalian counterparts. The mRNA expression patterns for the novel zebrafish genes we identified, *scube*1 and *scube*3 overlap significantly with the published expression of *scube*2, (Hollway et al., 2006; Kawakami et al., 2005; Woods and Talbot, 2005). The three *scube* genes are collectively expressed in the floorplate, lateral ventral neural tube, the presomitic mesoderm and within the developing myotome, regions of the embryo known to be capable of responding to Hedgehog signaling. However, expression is also evident in the notochord and precaudal plate mesoderm, tissues known to secrete HH signals and the floor plate itself is both patterned by and secretes HH ligands. Additionally, other expressing tissues, such as the dorsal neural tube in the case of *scube2* and the hind brain in the case of *scube1*, neither secrete nor are patterned by HH signals. Hence, the collective expression of the *scube* gene family in zebrafish suggests a potential role in both secretion and reception of HH ligand, and likely developmental roles independent of HH signal transduction in a number of tissues. Examination of HH target gene expression, in embryos knocked down for Scube1, 2 and 3 proteins, demonstrated a more severe loss of HH target gene expression in triple knockdown embryos than in *scube2* mutant embryos alone both in the forming myotome and the neural epithelium. The triple morpholino knockdown embryos phenotypically resembled cyclopamine-treated embryos, a context in which all HH signaling is believed to be blocked, strongly suggesting all *scube* genes are involved in HH signaling. The severe loss of HH target gene expression in the myotome and neural tube and the lack of slow MyHC-positive slow muscle fibers, in the triple knockdown morphants also suggests that at least one functional *scube* gene is required for any HH signaling in the myotome.

Interestingly, the three *scube* genes do not appear to be "equal" in their capacity to compensate and display a regional requirement along the embryonic axis. These results suggest that *scube2* activity is the most important family member of the group in modulating HH signaling, as defects in HH sensitive cell types are only evident when *scube1* or *scube3* are knocked down in combination with *scube2*. The anterior–posterior variability in the severity of slow MyHC fiber loss between the various *scube* knockdown combinations is also suggestive of a more complex relationship than simply functional equivalence of the three Scube proteins. There is evidence from *in vitro* experiments, that the Scube proteins are capable of both homo- and heterodimerization (Wu et al., 2004; Yang et al., 2002). The overlapping expression patterns of the three zebrafish *scube* genes suggest this might also occur *in vivo* in zebrafish. Given that *scube1* and *scube3* cannot fully compensate for the role of *scube2* in slow muscle development, there could be a functional difference between the three Scube proteins in their ability to transduce HH signaling. A heterodimer containing Scube2 could be the central mediator of HH signaling in the zebrafish myotome. The anterior–posterior restriction of the *scube1* and *scube3* fiber loss phenotypes, in the context of the lack of Scube2 activity, suggests a regional requirement for obligate heterodimerization of Scube1 and Scube3 with Scube2 to mediate HH signal transduction.

In light of previous experiments, where the requirement for *scube*2 was demonstrated in ligand receiving cells for HH signal transduction (Hollway et al., 2006; Woods and Talbot, 2005), the ability of ectopic *shh* mRNA to facilitate slow muscle development in the absence of *scube* gene function was unexpected. If, in the developing myotome, HH receiving cells require Scube function to mediate the HH signal and induce slow muscle cell fates, ectopic SHH would not be expected to restore HH signaling in *scube* triple knockdown embryos. The ability of SHH to induce ectopic slow muscle formation both cell autonomously and non cell autonomously in the *scube* triple *MO* context indicates that a lack of Scube activity can be overcome given sufficiently high levels of ligand. This implies that Scube activity is not obligate for downstream signaling and that it may be required to regulate the "bioavailability" of the SHH ligand within responding cells. Collectively this data is in line with our previously stated model for Scube function, which was based on the homology between the Scube protein family and the endocytic receptor Cubilin (Hollway et al., 2006). An N-terminal fragment of Cubilin encompassing only the EGF repeats and the first two CUB domains, is strikingly similar to that encoded by the three *scube* ORFs, and is sufficient to bind the endocytic receptor Megalin a known SHH binding protein (Ahuja et al., 2008; Yammani et al., 2001). On this basis we suggested a possible endocytic role for Scube proteins in facilitating internalization of the HH ligand in responding cells, a process that would be essential for generating signal activation utilizing endogenous ligand. Confirmation of this model awaits the results of ongoing studies.

Materials and methods

Zebrafish strains

Zebrafish *you*^{ty97} mutant strain was obtained from H.G. Fronhoefer at the Tuebingen Zebrafish Stock Center. TE wildtype zebrafish were used for morpholino and RNA injections as well as cyclopamine manipulation.

In situ hybridization, histology and immunohistochemistry

In situ hybridization was performed according to previously published methods (Oxtoby and Jowett, 1993). Probes used for *in situ* hybridization were: *engrailed-1* (Hatta et al., 1991); *patched-1* (Concordet et al., 1996); *shh* (Krauss et al., 1993); *myod* (Weinberg et al., 1996); *foxa2* (Strähle et al., 1996); *scube-1* (Fwd: 5′-GACGCCAAGTGCCACAT GAGAC-3′, Rev: 5′-GCAACTTCATATGCGGTGCCAG-3′) and *scube-3* (Fwd: 5′-GAGAGCTGTTCCCTCACGTGTG-3′, Rev: 5′-AGCTCCAGAGTAATGCGCGTCA-3′). Following *in situ* hybridization, embryos were wholemount photographed or wax embedded using standard procedures (Kiernan, 2005) and 12–14 μm sections cut using a Leica DCS1 microtome (Leica Microsystems). Embryos were prepared for immunohistochemistry as described by Blagden et al. (1997). Antibodies used; F59 (1:50) (Developmental Studies Hybridoma Bank, University of Iowa, USA) anti-acetylated alpha tubulin (1:1000, Sigma), anti-gamma tubulin (1:800, Sigma) and Alexa Fluor 488 conjugated donkey anti-mouse (1:400) (Molecular Probes, USA).

Fiber counting

To quantify the effects of the MO knockdown of *scube1* and *scube3* on slow muscle fiber formation, F59-positive slow muscle fibers were counted in ~30 hpf embryos. Embryos analyzed: wildtype ($n=5$), *you*^{ty97} ($n=8$), *you*^{ty97}+*scube1*MO ($n=44$), *you*^{ty97}+*scube3*MO ($n=30$), *you*^{ty97}+*scube1*MO+*scube3*MO ($n=25$), *scube* triple MO (*scube1*MO+*scube2*MO+*scube3*MO) ($n=34$). Analysis of variance (ANOVA) was performed using STATA Statistics/Data Analysis version 9.2 (StataCorp), Bonferroni multiple comparison was used to compare means between the groups.

Immunoprecipitation and western blotting

Scube2-Myc is a C-terminally tagged zebrafish Scube2 construct and Shh-GFP was a gift from Carol Wicking (IMB, Queensland, Australia). COS-1 cells, purchased from ATCC, were maintained in DME, 10% (vol/vol) fetal calf serum containing 2 mM glutamine, and transfected using the DEAE-dextran procedure and allowed to

grow for 2 day (Sambrook and Russell, 2001). Briefly, cells were washed with ice-cold PBS and harvested with ice-cold lysis buffer (1% Triton X-100, 0.5 M NaCl, 50 mM Tris pH 8 and cocktail inhibitor tablets (Promega)) for 2 h at 4 °C with agitation. Lysates were centrifuged at 15,400*g* for 10 min to obtain the Triton X-100—soluble supernatant which was pre-cleared with Protein A Sepharose for 1 h at 4 °C with agitation, followed by immunoprecipitation using tag specific antibodies and Protein A Sepharose for 2 h at 4 °C with agitation. Immunoprecipitates were separated by SDS-PAGE, subject to Western transfer and immunoblotted with tag specific antibodies, HA antibody (Covance) and Myc antibody (Santa Cruz).

Microinjection and embryo manipulation

Embryos were injected according to established methods (Concordet et al., 1996) using a Narishige IM 300 microinjector (Leica Microsystems). Morpholinos (GeneTools, USA) scube1MO (5′-CCAGGAGAAGCAGGCAGGACCCATG-3′), scube3MO (5′-TGCATGAACATCATCGCAAAACACA-3′). Non-overlapping 'B' morpholinos were also tested for phenotype consistency: scube1MOB (5′-TTAGAGGCTGTGAGTGAATGGG TCT-3′) scube3MOB (5′-AAACACCTTGAATCTTCAGTGAGAC-3′). Five-nucleotide mismatch control morpholinos (mismatch nucleotides are lower case) scube1-mismatchMO (5′-TGgATGAAgATgATCcCAAAAgACA-3′) and scube3-mismatchMO (5′-CCAcGAcAAGCAGcCAGcACCgATG-3′), were used to confirm specificity. Varying amounts of each morpholino were injected; the lethality and phenotypes displayed were compared to mismatch controls of similar amounts to determine the amount of morpholino used in this work. 13.9 ηg of scube1MOA and 41.3 ηg of scube3MOA were injected, unless otherwise indicated. scube2MO injections were performed at 41.1 ηg, equivalent to previously published concentrations (Hollway et al., 2006) and phenocopied *you*^{ty97} mutant embryos.

Capped RNA and DNA injection

Capped RNA for *shh* (Krauss et al., 1993) and dominant negative PKA (*dnPKA*) (Hammerschmidt et al., 1996) were *in vitro* transcribed using mMessage Machine SP6 kit (Ambion). 0.655 ηg of *dnPKA* RNA was injected at 100 μg/mL, *shh* RNA injected at 25, 100 and 250 μg/mL, was equivalent to 0.16 ηg, 0.65 ηg and 1.63 ηg. 0.655 ηg of Pax-3 promoter DNA constructs were injected at 100 ug/ml. All injections were performed into one of the developing blastomeres at the one or two-cell stage.

Cyclopamine treatment

Cyclopamine (Calbiochem) resuspended in 95% ethanol at 10 mM was diluted in aquarium water to 100 μM or 200 μM and added to late epiboly stage embryos in 12 well plates. An equal volume of 95% ethanol was diluted in aquarium water aquarium and added to control embryos. Plates were wrapped in parafilm and foil to reduce evaporation and exposure to light.

Rapid amplification of cDNA ends (RACE)

5′ and 3′ rapid amplification of cDNA ends (RACE) was performed according to manufacturers protocols for the BD SMART RACE cDNA amplification kit (BD Biosciences Clontech). RACE fragments were cloned into pGEM-T Easy and sequenced.

Genotyping

Genomic DNA was extracted from MO injected embryos following immunohistochemistry. Individual embryos were incubated at 50 °C for 6 h in PCR extraction buffer (10 mM Tris pH 8, 2 mM EDTA, 0.2% Triton X-100, 200 μg/mL proteinase K). Proteinase K was heat inactivated and samples briefly centrifuged. A 484 bp region of *scube2* encompassing the *ty97* mutation was amplified using primers 5′-GATGGAAGTGGGCATTGTAGAC-3′ and 5′-CTAGTTCAACCATTGGGTGTC-3′. PCR products were sequenced with a nested primer 5′-TCCGTACTCTTCGCAAGTC CA-3′.

Cloning of the Pax3a-Shh-GFP expression construct

The zebrafish *pax3a* promoter was sub-cloned from BAC clone DKEY-20F20 (BACPAC Resources Center, Oakland). Briefly, since the full sequence of the BAC containing Pax3a exon1 was unavailable we used a process of semi-random, single primer PCR to isolate the promoter fragment. The single, low annealing temperature primer 5′-GCGCATCATCCTGGAAAT-3′ was designed to extend in the upstream direction from the first exon. PCR reactions were performed using decreasing annealing temperatures at 2 degree increments between 56 and 42 degrees, such that, at low annealing temperatures the single primer produced amplicons by sporadic mispriming in the region upstream of the initiation codon. One robust band of 6.5 kb was extracted, TA cloned into pGEMT easy (Promega) and end sequenced for verification. This fragment was then subcloned into the gateway compatible vector pDONRp4-p1R (Invitrogen) to give the plasmid p5E-Pax3a. The mouse Shh-GFP cassette (a kind gift of Carol Wicking, IMB Queensland) was cloned into pDONR221 to make pME-Shh-GFP. Both plasmids were recombined in a single reaction with pDEST-Tol2-pA2 and p3E-pA from the Tol2 kit to produce the Pax3a-Shh-GFP cassette.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.05.007.

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