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The Zebrafish-Secreted Matrix Protein You/Scube2 Is Implicated in Long-Range Regulation of Hedgehog Signaling

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Summary

The Hedgehog (Hh) signal plays a pivotal role in induction of ventral neuronal and muscle cell types around the midline during vertebrate development [1]. We report that the gene disrupted in zebrafish *you* mutants, in which Hh signaling is impaired, encodes the secreted matrix protein Scube2. Consistently, epistasis analyses suggested that Scube2 functions upstream of Hh ligands or through a parallel pathway. In addition, overexpression analyses suggested that Scube2 is an essential, but a permissive, mediator of Hh signaling in zebrafish embryos. Surprisingly, the *you* gene is expressed in the dorsal neural tube, raising the possibility that Scube2 could indirectly act via a long-range regulator of Hh signaling. The dorsal Bmps have a long-range and opposing influence on Hh signaling [2–5]. We show that neural plate patterning is affected in *you* mutants in a way that is consistent with the aberrant long-range action of a Bmp-dependent signal. We further show that Bmp activity can be attenuated by the coexpression of Scube2. Our data support the idea that Scube2 can modulate the long-range action of Bmp-dependent signaling in the neural tube and somites.

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Results and Discussion

Defect in Hh Signal Activation in Zebrafish *you* Mutants

Large-scale mutagenesis has been used to generate mutations in a variety of genes in zebrafish, including the midline mutations [6] and the *you*-type mutations [7], which are thought to lead to defects in Hedgehog (Hh) signaling [8–15]. The zebrafish *you* mutants have morphological traits in common with other Hh pathway mutants, such as a curled tail, weak cyclopia, and U-shaped somite boundaries. We performed a detailed characterization of zebrafish *you* mutants to determine whether the *you* gene product does indeed regulate Hh signaling. During the early stage of somitogenesis in *you* mutants, the expression of *myoD*, a gene activated in the adaxial cells by Hh signaling from the notochord, was severely downregulated (Figures 1A and 1B, arrows) [16]. In the subsequent stages, the midline-derived Hh signal normally induces the expression of the *engrailed1* (*en1*) gene in the muscle pioneer cells (MPs) and the medial fast fibers (MFFs) in wild-type embryos [17]. In *you* mutants, *en1* expression was completely lost in these cells (Figures 1C and 1D), though in some *you* mutant embryos weak *en1* expression was retained in a few segments of the posterior somites (data not shown). In addition, *you* mutants lacked the border of dorsal and ventral subdivision of the somites (Figure 1D) and later formation of the myoseptum (data not shown), which led to aberrant navigation of the lateral line axons in the somites (Figures 1E and F) [18].

Consistent with the phenotype in the somites, there was disrupted activation of Hh target genes in the neural tube of *you* mutants. The expression of *nk2.2* and *islet2* (*isl2*), both of which are positively regulated by Hh signaling, was downregulated in *you* mutants (Figures 1G–1J, arrows). Despite these defects in the trunk level, the expression of *nk2.2* in the anterior regions from the brain to the level of the yolk extension was nearly normal (arrowhead in Figure 1H). This is in marked contrast to other Hh signal mutants [8–15] in which *nk2.2* expression is severely downregulated in the brain and completely lost in the trunk level [11, 14]. Except for this defect in *nk2.2, isl2*, and *forkhead4* expression in the lateral floor plate [19], we did not see significant changes in gene expression in the dorsal or intermediate regions of the neural tube (see Figures S1 and S4 in the Supplemental Data available with this article online).

Additionally, the defect in Hh signal activation in both the ventral neural tube and somites in *you* mutants is also evident by the downregulation of *patched1* (*ptc1*), a sensitive marker of Hh signal activation [16] (Figures 1K and 1L). Taken together, these results suggest that *you* mutants have an apparent defect in Hh signal activation, although this defect is relatively mild in the ventral neural tube.

Epistatic Relationship of *you* Gene Product with Hh Signaling Pathway

To investigate the epistatic relationship between the *you* gene and Hh signaling, we made a series of compound

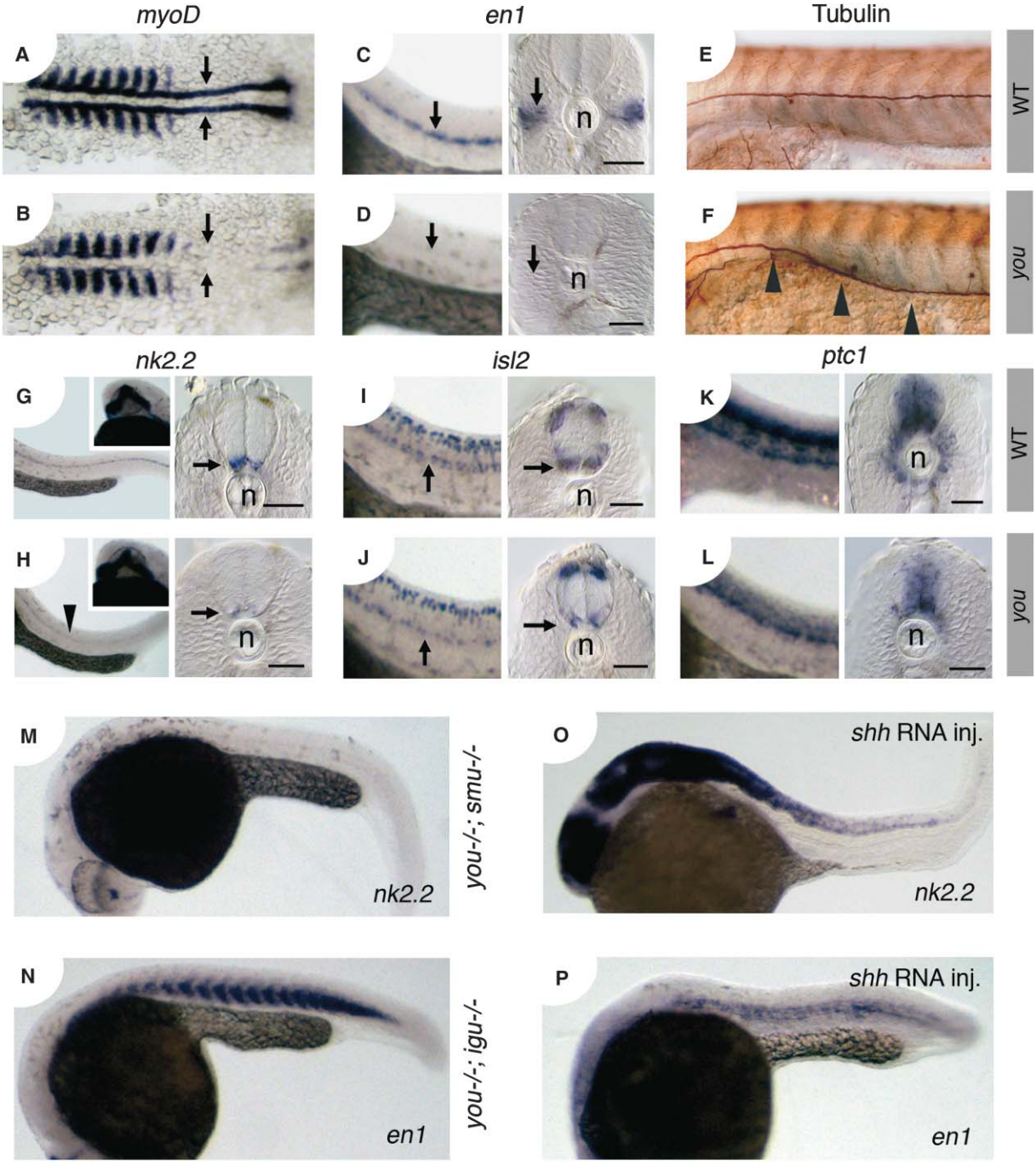

Figure 1. Phenotypes of *you* Mutants and Epistasis Analyses

(A–L) The *you* mutant phenotype. The wild-type phenotype (A, C, E, G, I, and K) was compared with that of *you* mutant embryos (B, D, F, H, J, and L). (A and B) *myoD* expression at the eight-somite stage. Dorsal views of embryos. *myoD* expression is reduced in the adaxial cells of *you* mutants. Arrows indicate the positions of the adaxial cells. (C, D, and G–L) Lateral views of embryos (anterior to the left) at the trunk region of 28 hpf embryos (left panels) and respective cross-sections at the level of yolk extension (right panels). The expression of *en1* (C and D, arrows), *nk2.2* (G and H, arrows), *isl2* (I and J, arrows), and *ptc1* (K and L) is downregulated in *you* mutants. An arrowhead in (H) indicates the posterior limit of *nk2.2* expression in *you* mutants. Insets in (G) and (H) show *nk2.2* expression in the head region, which is unaffected in *you* mutants. Scale bars in the sections represent 20 μm. (E and F) Lateral line axons stained with acetylated tubulin antibody in 72 hpf embryos. Arrowheads in (F) indicate the axons running at the ectopic ventral position. n, the notochord.

(M and N) Phenotypes of compound mutants. *you;smu* (M) and *you;igu* mutants (N). These compound mutants have identical phenotypes with those of *smu* and *igu* mutants, respectively [14].

(O and P) Effect of Shh overexpression in *you* mutants. Injection of *shh* mRNA induced the expanded and ectopic expression of *nk2.2* (O) and *en1* (P) in *you* mutants.

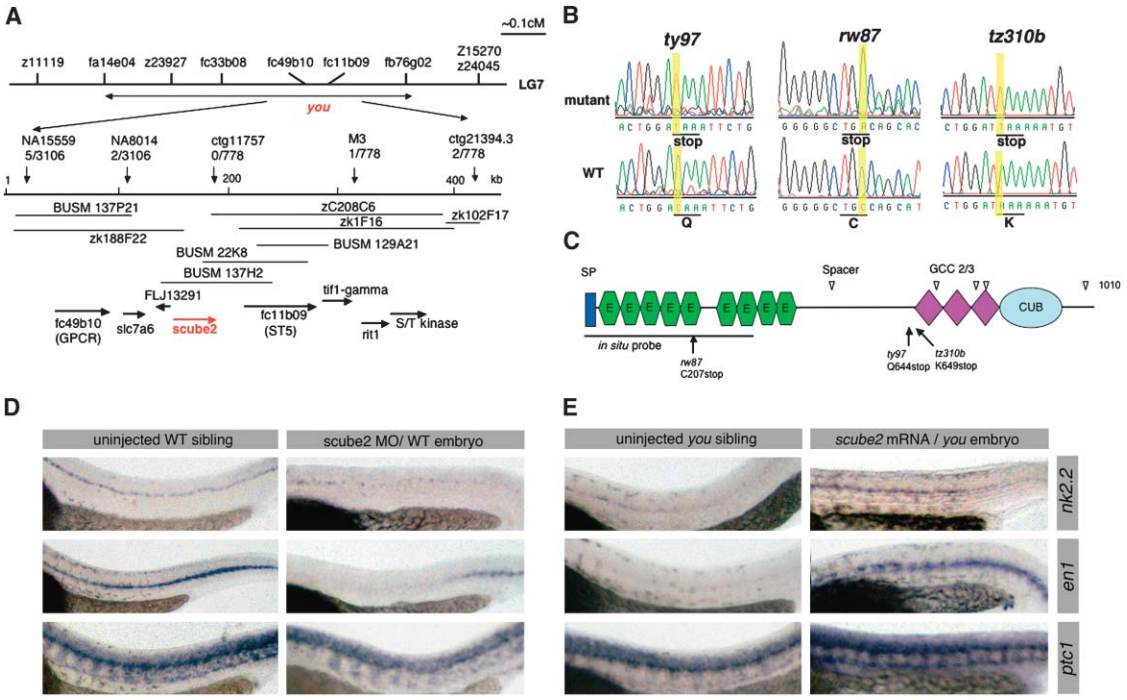

Figure 2. Positional Cloning of the *you* Gene

(A) Map of the region containing the *you* gene on the zebrafish linkage group 7. The DNA markers used to score the recombination frequency are indicated in the upper part. The close-up view of the *you* region is shown in the middle part. The DNA markers and their recombination frequencies are indicated. The BAC and PAC genomic clones and predicted ORFs are indicated in the bottom part. Among the eight ORFs, genes encoding the homologs of amino acid transporter SLC7A6 (GenBank, AAH28216), FLJ13291 (AAH13778), SCUBE2 (NP_066025), and tumor suppressor 5 (ST5) (NM_213618) are within the critical region. The EST, *fc49b10*, encodes a G protein-coupled transmembrane receptor protein.

(B) Sequence chromatograms of mutated sites. Nonsense mutations were found within the *scube2* gene in the respective *you* mutant alleles.

(C) Domain structure of the Scube2 protein. The positions of mutations are shown below. Open triangles indicate the potential glycosylation sites. SP, signal peptide; E, EGF-like domains.

(D) Knockdown of Scube2 leads to a *you*-like phenotype. The injection of *scube2*-MO into wild-type embryos (right panels) resulted in the downregulation of *nk2.2* in the ventral neural tube, *en1* in the somite, and *ptc1* around the midline. Uninjected wild-type siblings are shown in the left panels.

(E) Rescue of the *you* mutant phenotypes by *scube2* mRNA overexpression. The injection of synthetic *scube2* mRNA into *you* mutants (right panels) rescued the defects in *nk2.2*, *en1*, and *ptc1* expression. Uninjected *you* mutant siblings are shown in the left panels. All images in (D) and (E) are lateral views of embryos with anterior to the left.

mutants. The compound mutant, *you;smu*, in which both You and the Smoothened (Smo) signaling protein are disrupted [10, 11], showed a complete loss of *nk2.2* gene expression, as in *smu* mutants (Figure 1M), suggesting that the residual *nk2.2* expression in *you* mutants remains dependent upon the signal mediated by Smo. In another compound mutant, *you;igu*, in which both You and the Igu/Dzip1intracellular regulatory protein that affects the activation of Gli transcription factors are disrupted [14, 15], double homozygous mutants showed the expanded *en1* expression, as in *igu* mutants (Figure 1N). Because the *igu* mutation causes a constitutive weak activation of Gli transcription factors [14], these results suggest that the Hh pathway downstream of Gli proteins may not be disrupted in *you* mutants. Furthermore, the injection of *shh* mRNA (250 pg per embryo) into *you* mutant embryos induced ectopic *nk2.2* expression in the neural tube (in 28 *you* mutants) and *en1* expression in the somites (in 20 *you* mutants) (Figures 1O and 1P), indicating that the Hh signaling pathway in *you* mutants can be activated in response to Hh ligands. Taken together, these data suggest that the *you* mutations impair Hh signaling either by acting upstream of Hh or through a parallel pathway.

you Gene Encodes Scube2 Protein

To characterize the You protein, we first identified the gene through a positional cloning approach [20]. By scoring meiotic recombination frequency, we initially mapped the *you* mutation between the markers z11119 and z24045 on the zebrafish linkage group 7 (Figure 2A). By using a number of DNA markers mapped in this region (http://zfin.org/), we narrowed the critical region between the EST markers fc11b09 and fc49b10. We identified a number of BAC and PAC clones in the region by PCR-based screening and searches of the GenBank sequence database. The sequenced BAC clone, zC208C6, covered a long genomic region containing the fc11b09 marker (Figure 2A). In addition, we determined the sequence of BAC clone, zk188F22, which contained the fc49b10 marker (Figure 2A). Based on these sequences, eight ORFs were predicted by GenScan software (Figure

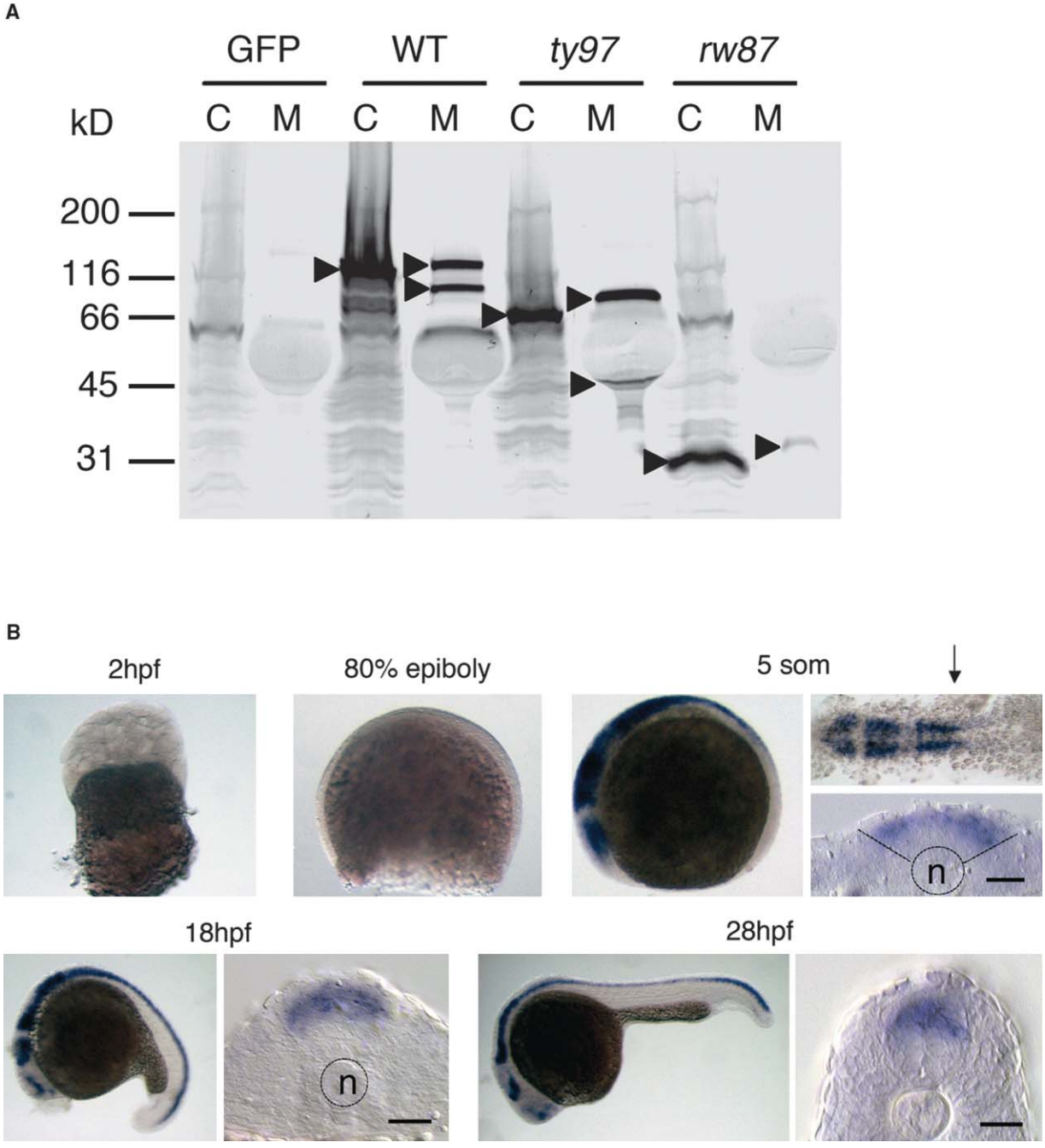

Figure 3. Scube2 Is a Secreted Protein in the Dorsal Neural Tube

(A) Western blot analysis of Scube2 proteins expressed in HEK293T cells. Full-length (WT) and truncated Scube2 proteins (*ty97* and *rw87*) were tagged with V5/His epitope at their C termini. The pcDNA-EGFP (Invitrogen) that expresses the enhanced green fluorescent protein (GFP) was used as a negative control. The lanes represent: C, whole cells; M, concentrated culture media. The filled triangles indicate the Scube2 proteins. Note that smaller molecular weight forms were seen in the respective culture media of wild-type and *ty97* Scube2 proteins (M lanes), suggesting that a possible proteolytic cleavage occurs in both the wild-type and *ty97* mutant proteins.

(B) Expression of the *scube2* gene during development. Lateral views of embryos hybridized with *scube2* probe. Cross-sections of five-somite-, 18-hpf-, and 28-hpf-stage embryos are shown in the right panels. The upper right panel of the five-somite stage is a dorsal view in which an arrow indicates the position of the cross-section. Note that little *scube2* is expressed in the mesoderm and surface ectoderm in all stages. n, notochord. Scale bars, 20 μm.

2A). Among them, we determined the full-length cDNA sequences of *fc49b10*, *slc7a6*, *FLJ13291*, *scube2*, and *fc11b09* and compared them between the wild-type and *you* mutant alleles. Through this sequence analysis, we identified nonsense mutations in the *scube2* gene in all three alleles of *you* mutants (Figure 2B).

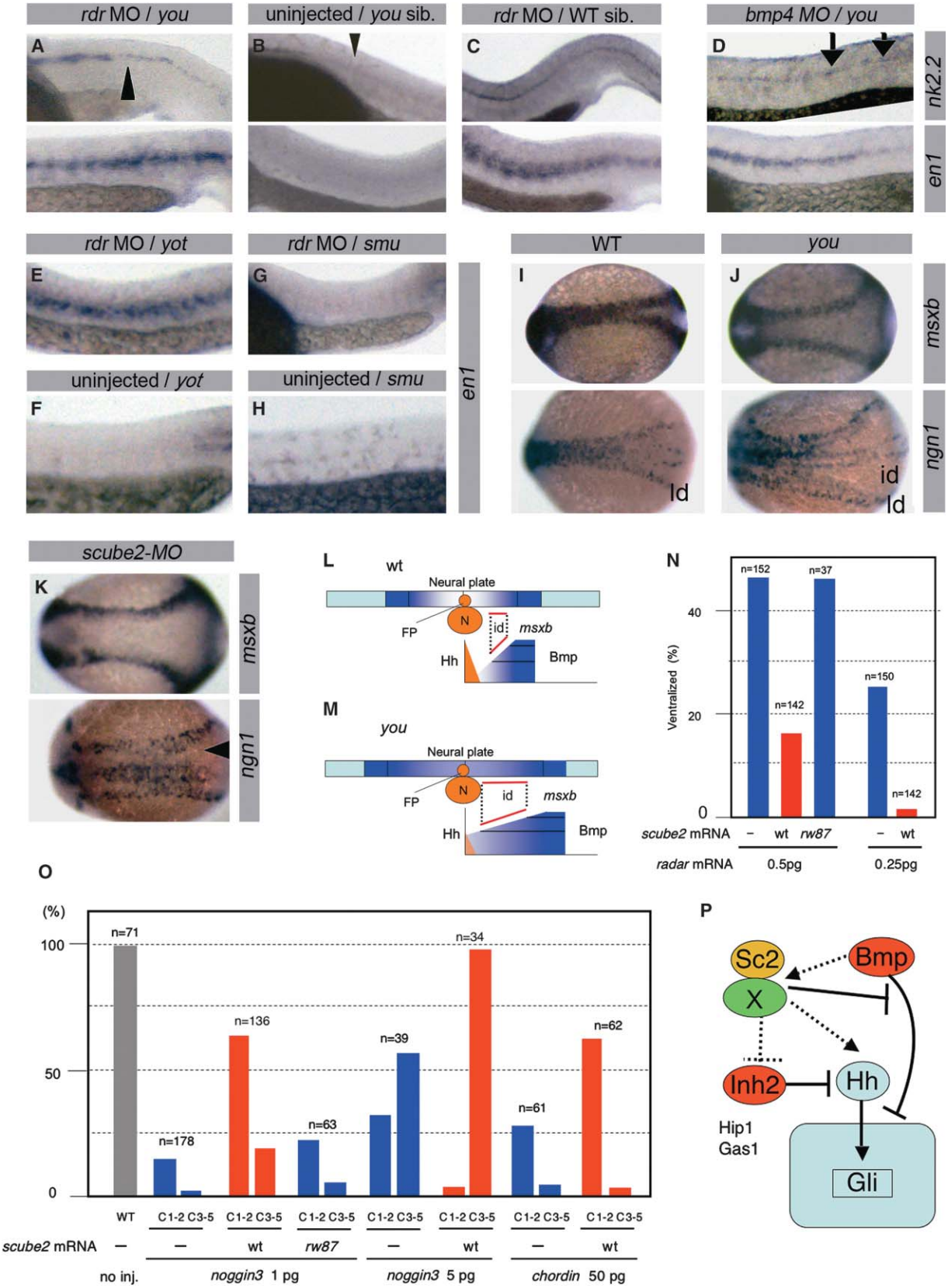

Figure 4. Long-Range Influence of Dorsal Bmp Signal on Hh Signal Activation and Interaction With Scube2

(A–H) Rescue of Hh signaling defect by the knockdown of Bmps. Lateral view of 28 hpf embryos with anterior to the left. (A) Radar/Gdf6a knockdown in *you* mutants. Injection of *radar*-MO (5 ng) rescued the defects in expression of *nk2.2* and *en1* in *you* mutants. Arrowhead

To further confirm the identity of the *you* and *scube2* genes, we knocked down Scube2 expression with antisense morpholino oligonucleotide targeted to the translation initiation site (*scube2*-MO). Injection of *scube2*-MO produced a *you*-like downregulation of the Hh target genes, *nk2.2*, *en1*, and *ptc1* (Figure 2D and Table S1). We also observed that *myoD* expression was downregulated in the adaxial cells at the five-somite stage by *scube2*-MO (data not shown). Conversely, the injection of wild-type *scube2* mRNA into *you* mutant embryos effectively rescued the expression of Hh target genes (Figure 2E and Table S2). Accordingly, these results support that *scube2* is the responsible gene disrupted in *you* mutants and further suggest that the respective *you* mutations are null or hypomorphic alleles.

Importantly, we did not see any dominant effect of Scube2 overexpressed in *you* mutants (Figure 2E). This was further confirmed by injecting a large amount of *scube2* mRNA (200 pg) into wild-type embryos. The embryos injected with wild-type *scube2* mRNA had a normal morphology and normal expression of *nk2.2, en1*, and *ptc1* (pictures not shown; Table S3). Similarly, the injection of mRNA encoding the truncated Scube2 protein (*rw87* allele) did not have any effect in wild-type embryos (Table S3). These results suggest that the Scube2 protein is an essential, but a permissive, regulator of Hh signaling in zebrafish embryos.

Scube2 Is a Secreted Protein Expressed in the Dorsal Neural Tube

Previous studies have demonstrated that SCUBE2 is a secreted cell surface protein that has nine EGF-like repeats and a CUB domain [21–23]. A search of the protein families database (Pfam) (http://www.sanger.ac.uk/Software/Pfam/) also identified additional GCC2/3 domain repeats in Scube2 (Figure 2C and Figure S2). These domains are found in a group of secreted matrix proteins in combination with CUB and/or EGF-like domains (see Pfam website). There are nonsense mutations at the N-terminal end of the GCC2/3 domains in the *ty97* and *tz310* alleles of *you* mutants and within the fifth EGF-like repeat in the *rw87* allele (Figure 2C). The Scube2 protein encoded on the *rw87* allele has only 207 amino acids corresponding to 4 EGF-like domains and is therefore likely to be a null allele. Accordingly, the *ty97* allele, which has the same phenotype as the *rw87* alleles, may also be a null allele, suggesting that the GCC2/3 and CUB domains are essential for Scube2 function.

We next examined whether the *you* mutations affect the distribution of Scube2 protein in cultured cells. Western blot analysis of Scube2 proteins expressed in HEK293T cells showed that the wild-type and the truncated (*ty97* allele) Scube2 proteins are secreted into the culture media at a similar efficiency (Figure 3A), whereas the shortest Scube2 mutant protein (*rw87* allele) is only secreted at very low levels (Figure 3A). In addition, both the wild-type and *ty97* Scube2 proteins appear to have been processed into smaller-molecular weight forms before being secreted (Figure 3A). These observations are consistent with those of human SCUBE proteins [21, 23]. Because there were no marked differences in protein distribution or processing between the wild-type and *ty97* Scube2 proteins (Figure 3A and Figure S3), the

indicates a mosaic expression of *nk2.2*. (B) Uninjected *you* mutant siblings. Arrowhead marks the posterior limit of *nk2.2* expression. (C) Wild-type embryos injected with *radar*-MO. The embryos have almost normal morphology but show slightly expansion of *en1* expression in the somites (bottom). (D) Bmp4 knockdown in *you* mutants. The injection of *bmp4*-MO (5 ng) rescued the expression of *nk2.2* (G) and *en1* (H), though the effect was weaker than that of *radar*-MO. Note that *nk2.2* expression is partially rescued by *bmp4*-MO (arrows), although most *you* embryos injected with *bmp4*-MO were judged as "*nk2.2* down" in Table S4. (E–H) Knockdown of Radar/Gdf6a in *yot*/*gli2*and *smu*/*smo*. Injection of *radar*-MO rescued *en1* expression in *yot* mutants (E), but not in *smu* mutants (G). Respective uninjected mutants are shown in (F) and (H), respectively. Genotypes were confirmed in respective embryos by sequencing the mutant alleles of genomic DNA.

(I and J) Neural plate patterning in wild-type (I) and *you* mutants (J). Dorsal views of five- to eight-somite-stage embryos. The expression *msxb* (upper panels) in the lateral domain of the neural plate (ld; prospective dorsal domain) is relocated laterally in *you* mutants. Consistently with this, *ngn1* expression (lower panels) is expanded in the lateral and intermediate domains (id) in *you* mutants. The number of somites was counted by looking at the expression of *myoD* in the sibling embryos and confirmed that the developmental stages were not significantly delayed in *you* mutants.

(K) Phenocopy of the neural plate phenotypes by Scube2 knockdown. The injection of *scube2*-MO (5 ng) into wild-type embryos produced a range of *you*-like patterns of expression of *msxb* (top) and *ngn1* (bottom). Arrowhead indicates the expanded *ngn1* expression on one side of the embryos.

(L and M) Schematics to explain the altered intermediate domain of the neural plate in *you* mutants. The diagram is a representational cross-section of the neural plates at the five- to eight-somite stage. In the wild-type embryo (L), the Bmp-dependent signal derived from the neural plate margin and ectoderm forms a gradient of positional information across the entire neural plate and somites. If the dorsal Bmp signal extends over a longer-range in *you* mutants (M), it may impair activation of the Hh signal around the midline. Additionally, because the intermediate domain (id) of the neural plate may be induced between specific thresholds on a gradient of Bmp signal, the extended range of Bmp signal may also result in an expanded intermediate domain. This is consistent with the observed phenotypes in *you* mutants. N, notochord; FP, floor plate.

(N and O) Effect of Scube2 on Bmp activity. (N) Coexpression of Scube2 with Radar/Gdf6a. The ventralization of embryos induced by the injection of *radar*/*gdf6a* mRNA (0.5 pg or 0.25 pg) was significantly reduced by the coinjection of wild-type (wt; red bars) *scube2* mRNA (50 pg), whereas the mutant version of mRNA (*rw87*) did not affect Radar/Gdf6a activity. Embryos were scored at 26–32 hpf according to the criteria of Kishimoto et al. [30]. Respective bars represent the total percentage of ventralized embryos. Total numbers of embryos were indicated above the bars. (O) Enhancement of Noggin or Chordin action by Scube2 coexpression. The dorsalization of embryos induced by the injection of *noggin3* (1 pg and 5 pg) or *chordin* mRNA (50 pg) was enhanced by the coinjection of wild-type (wt; red bars) *scube2* mRNA (50 pg), but not by mutant *scube2* mRNA (*rw87*). Respective bars represent the ratios of dorsalized embryos with mild (C1–C2) and severe (C3–C5) phenotypes [30]. Total numbers of embryos were indicated above the bars. All uninjected siblings (WT) had normal wild-type morphology.

(P) A model of Scube2 action. Scube2 may require another cofactor for its action (X). Scube2 positively regulates Hh signaling by affecting the long-range Bmp-dependent signal. It is also possible that Scube2 may affect other pathways in addition to the Bmp-dependent signal. Sc2, Scube2; Inh, Hh-inhibiting molecules such as Gas1 and Hip1 [31, 32].

GCC2/3 and CUB domains probably do not function primarily in the regulation of protein secretion or processing.

We investigated the pattern of *scube2* expression during early zebrafish development by whole mount in situ hybridization. There are only minimal amount of maternal *scube2* transcripts, and zygotic *scube2* expression remains low until the end of the epiboly stages (Figure 3B). Consistent with the low maternal expression of *scube2,* the maternal and zygotic *you* mutants that were obtained from incrosses of homozygous *you* mutant fish have the same phenotype with the zygotic *you* mutants (Figure 2E), confirming that Scube2 has little maternal role during development. There is a marked increase in *scube2* expression in the early segmentation stage, which begins as bilateral stripes at the converging neural edges (see the section of five-somite stage in Figure 3B). Importantly, this bilateral expression of *scube2* is not in the paraxial mesodermal cells, but confined to the neuroectodermal cells. Subsequently, these stripes merged at the midline to be integrated into the dorsal region of the neural tube (28 hpf in Figure 3B). Thus, the *scube2* gene expression is distal to the site of Hh signaling during zebrafish development. Because it has been suggested that Scube2 proteins are tethered to the cell surface in oligomers [21], Scube2 is likely to regulate Hh signaling via some second highly diffusible molecule.

Long-Range Influence of Dorsal Bmps on Hh Signaling in *you* Mutants

Several in vivo and in vitro studies have suggested that Bmp signaling has an antagonistic effect on Hh signaling and that the dorsal neural tube and surface epithelium are the sources of Bmp-related molecules [2–5, 24–26]. As well as in the mouse and chick, several *bmp* family genes including *radar*/*gdf6a* and *bmp4* are abundantly expressed during early zebrafish development and continue to be expressed in the roof plate and surface epithelium until later stages [27] (Figure S4). We therefore suspected that Bmp-dependent signaling might be affected in *you* mutants and perhaps could provide the link between Scube2 and Hh signaling.

To determine whether the dorsal Bmp signal affects Hh signaling in zebrafish, we knocked down Radar/Gdf6a with antisense morpholino oligonucleotide (MO). To minimize any early effect on ectodermal patterning, we used MO specifically targeted to the zygotic *radar*/*gdf6a* mRNA, but not to the maternal *radar*/*gdf6a* mRNA [28]. Injection of *radar*-MO into embryos led to a dramatic restoration of *en1* and *nk2.2* expression in *you* mutants (Figure 4A and Table S4). In the wild-type siblings, injection of *radar*-MO induced a slight expansion of *en1* expression in the somites in comparison with normal embryos (Figure 4C), suggesting that reduced Bmp signaling may upregulate Hh signaling. Injection of *bmp4*-MO gave similar but less efficient rescue of *nk2.2* expression (Figure 4D and Table S4). Furthermore, *radar*-MO also rescued *en1* expression in *yot*/*gli2* mutants but showed less efficient rescue in the severer Hh mutant, *smu*/*smo* (Figures 4E and 4G and Table S4). Previous study in zebrafish has shown that a cell in the notochord ectopically expressing Dorsalin-1, a Bmp4-related molecule, blocks the Engrailed (En) expression in MP cells in the franking two to four somites [26]. Together with this gain-of-function experiment, our loss-of-function data strongly support the idea that Bmp-dependent signaling from the dorsal neural tube and/or surface epithelium has a long-range and opposing effect on Hh signaling.

Neural Plate Patterning in *you* Mutants

Given the long-range influence of dorsal Bmps on Hh signaling and the overlapping expression between Scube2 and Bmps including Radar and Bmp4 (Figure 3B and Figure S4), Bmp-dependent signaling is the most likely candidate of long-range mediator of Scube2 function. It has been suggested that the Bmp-dependent signal affects neural differentiation at all dorso-ventral (DV) levels of the neural plate [29]. If the dorsal Bmp-dependent signal is affected in *you* mutants, then the neural plate patterning should be altered.

We looked at the expression of neurogenic genes including *msxb* and *neurogenin1* (*ngn1*) at the five- to eight-somite stage and found that the intermediate domain (id; prospective ventral and intermediate neural tube) of the neural plate is expanded in *you* mutants (Figures 4I and 4J). The lateral domain (ld; prospective dorsal neural tube) and medial domain were not significantly altered. Because *you* mutant embryos have the same number of somites as wild-type siblings and somites are formed at the normal lateral position (Figures 1A and 1B; also confirmed in the sibling *you* mutants), this phenotype is neither due to a delay in development nor to dorsalization of the embryos. Furthermore, we did not see this phenotype in other Hh signal mutants, *yot*/*gli2* and *smu*/*smo* (data not shown). An equivalent expansion of the intermediate domain was induced by injection of *scube2*-MO into wild-type embryos (Figure 4K), confirming that loss of Scube2 is responsible for this phenotype. Such an alteration of early neural plate patterning can be explained by a model in which an extended influence of Bmp-dependent signal interferes Hh signaling around the midline and expands the region of signal concentration sufficient to induce intermediate domain (Figures 4L and 4M).

At later stages of development, the phenotype in the dorsal and intermediate regions of the neural tube was not evident (Figures S1 and S4). Because loss of Scube2 function may extend the effective range of Bmp signaling, but not enhance the maximum level of action at the most dorsal region (Figures 4L and 4M), the dorsal and intermediate regions of the neural tube may be less susceptible than the ventral region in *you* mutant embryos. In addition, it is also possible that other signals and/or the regulatory networks of transcription factors during later neural tube development could compensate the neural tube patterning.

Scube2 Attenuates the Activity of Bmps

To test the possible influence of Scube2 on Bmp activity, we coexpressed *scube2* with various doses of *radar*/*gdf6a* in embryos and examined the well-characterized *radar*/*gdf6a* mRNA dose-dependent ventralization phe-

notype [28, 30]. This assay was predicted to be able to detect even small fluctuations in Radar/Gdf6a. Coinjection of wild-type *scube2* mRNA with *radar/gdf6a* mRNA led to a significant attenuation of the ventralization phenotype (Figure 4N). The truncated *rw87* Scube2 protein did not affect the Radar/Gdf6a-dependent ventralization, indicating this was a specific action of Scube2.

To further confirm the effect of Scube2 on Bmp activity, we also performed a complementary experiment in which embryos were injected with mRNA of the Bmp antagonist, Noggin, to reduce endogenous Bmp activity. Under conditions that produce a dose-dependent dorsalization of the embryos, coexpression of Scube2 led to a significant enhancement of the dorsalization phenotype (Figure 4O). Similar enhancement of the dorsalization phenotype also occurred when Scube2 was coexpressed with another Bmp antagonist, Chordin (Figure 4O). Taken together, these data strongly suggest that Scube2 can attenuate Bmp activity.

Further study is required to fully explain why overexpression of Scube2 alone had little apparent effect on early DV patterning of embryos, whereas it was able to counteract the ventralizing effect of cooverexpressed Bmps or facilitate dorsalization when activity of intrinsic Bmps were reduced by Noggin. Because of permissive involvement of Scube2 in the regulation of Hh signal, the anti-Bmp activity of Scube2 may depend on the simultaneous presence of some as-yet-unidentified cofactors (Figure 4P). Therefore, because of the possible low expression of cofactor(s) in the early stages of embryonic development, overexpressed Scube2 may not act efficiently enough to eliminate the maternal contribution of Radar/Gdf6a [28]. Furthermore, if such cofactor(s) are induced by Bmp signals, ectopic Scube2 may only exert its anti-Bmp function when these cofactors are sufficiently induced by the cooverexpression of Bmps. It is also possible that Scube2 may not equally counteract all types of endogenous Bmps in the early stages of embryonic development.

Conclusion

In summary, our data demonstrate that the dorsal Bmps, including Radar/Gdf6a and Bmp4, have an opposing effect on Hh signaling and that the function of the secreted matrix protein Scube2 may regulate Hh signaling through attenuation of the Bmp-dependent signal derived from the dorsal neural tube and surface ectoderm. The interaction between Scube2 and the Bmp signal may require some as-yet-unidentified localized cofactors.

How can Bmp signaling interfere with Hh signaling? It has been suggested that the truncated Gli3 interacts directly with Smad proteins, downstream transcription factors of the Bmp pathway [31]. Hence, the interference between the Hh and Bmp pathway might occur at the level of Gli transcription factors.

Previous studies have identified that there are a number of negative regulators of Hh ligands, such as Gas1 and Hip1 [32, 33], and positive regulators, such as Ext and heparan sulfate proteoglycan (HSPG) [34, 35]. These molecules function in the long-range actions of Hh and/or other signals including Bmp and Wnt. Therefore, we cannot completely rule out the possibility that Scube2 could affect Hh ligands by interacting with such molecules (Figure 4P). These putative interacting molecules need to be identified and characterized to reveal the exact mechanism of Scube2 action.

Supplemental Data

Supplemental Data include four figures and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/5/480/DC1/.

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Note Added in Proof

During the revision of this paper, a paper describing the cloning of the *you* gene was published online: Woods, I., and Talbot, W.S. (2005). The *you* gene encodes an EGF-CUB protein essential for hedgehog signaling in zebrafish. PLoS Biol 3(3): e66 DOI:10.1371/journal.pbio.0030066. By cell transplantation experiments, they have shown that *you* act non-cell autonomously in MP cell differentiation and that wild-type cells present in the non-MP regions of *you* mutants can rescue the Engrailed expression in MP cells. Though their interpretation was that *you* is essential for the transport or stability of Hh signals, the data are also consistent with our model that the Scube2 can suppress an Hh-inhibitory signal in *you* mutants.

This article contains some minor differences from the Immediate Early Publication published online on February 10, 2005. A citation for Figure 2C has been added. References 28 and 29 as well as their citations have been swapped. Two citations for reference 29 have been changed to 30. In the Figure 4 legend, *noggin* has been changed to *noggin3*. The accession number for zebrafish *slc7a6* in the Supplemental Data has been changed.