Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein

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The Hedgehog signalling pathway is essential for the development of diverse tissues during embryogenesis[1]. Signalling is activated by binding of Hedgehog protein to the multipass membrane protein Patched (Ptc)[2,3]. We have now identified a novel component in the vertebrate signalling pathway, which we name *Hip* (for *Hedgehog-interacting protein*) because of its ability to bind Hedgehog proteins. *Hip* encodes a membrane glycoprotein that binds to all three mammalian Hedgehog proteins with an affinity comparable to that of Ptc-1. *Hip*-expressing cells are located next to cells that express each *Hedgehog* gene. *Hip* expression is induced by ectopic Hedgehog signalling and is lost in *Hedgehog* mutants. Thus, *Hip*, like *Ptc-1*, is a general transcriptional target of Hedgehog signalling. Overexpression of *Hip* in cartilage, where *Indian hedgehog* (*Ihh*) controls growth[4], leads to a shortened skeleton that resembles that seen when *Ihh* function is lost (B. St-Jacques, M. Hammerschmidt & A.P.M., in preparation). Our findings support a model in which Hip attenuates Hedgehog signalling as a result of binding to Hedgehog proteins: a negative regulatory feedback loop established in this way could thus modulate the responses to any Hedgehog signal.

All multicellular organisms require cell communication to regulate growth and differentiation in the embryo. One strategy for this is to establish discrete organizing centres that emit signals to coordinately control cell proliferation and cell fate determination. *Sonic hedgehog* (*Shh*), a member of the *Hedgehog* (*Hh*) family expressed in several vertebrate signalling centres, is a key signal in these patterning mechanisms[1–3,5,6]. The other two mammalian Hh members, *Indian hedgehog* (*Ihh*) and *Desert hedgehog* (*Dhh*), are principally expressed in cartilage and testes, respectively[4,7,8], where they are essential for the development of these structures (refs 4, 8, and B. St-Jacques, M. Hammerschmidt & A.P.M., in preparation).

Genetic and biochemical analysis of the Hh signalling pathway has established that two membrane proteins encoded by the segment polarity genes *patched* (*ptc*) and *smoothened* (*smo*)[2,3] are involved in ligand reception and signal transduction. Binding of Hh to Ptc relieves Ptc-mediated repression of Smo, thereby activating the signalling pathway. The targets transcriptionally activated in response to Hh signalling include *Ptc* itself, which creates a negative regulatory loop that may restrict further diffusion of Hh protein[9,10].

If we are to fully understand how Hh signalling is transformed into cellular responses, we must identify all the key components involved in the signalling process. As most components have been identified by genetic screening in the fruitfly *Drosophila*, we initiated a biochemical screen to identify novel regulatory factors required for transducing or modulating Hh signalling in vertebrates. We generated a biologically active fusion of the amino-terminal fragment of Sonic hedgehog (Shh-N) with alkaline phosphatase[11,12] (Shh-N::AP) to allow colorimetric detection of cells which bound Shh. Shh-N::AP was used to screen a complementary DNA (cDNA) expression library from a mouse limb bud obtained on day 10 postcoitum. After screening around 60,000 clones, we identified one positive pool that promoted cell-surface binding to Shh-N::AP and binding in subsequent experiments to alkaline phosphatase-tagged Ihh-N and Dhh-N (data not shown). From this pool, we isolated one positive clone containing a cDNA of 2.7 kilobases. As the protein encoded by the isolated clone binds to all three mammalian Hedgehog proteins, we have called this gene *Hedgehog-interacting protein* (*Hip*).

The complete *Hip* cDNA (2,669 base pairs) encodes a protein of 700 amino acids with a predicted relative molecular mass of 78,400 (Fig. 1a, c). The first 15 amino-acid residues of Hip constitute the first hydrophobic stretch, reminiscent of a signal peptide[13], while the last 22 amino-acid residues are predicted to form a hydrophobic membrane-spanning domain. These are four potential N-linked glycosylation sites and two domains resembling epidermal growth factor (EGF)[14] repeats that lie close to the carboxy terminus of Hip (Fig. 1a, c). The protein shares sequence similarity with the *Xenopus Gene 5* product (21% sequence identity)[15] and with two bacterial proteins, 345 and PCZA361.11 (15% and 16% sequence identity, respectively)[16] (Fig. 1b). Comparison of the mouse *Hip* sequence

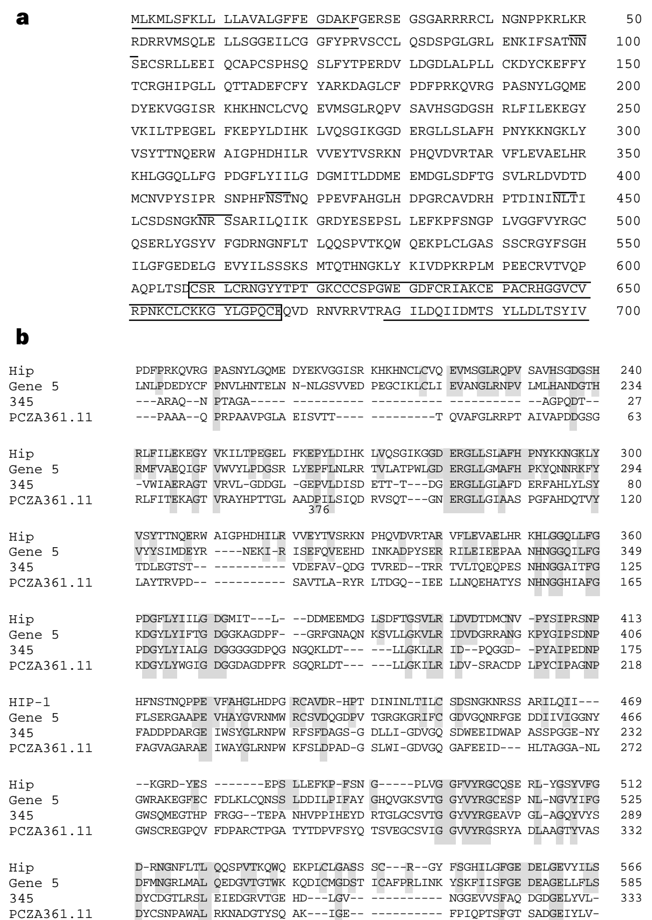

Figure 1 Sequence analysis of the *Hip* cDNA. **a**, Predicted translation product of the *Hip* cDNA. The hydrophobic stretches at the N- and C-termini are underlined, two EGF-like domains are boxed and four potential N-linked glycosylation sites are overlined. **b**, Amino-acid sequence alignment between Hip, Gene 5, 345 and PCZA361.11. Only regions with significant sequence identity are shown. Shaded regions represent sequences identical in all four proteins as well as sequences identical between only Hip and Gene 5. Numbers represent the amino-acid position in the corresponding protein. **c**, Schematic diagram showing the structure of Hip protein. I, hydrophobic stretch at the N terminus; II, EGF-like domains; III, hydrophobic stretch at the C terminus. Four potential N-linked glycosylation sites are represented by 'Y'.

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with that of zebrafish, chick and human (data not shown) indicates that the *Xenopus Gene 5* is not the *Xenopus Hip* orthologue.

The finding that Hip can cause Hedgehog::AP fusion proteins to be retained on the cell surface and the presence of a putative signal peptide and transmembrane domain strongly suggest that it is a membrane-associated protein. Consistent with this, when expressed in cultured cells, a Myc-epitope tagged form of Hip (Myc–Hip) associated preferentially with the cell pellet fraction (Fig. 2a, lane 2) and was absent from the supernatant (Fig. 2a, lane 1). As the last 22 amino-acid residues encode a hydrophobic region, we used a Hip protein lacking these residues (Myc–HipΔC22) to test the possibility that this sequence might function as a transmembrane domain. A significant fraction of Myc–HipΔC22 was detected in the supernatant, indicating that this domain is essential for membrane binding (Fig. 2a, lane 3). To rule out the possibility that Hip is anchored to the membrane through a glycosylphosphatidylinositol (GPI)-linkage, we tested its susceptibility to cleavage by phosphatidylinositol phospholipase C (PI-PLC). Whereas a control GPI-linked protein, RETL1 (ref. 17), was cleaved by PI-PLC and released into the medium (Fig. 2a lane 8), Myc–Hip remained associated with the cell (Fig. 2a, compare lanes 12 and 13). Thus, Hip is a type I transmembrane protein[18] with its last 22 amino-acid residues involved in membrane anchoring. The presence of four potential N-linked glycosylation sites suggests that Hip is a glycoprotein and, consistent with this view, treatment with endoglycosidase F to remove N-linked glycosylation produced a protein that migrated faster on SDS–polyacrylamide gel electrophoresis (Fig. 2a, lanes 14, 15). To determine whether Hip binds Shh-N directly as expected, we transfected cultured cells with the secreted form of Hip (Myc–HipΔC22), and then immunoprecipitated this from the medium with a Shh-N::IgG fusion protein (Fig. 2a, lane 5). Thus, Hip does interact directly with Shh-N.

Ptc-1 has been reported to confer cell-surface binding of Shh[19,20]. Using the cell-surface-binding assay, we found that Ptc-1, like Hip, also binds to all three mammalian Hedgehog proteins (data not shown). The dissociation constants (K_d) for Shh-N::AP binding to Hip and Ptc-1 were similar, approximately 5 nM and 4 nM, respectively (Fig. 2b). The finding that the K_d for Ptc-1 binding to Shh-N::AP is higher than the published value for Shh-N[19,20] (0.5–1.0 nM) probably reflects a weakened interaction of the biologically less active fusion protein[12].

We examined the temporal and spatial expression pattern of *Hip* in mouse embryos from 7.5 to 14.5 days post-coitum (dpc). When *Shh* and *Ptc-1* were first detected at 7.75 dpc[21,22], *Hip-1* expression was undetectable (data not shown). Subsequently, *Shh* expression was detected in several signalling centres, including the notochord, floor plate and zone of polarizing activity (ZPA) of the limb and in several endodermal derivatives[7,21]. In the caudal neural tube at 8.75 dpc, *Shh* expression, which is restricted to the notochord, is implicated in ventralizing the somites and neural tube. Here, *Hip* was activated at the ventral midline of the neural tube and in the ventral medial somites (Fig. 3a, b), next to *Shh*-expressing cells but anterior to where *Ptc-1* is first upregulated (data not shown). The close association of *Hip* and *Shh* expression in the notochord was maintained in the caudal region at tail bud stages (Fig. 3c, d). When *Shh* was induced in the floor plate at the ventral midline of the more rostral neural tube, *Hip* expression was lost in the floor plate but retained in the ventral half of the neural tube and in the sclerotome of the adjacent somites (Fig. 3a, b, e, f). In the midbrain, where *Shh* is implicated in the induction of dopaminergic neurons ventrally[1], *Hip* expression was confined to two lateral stripes immediately adjacent to *Shh*-expressing cells in the floor plate (Fig. 3e–h). *Hip* was also expressed in gut mesenchyme along the length of gastrointestinal tract next to *Shh*-expressing cells (Fig. 3g, h) and in

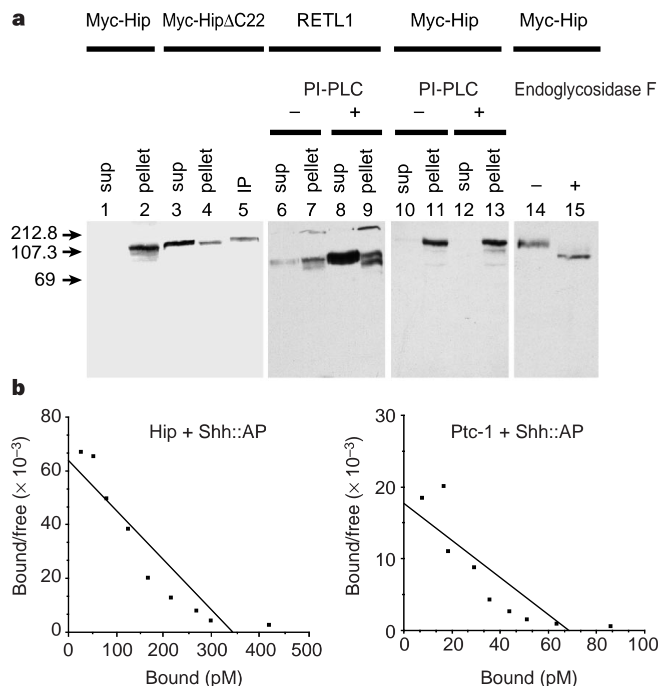

Figure 2 Analysis of the Hip protein. **a**, Lanes 1–5, western blot of the cell pellet and concentrated supernatant (sup) from COS-7 cells transfected with Myc-tagged Hip or Myc–HipΔC22, probed with an anti-Myc antibody. Whereas Myc–Hip is almost exclusively associated with the cell pellet fraction (lane 2), a significant portion of Myc–HipΔC22 is present in the medium (lane 3). Myc–HipΔC22 in the medium can be immunoprecipitated with Shh-N::IgG (lane 5). Lane 6–13, western blots of the cell pellet and supernatant from COS-7 cells transfected with RETL1 or Myc–Hip probed with an anti-RETL1 or anti-Myc antibody. As a result of PI-PLC treatment, a significant portion of RETL1, a GPI-linked protein, is released into the medium (lane 8). In contrast, Myc–Hip remains associated with the cell pellet fraction (lane 13). Lanes 14, 15, western blot of Hip protein in the absence or presence of endoglycosidase F. Treatment with endoglycosidase F results in a significantly faster migration (lane 15) on SDS–PAGE compared with untreated cells (lane 14). The location of protein size standards ($\times 10^{-3}$) are indicated by the arrows on the left. **b**, Scatchard analysis[11] of the dissociation constants (K_d) between Shh and two Hedgehog-binding proteins, Hip and Ptc-1.

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mesenchyme of the posterior half of the limb bud anterior to *Shh* expression in the ZPA (Fig. 3e, f). These results suggest that *Hip* is well placed to participate in *Shh* signalling. Further, as activation of *Hip* follows upregulation of *Ptc-1*, *Hip* may be a transcriptional target of *Shh* signalling, activated shortly after the initial response to *Shh*. Later in development, *Shh* is expressed in the epithelia of a wide variety of tissues[7], for instance the epithelium of the lung (Fig. 3i), gut (Fig. 3m), whisker (Fig. 3o), hair and rugae. In all cases, *Hip* was expressed in the underlying mesenchyme (Fig. 3j, n, p, and data not shown).

Expression of *Hip* was also detected in close association with sites of *Ihh* and *Dhh* expression. *Ihh* is expressed in the prehypertrophic chondrocytes, where it acts as a chondrocyte mitogen and controls the rate of chondrocyte differentiation (refs 4, 7, and B. St-Jacques, M. Hammerschmidt & A.P.M., in preparation). We found that *Hip*, like *Ptc-1*, was expressed in the perichondrium, including regions flanking *Ihh* expression in the appendicular (Fig. 3k) and axial skeleton (Fig. 3l). *Dhh* is expressed in Sertoli cells of the testis, where it is required for male germline development[8]. *Hip* and *Ptc-1* were expressed in the androgen-producing interstitial somatic cells (the Leydig cells) which are thought to respond to *Dhh* signalling (Fig. 4o and ref. 8). Thus, *Hip*, like *Ptc-1*, may be a transcriptional target of all mammalian Hh pathways and may have a general role in modulating all Hedgehog signalling.

We examined the regulation of *Hip* expression in transgenic mouse embryos expressing *Shh* ectopically, or in embryos carrying mutant *Hedgehog* genes. When *Shh* was ectopically expressed in the dorsal spinal cord[21] (arrows in Fig. 4b, f, compare with Fig. 4a, e), *Hip* (arrows in Fig. 4d, h, compare with Fig. 4c, g) and *Ptc-1* (arrow in Fig. 4j, compare with Fig. 4i) were ectopically expressed in regions immediately dorso-lateral to ectopic *Shh* expression. Further, expression of a transgene encoding a dominant-negative form of cyclic AMP-dependent protein kinase A in the dorsal neural tube, which ectopically activates Hh targets such as *Ptc-1*[23], also led to ectopic *Hip* expression (arrow in Fig. 4l). Finally, in *Shh* (Fig. 4n) and *Dhh*[8] mutants (Fig. 4p), *Hip* expression was absent in Hh-responsive cells (Fig. 4m, o). We conclude that *Hip*, like *Ptc-1*, is a general transcriptional target of Hh signalling.

The fact that Hip binds Hedgehog proteins directly and that its expression requires Hh signalling suggests that *Hip* may modify Hh

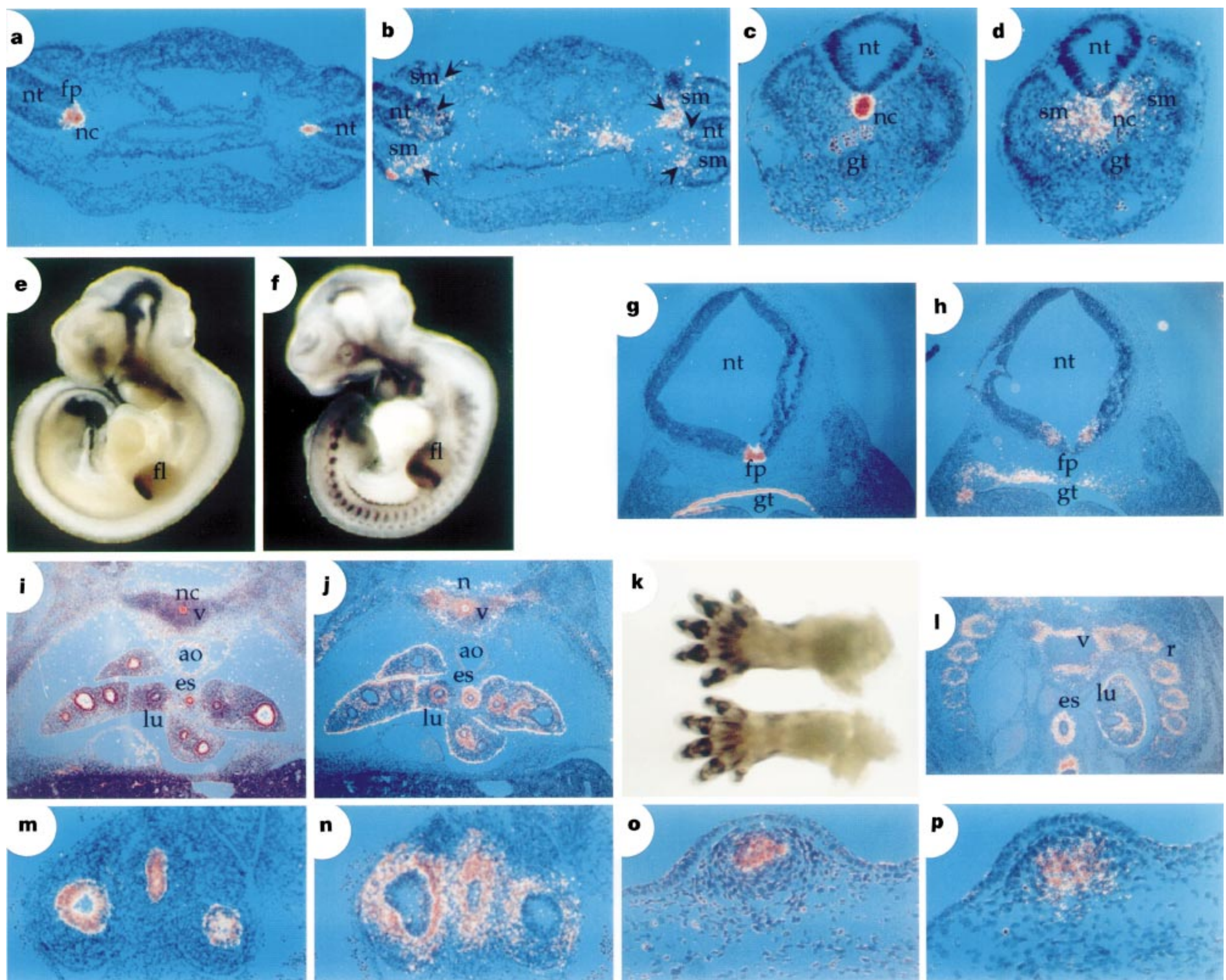

Figure 3 Expression of *Hip* during embryogenesis. See text for details of expression pattern. **e**, **f**, **k**, Whole-mount *in situ* hybridization. **a**–**d**, **g**–**j**, **l**–**p**, *In situ* hybridization performed on sectioned tissue. **a**, **c**, **e**, **g**, **i**, **m**, **o**, *In situ* hybridization to mouse *Shh* messenger RNA (mRNA). **b**, **d**, **f**, **h**, **j**, **k**, **l**, **n**, **p**, *In situ* hybridization to mouse *Hip* mRNA. **a**/**b**, **c**/**d**, **g**/**h**, **i**/**j**, **m**/**n**, **o**/**p**, Each pair represents adjacent sections. **a**, **b**, Section through a 9.0-dpc embryo. Arrowheads indicate *Hip* expression in the neural tube and arrows point to *Hip* expression in the somite. **c**, **d**, Section through a 10.5-dpc embryo. **e**, **f**, Lateral view of 10.5-dpc embryos. **g**, **h**, Transverse section through the midbrain of a 10.5-dpc embryo. **i**, **j**, Section through the thoracic region of a 14.5-dpc embryo. **k**, Dorsal view of forelimb dissected at 14.5 dpc. **l**, Transverse section through the rib cage of a 14.5-dpc embryo. **m**, **n**, Transverse section through the gut of a 14.5-dpc embryo. **o**, **p**, Section through the skin of a 14.5-dpc embryo. ao, Aorta; es, oesophagus; fl, forelimb; fp, floor plate; gt, gut; lu, lung; nc, notochord; sm, somite; nt, neural tube; r, rib; v, vertebral body.

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signalling. *Hip* could function either in a negative manner to attenuate a Hh signal or positively to facilitate signal transduction. In the first model, overexpression of *Hip* is likely to result in loss of Hh signalling by competition with Ptc for Hh binding. In the second model, *Hip* overexpression may enhance signal transduction, perhaps by facilitating Hh protein diffusion or increasing the affinity of Hh for other proteins on the membrane such as Ptc. To distinguish between these models, we expressed *Hip* ectopically in the developing endochondral skeleton using a rat α1(II) collagen promoter/enhancer[24]. α1(II) collagen is expressed in proliferating chondrocytes before hypertrophic differentiation[25]. Loss-of-function studies in the mouse have demonstrated that *Ihh* is essential for proliferation of chondrocytes (B. St-Jacques, M. Hammerschmidt & A.P.M., in preparation). *Ihh* mutants displayed a markedly shortened skeleton, most pronounced in the limbs (compare Fig. 5a and 5c). Interestingly, transgenic animals expressing the *Hip* transgene (*HipTg*) showed severe skeletal defects, including short-limbed dwarfism (Fig. 5b) remarkably similar to that observed in *Ihh* mutants. Histological analysis of *HipTg* animals revealed a marked reduction in the zone of undifferentiated chondrocytes (compare Fig. 5d, e and arrows in Fig. 5g, h), similar to *Ihh* mutants (Fig. 5f and arrows in Fig. 5i) but opposite to the expansion of proliferating, undifferentiated chondrocytes observed in animals overexpressing *Ihh*[4]. In addition, ectopic calcification occurred in similar regions of the skeleton as those observed in *Ihh* mutants (B. St-Jacques, M. Hammerschmidt & A.P.M., in preparation) and *HipTg* animals (data not shown). Finally, *Ptc-1* expression in proliferating chondrocytes was greatly reduced in *HipTg* animals whereas *Ihh* expression in prehypertrophic chondrocytes was unaltered (data not shown). Thus, overexpression of *Hip* diminishes *Ihh* signalling, consistent with a general model in

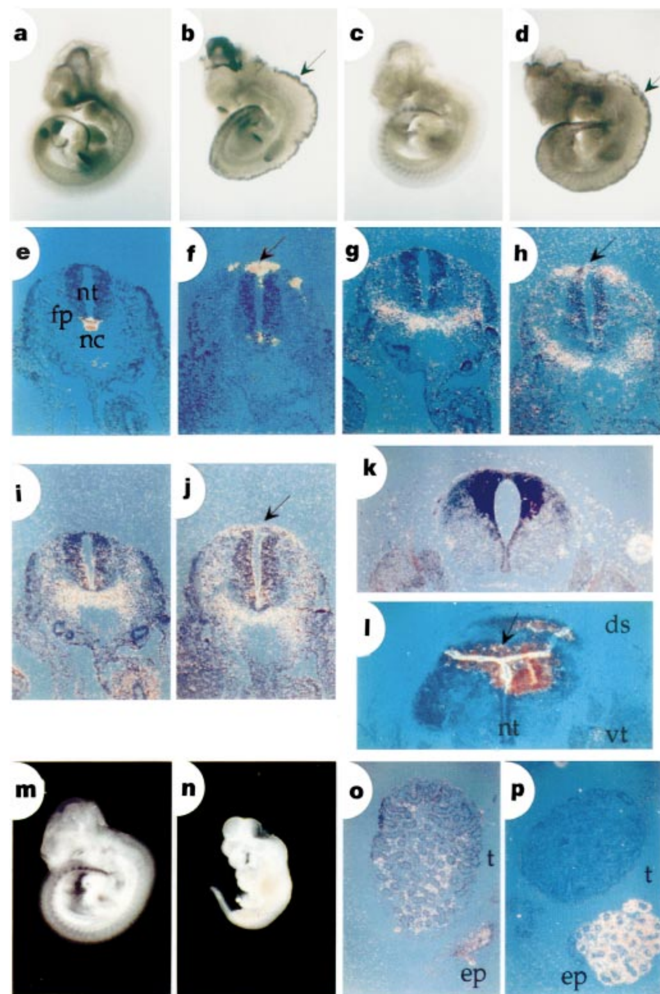

Figure 4 Expression of *Hip* transcripts in animals with ectopic or defective Hedgehog signalling. **a**–**d**, **m**, **n**, Whole-mount; **e**–**l**, **o**, **p**, section *in situ* hybridization. **a**, **b**, **e**, *In situ* hybridization to mouse *Shh* mRNA. **f**, *In situ* hybridization to chick *Shh* mRNA. **c**, **d**, **g**, **h**, **k**, **l**, **m**, **n**, **o**, **p**), *In situ* hybridization to mouse *Hip* mRNA. **i**, **j**, *In situ* hybridization to mouse *Ptc-1* mRNA. **a**–**d**, Lateral view of two 10.5-dpc wild-type (**a**, **c**) and two transgenic animals (**b**, **d**) expressing mouse *Shh* under the control of a *Wnt-1* enhancer. **e**–**j**, Section through the spinal cord of wild-type (**e**, **g**, **i**) and transgenic (**f**, **h**, **j**) embryos (10.5 dpc) expressing chick *Shh* under *Wnt-1* control. Ectopic *Hip* (arrows in **d** and **h**) and *Ptc-1* (arrow in **j**) expression is seen in the dorsal neural tube where *Shh* is ectopically expressed (arrows in **b** and **f**). **k**, **l**, Section through the spinal cord at 12.5 dpc of a wild-type (**k**) and transgenic embryo (**l**) carrying a transgene in which dominant-negative PKA is under *Wnt-1* control. Ectopic *Hip* expression (arrow in **l**) is seen in the dorsal neural tube where the Hh pathway is ectopically activated. **m**, **n**, Lateral view of a 10.5-dpc wild-type (**m**) and *Shh* mutant (**n**) embryo in which *Hip* expression is lost. **o**, **p**, Section through the testes of a wild-type (**o**) and *Dhh* mutant animal (**p**) (3 weeks *post partum*). Whereas *Hip* is expressed in the Leydig cells of wild-type testes (**o**), its expression is lost in a *Dhh* mutant (**p**). Mesenchymal expression of *Hip* is maintained in the epididymis, where *Shh* expression in the epithelium of *Dhh* mutant testes (**p**) is unaltered. nt, Neural tube; fp, floor plate; nc, notochord; ds, dorsal; vt, ventral; ep, epididymis; t, testis.

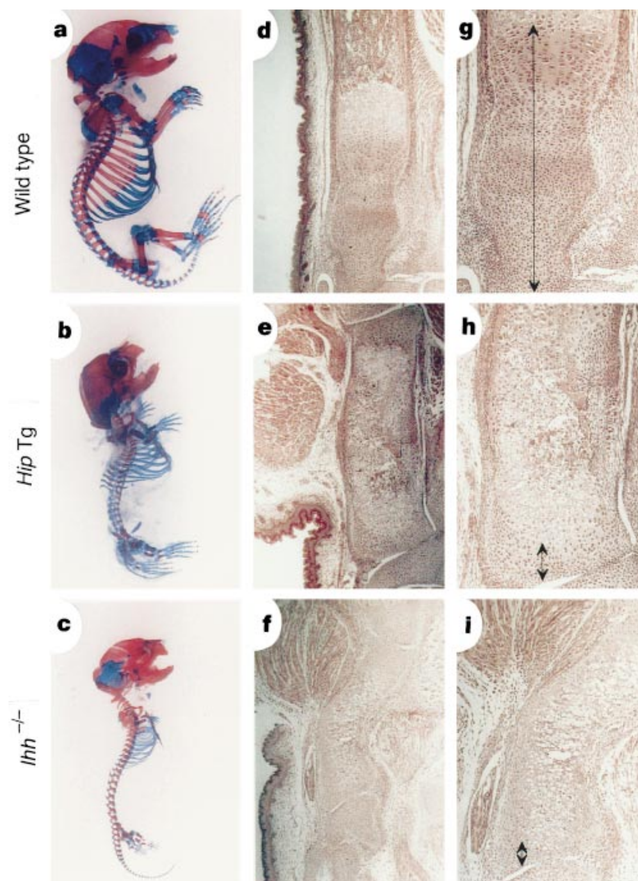

Figure 5 Analysis of skeleton in animals overexpressing *Hip* in chondrocytes. **a**–**c**, Skeleton of a wild-type (**a**), *Hip* transgenic (*HipTg*) (**b**) and *Ihh* mutant (**c**) 18.5-dpc embryo stained with Alcian blue and Alizarin red. Short-limbed dwarfism with general reduction of Alizarin red S staining is seen in the transgenic embryo, similar to that observed in *Ihh* mutants. **d**–**i**, Haematoxylin- and eosin-stained sections through the radius of a wild-type (**d**, **g**), *HipTg* (**e**, **h**) and *Ihh* mutant (**f**, **i**) 18.5-dpc embryo. A marked reduction of immature chondrocytes is observed in *HipTg* animals (arrows in **h**) and *Ihh* mutants (arrows in **i**) compared with that seen in the wild type (arrows in **g**). Photographs of **d**–**f** and **g**–**i** were taken at the same magnification, respectively.

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which upregulation of *Hip* by Hedgehog signals attenuates further Hedgehog signalling. Attenuation of signalling by physical binding of ligand may then allow differential responses to be generated within a field of competent cells. In *Drosophila*, a single *Hedgehog* gene mediates all Hedgehog signalling whereas in mammals three *Hedgehog* genes have largely non-overlapping activities. We have failed to identify a *Hip* orthologue in *Drosophila*. This suggests that in addition to diversification of Hedgehog signals, vertebrates may have evolved novel mechanisms of Hedgehog signal modulation that could facilitate distinct aspects of vertebrate development. □

Methods

Standard molecular biology and protein biochemistry techniques were performed as described[26].

Database searches. The Hip protein sequence was used to search the National Center for Biotechnology Information database using the BLAST program. Motif analysis of Hip and sequence alignments from Hip, Gene 5, 345 and pCZA361.11 were done with the GeneWorks program (IntelliGenetics). Transmembrane prediction was performed by SOSUI algorithm[27].

Epitope tagging, co-immunoprecipitation, endoglycosidase and PI-PLC treatment. To generate Myc–Hip, *Hip* cDNA was cloned into pcDNA3 (Invitrogen) and a Myc epitope was inserted between amino-acid residues 23 and 24. To generate Myc–HipΔC22, a stop codon was introduced 5′ to the last 22 residues in Myc–Hip. Shh-N::IgG was generated by cloning a DNA fragment encoding Shh-N into the pCD5IgG1 vector. To determine the localization of Myc–Hip and Myc-HipΔC22, COS-7 cells transfected with Myc–Hip or Myc–HipΔC22 using LipofectAMINE were collected and lysed 48 h after transfection. The supernatant was filtered and concentrated by precipitation with trichloroacetic acid (TCA). To immunoprecipitate Myc-HipΔC22, unconcentrated supernatant from COS-7 cells transfected with Myc–HipΔC22 was incubated with Shh-N::IgG bound to Protein A beads for 4 h at 4 °C. After extensive washing with buffer (25 mM HEPES (pH 7.6), 0.1 mM EDTA, 100 mM NaCl and 0.1% NP40), the beads were resuspended in sample buffer. Protein was run on SDS–PAGE and western blotting was done using anti-Myc antibody. To examine glycosylation of Hip, COS-7 cells transfected with Myc–Hip were lysed in RIPA buffer 48 h after transfection. The supernatant was mixed with an equal volume of buffer (20 mM sodium phosphate (pH 6.8), 40 mM EDTA, 0.1% SDS and 20 mM β-mercaptoethanol), boiled for 10 min and 0.4 U of endoglycosidase F (Boehringer Mannheim) was then added. The mixture was incubated at 37 °C for 24 h and analysed by western blotting using an anti-Myc antibody.

***In situ* hybridization.** Whole-mount *in situ* hybridization using digoxigenin-labelled probes and section *in situ* hybridization using ^{35}S-labelled probes were performed as described[28].

Generation of transgenic animals. To mis-express *Hip* under the α1(II) collagen promoter/enhancer, the full-length *Hip* cDNA was cloned into the α1(II) collagen expression vector. Transgenic animals were generated by pronuclear injection as described[29]. Staining of skeleton for bone and cartilage was as described[29].

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The Rab5 effector EEA1 is a core component of endosome docking

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Intracellular membrane docking and fusion requires the interplay between soluble factors and SNAREs. The SNARE hypothesis[1] postulates that pairing between a vesicular v-SNARE and a target membrane z-SNARE is the primary molecular interaction underlying the specificity of vesicle targeting as well as lipid bilayer fusion. This proposal is supported by recent studies using a minimal artificial system[2]. However, several observations demonstrate that SNAREs function at multiple transport steps and can pair promiscuously, questioning the role of SNAREs in conveying vesicle targeting[3–6]. Moreover, other proteins have been shown to be important in membrane docking or tethering[7–9]. Therefore, if the minimal machinery is defined as the set of proteins sufficient to reproduce *in vitro* the fidelity of vesicle targeting, docking and fusion as *in vivo*, then SNAREs are not sufficient to specify vesicle targeting. Endosome fusion also requires cytosolic factors and is regulated by the small GTPase Rab5 (refs 10–20). Here we show that Rab5-interacting soluble proteins can completely substitute for cytosol in an *in vivo*

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