RESEARCH COMMUNICATION

Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung

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Hedgehog (Hh) signaling plays a major role in multiple aspects of embryonic development. A key issue is how negative regulation of Hh signaling might contribute to generating differential responses over tens of cell diameters. In cells that respond to Hh, two proteins that are up-regulated are Patched1 (Ptch1), the Hh receptor, a general target in both invertebrate and vertebrate organisms, and Hip1, a Hh-binding protein that is vertebrate specific. To address the developmental role of Hip1 in the context of Hh signaling, we generated *Hip1* mutants in the mouse. Loss of *Hip1* function results in specific defects in two Hh target issues, the lung, a target of Sonic hedgehog (Shh) signaling, and the endochondral skeleton, a target of Indian hedgehog (Ihh) signaling. Hh signaling was up-regulated in *Hip1* mutants, substantiating Hip1's general role in negatively regulating Hh signaling. Our studies focused on Hip1 in the lung. Here, a dynamic interaction between Hh and fibroblast growth factor (Fgf) signaling, modulated at least in part by Hip1, controls early lung branching.

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The Hedgehog (Hh) signaling pathway plays a central role in the regulation of invertebrate and vertebrate development (for reviews, see Ingham and McMahon 2001; McMahon et al. 2003). Several components of the pathway have been identified that are essential for transducing the Hh signal; these include membrane proteins, cytoplasmic components, and transcriptional activators. Hh signal transduction is regulated by many cellular processes, including proteolysis, phosphorylation, and transcriptional activation of negative regulators. A Hh signal is transduced on binding of Hh ligand to its receptor, Patched1 (Ptch1), a multipass transmembrane protein. Genetic and molecular studies suggest that Ptch1 inhibits the signaling activity of Smoothened (Smo), a seven-transmembrane protein that shares sequence similarity with G-protein coupled receptors. Though the precise molecular mechanism remains to be elucidated, Hh binding to Ptch1 appears to relieve a Ptch1-mediated repression of Smo, resulting in elevated levels of a phosphorylated form of Smo at the surface of the cell. In its activated form, Smo can initiate the signaling cascade, activating transcriptional targets of the Hh signaling pathway.

An important aspect of Hh signaling is induction of the genes encoding Hh-binding proteins, *Ptc/Ptch1* and Hedgehog-interacting protein 1 (*Hip1*; for review, see Ingham and McMahon 2001). Whereas up-regulation of *Ptc/Ptch1* in target cells is a highly conserved response, no *Hip1* homologs have been identified in invertebrates. Increased Ptc on the cell surface of Hh-responsive cells sequesters Hh signal, limiting the range of Hh action in its target field, and may help shape the cell's response to Hh signaling (see Ingham and McMahon 2001). How Hip1 activity features in Hh signaling is less well understood.

Hip1 encodes a membrane-bound protein that directly binds all mammalian Hh proteins (Chuang and McMahon 1999). Like *Ptch1*, *Hip1* is transcriptionally activated in response to Hh signaling, overlapping the expression domains of *Ptch1* (Goodrich et al. 1996; Chuang and McMahon 1999). Further, gain-of-function experiments indicate that Hip1 binding of Hh ligands attenuates Hh signaling (Chuang and McMahon 1999). Here we demonstrate that loss-of-function mutants in *Hip1* result in an up-regulation of Hh signaling in the mouse embryo, disrupting cell interactions essential for the normal morphogenesis of the lung and skeleton (see Supplemental Material).

Results and Discussion

Targeted disruption of Hip1 results in neonatal lethality with respiratory failure

To generate a null allele of the *Hip1* gene in mice, a standard positive/negative targeting vector was constructed. The details are described in the Supplemental Material and Supplementary Figure 1A. Loss of *Hip1* activity leads to recessive postnatal lethality. The ratio of *Hip1*+/+:*Hip1*+/−:*Hip1*−/− (55:118:51) newborn pups approximates a 1:2:1 Mendelian distribution (Supplementary Fig. 1B), but all homozygous *Hip1* mutant pups die a few hours after birth due to respiratory failure. *Hip1* mutants are superficially identical to their wild-type littermates, indicating that *Hip1* activity does not appear to be essential for normal patterning of limbs, hair, or whisker, all of which are regulated by Hh signaling (Supplementary Fig. 1C; McMahon et al. 2003). Histological analysis revealed that dorsal-ventral patterning of the neural tube, development of the somites, and the organization of most internal organs appeared grossly normal in *Hip1* mutants (data not shown). In contrast, *Hip1* mutants have only one right and one left lung lobe rather than the five lobes (four on the right side and one

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on the left side) typical of wild-type mice (Fig. 1A–B,E–L). Mutant lungs do not inflate after birth and *Hip1* mutants die of respiratory failure.

To investigate the nature of the lung defects in *Hip1*–/– embryos, lungs were collected from embryos between 9.5 and 18.5 days postcoitus (dpc) (see Supplementary Fig. 1A for genotyping data). Lung development initiates at 9.5 dpc with outgrowth of paired buds of ventral foregut endoderm into surrounding splanchnic mesenchyme (for review, see Hogan 1999). At 10.5 dpc, two primary lung buds elongate, the right more rapidly than the left, and undergo further branching in an invariant pattern that is species specific, giving rise to five buds, four on the right and one on the left of the mouse embryo (Fig. 1E). In contrast, although both the left and right buds grew out in *Hip1* mutants, and the left-right asymmetry in their growth was conserved, the initial stereotyped branching from the two primary buds was absent in *Hip1*–/– lungs (Fig. 1F). These results suggest that, in the absence of *Hip1* activity, there is a failure to specify the early, invariant lateral branches that determine the lobulation pattern. At 11.5 dpc, the *Hip1*–/– lung exhibits two prominent lateral bulges, one on each lobe, indicating that partial secondary branching has begun (Fig. 1H). But the characteristic branching pattern (Fig. 1G) was never generated in *Hip1* mutant lungs (Fig. 1H). Thus, the failure to generate a complete respiratory tree explains the much smaller lung of mutants that are only 1/4 to 1/3 the size of wild-type lungs at birth (Fig. 1, cf. A and B). However, the single left lobe is also significantly reduced, indicating that Hip1 is also likely to play a later role in the branching process. Interestingly, histological analysis of the *Hip1*–/– lungs at these later stages revealed reduced airway space and an increased number of mesenchymal cells (Fig. 1, cf. C and D; data not shown for 16.5 and 17.5 dpc), but proximodistal epithelial differentiation appeared normal, as judged by histological and marker analysis with a number of regional or cell type-specific markers, including *CC10*, *SP-A*, *SP-B*, *SP-C*, and *CFTR* (data not shown). Thus, *Hip1* is required for normal branching morphogenesis of the airways, but not for proximodistal patterning.

Hedgehog signaling is up-regulated in Hip1 mutants

As discussed earlier, initial studies suggested that Hip1 may directly antagonize Hh signaling (Chuang and McMahon 1999). If correct, Hh targets, which include *Ptch1* and *Hip1*, would be expected to be transcriptionally up-regulated in the absence of Hip1 function. In the wild-type lung at 10.5 dpc, *Ptch1* (Fig. 2C) is expressed in the mesenchyme surrounding the developing airway epithelium where *Sonic hedgehog* (*Shh*) is expressed (Fig. 2A), but expression is elevated at the distal tips of the lung buds in response to an up-regulation of *Shh* expression in the adjacent epithelium (Litingtung et al. 1998; Pepicelli et al. 1998). In *Hip1*–/– lungs, *Shh* expression was unaltered (Fig. 2B) but *Ptch1* expression in the mesenchyme was moderately increased and expanded (Fig. 2D). In wild-type lungs at 11.5 dpc, higher levels of *Ptch1* expression are found at the tips of the newly formed secondary buds as compared with the regions between buds (arrows in Fig. 2G). In contrast, *Ptch1* expression remained quite uniform in the mesenchyme of the two primary lung buds of *Hip1* mutants (Fig. 2H). As development proceeds, *Ptch1* expression is further down-regulated in the wild-type lungs (Fig. 2I), yet *Ptch1* expression remained high in *Hip1*–/– lungs throughout later embryonic development (Fig. 2J; data not shown for 16.5 and 18.5 dpc). These results suggest that *Shh* signaling is up-regulated in the absence of *Hip1*. Consistent with this model, β-galactosidase activity at the targeted *Hip1* locus indicated that *Hip1* expression was also up-regulated in *Hip1* mutant lungs from 10.5 dpc, even when taking

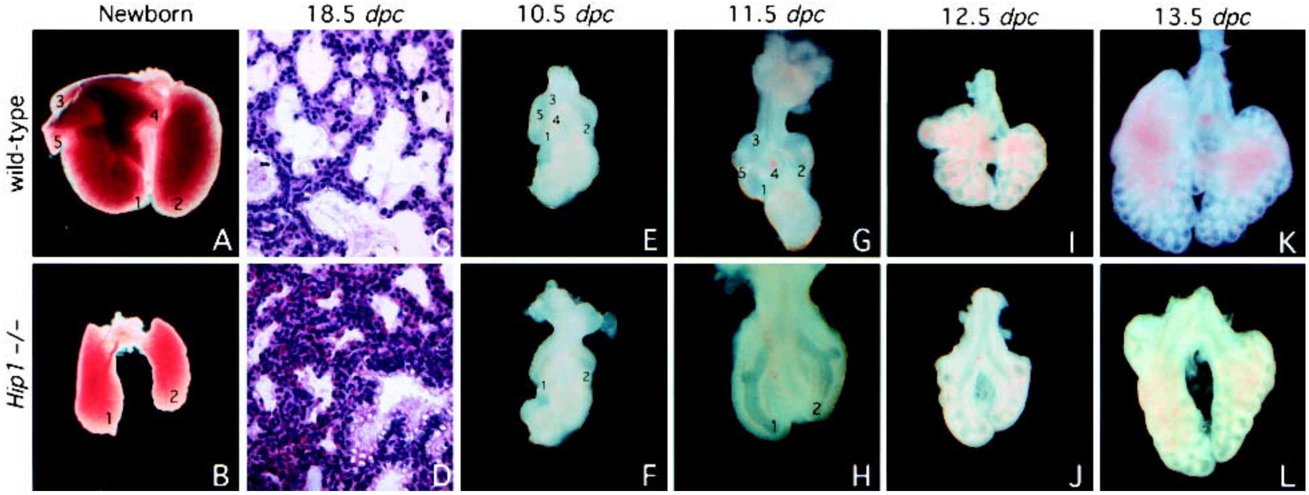

Figure 1. Defective branching morphogenesis in Hip1 mutant lungs. (*A–B,E–L*) Ventral view of lungs dissected at indicated stages from wild-type (*A,E,G,I,K*) and *Hip1* mutant (*B,F,H,J,L*) embryos or pups. (*C,D*) Hematoxylin/eosin-stained sections of wild-type (*C*) and *Hip1* mutant (*D*) lungs at 18.5 dpc. (*D*) A decrease in airway space and a relative increase in the number of mesenchymal cells was observed in *Hip1* mutant lungs. (*E*) The primary buds (labeled 1 and 2) in the wild-type lung can be seen at 10.5 dpc as well as the initial swellings (labeled 3–5) associated with the secondary branches of the right bud that establish the additional lobes of the right lung. (*G*) These elongate, generating three clear secondary branches from the right bud by 11.5 dpc. (*F,H*) The primary buds (labeled 1, 2) form in *Hip1* mutants, but initial secondary branching fails to occur during specification of lobulation. The lung bud of *Hip1* mutants in *H* was taken at a higher magnification in order to clearly visualize the branching pattern in *Hip1* mutant lungs. (*B*) As a consequence, only one lobe is generated from the right lung bud. (*I,K*) Extensive dichotomous branching ensues, giving rise to the respiratory tree. Dichotomous branching occurs at a slower rate in *Hip1* mutant lungs, resulting in a smaller sized left lung lobe and single right lobe and a generally stunted respiratory tree (cf. *B* and *A*).

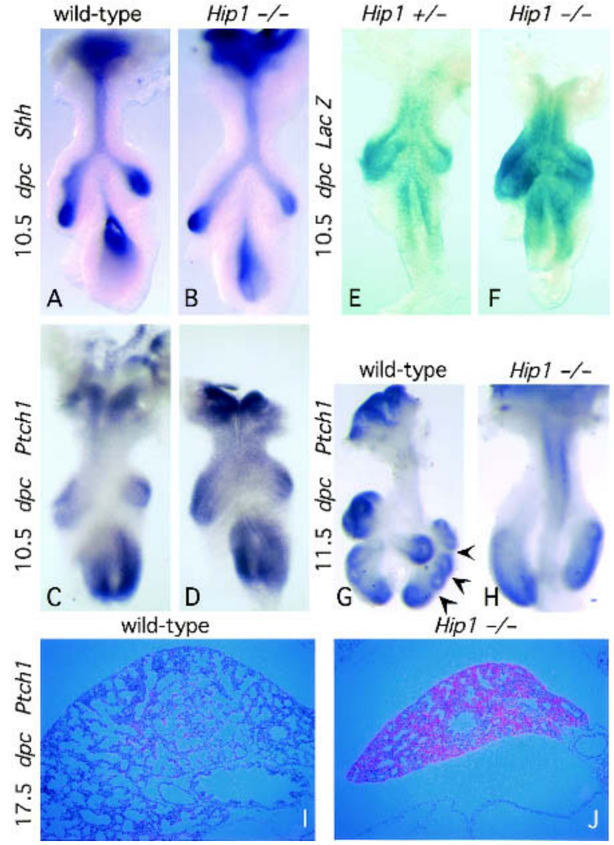

Figure 2. A molecular analysis of branching morphogenesis in *Hip1* mutant lungs. (*A–D*,*G–H*) Whole-mount in situ hybridization, using digoxigenin-labeled ribo-probes, on wild-type (*A*,*C*,*G*) and *Hip1* mutant (*B*,*D*,*H*) lungs (ventral view) at 10.5 and 11.5 dpc. (*A*) *Shh* expression in the epithelium increases at the distal tips of the wild-type lung buds. The expression pattern of *Shh* in the *Hip1* mutant lung buds (*B*) is similar to that of a wild-type embryo (*A*). In contrast, at 10.5 dpc, *Ptch1* expression in the mesenchyme is significantly increased and expanded in the *Hip1* mutant lung (*D*) as compared to that of the wild-type embryo (*C*). At 11.5 dpc, *Ptch1* expression becomes localized to the mesenchyme at the tips of the most recently developed buds in the wild-type lungs (arrowheads point to interbud regions where *Ptch1* expression is down-regulated; *G*), yet *Ptch1* expression remains uniformly expressed throughout the mesenchyme in *Hip1* mutant lung buds (*H*). As development proceeds, *Ptch1* expression is further down-regulated in the wild-type lungs (*I*; data not shown), yet *Ptch1* expression remains high in *Hip1*–/– lungs throughout embryonic development (*J*; data not shown). (*E–F*) β-Galactosidase staining of lungs (ventral view) dissected from *Hip1* heterozygous (+/–) and homozygous (–/–) embryos. The bacterial *LacZ* gene, inserted into the *Hip1* locus, is expressed under the *Hip1* promoter. Cells transcribing the *LacZ* gene can be viewed by histochemical staining for β-galactosidase activity. In the heterozygous lungs (*E*), *Hip1* transcripts are mainly confined to the mesenchyme at the tips of the lung buds, whereas in the *Hip1* mutant lungs (*F*), there is a dramatic increase in signal throughout the mesenchyme of the buds. This increase in signal seems to be greater than that due to the presence of an additional copy of the *LacZ* gene in *Hip*–/– lungs and indicates an up-regulation of the *LacZ* transcript level (*E*,*F*; data not shown).

into account differences in *lacZ* gene dosage (Fig. 2E,F; data not shown).

Defective secondary branching in Hip1 mutant lungs is due to the loss of Fgf10 signaling

Reciprocal interactions between the epithelium and the surrounding mesenchyme play a key role in inducing lung branching in a temporal- and spatial-specific manner (for reviews, see Hogan 1999; Warburton et al. 2000). Both loss-of-function and gain-of-function studies indicate that initial branching of the lung epithelium is controlled by a mesenchymal production of fibroblast growth factor 10 (Fgf10). Expression of *Fgf10* is initiated in mesenchymal cells some distance from the epithelium at focal sites adjacent to where branches later emerge (Bellusci et al. 1997). *Fgf10* knockout mice exhibit a lungless phenotype and die shortly after birth due to respiratory failure (Min et al. 1998; Sekine et al. 1999).

Analysis of *Shh* mutants indicates that Shh signaling plays an important role in the regulation of *Fgf10* expression in the mesenchyme, and, consequently, of the branching process (Litingtung et al. 1998; Pepicelli et al. 1998). In the wild-type lung at 10.5 dpc, *Fgf10* is expressed in mesenchyme cells at the distal tips of the primary lung buds and is localized to the prospective sites of lung bud formation (Bellusci et al. 1997; Fig. 3A,G). In *Shh* mutant lungs, *Fgf10* expression is broadly up-regulated in the lung mesenchyme and expression is observed in mesenchyme cells immediately adjacent to the lung epithelium (Pepicelli et al. 1998). Thus, *Shh* signaling to the mesenchyme negatively regulates *Fgf10* expression, and the resulting misregulation of *Fgf10* in *Shh* mutant lungs most likely accounts for the observed failure of secondary branching.

In contrast to delocalized *Fgf10* expression in *Shh* mutant lungs, *Fgf10* expression was slightly down-regulated at the distal tips of the primary lung buds in *Hip1*–/– lungs at 10.5 dpc, but completely absent from the mesenchyme where secondary branching normally initiates (Fig. 3B). At 11.5 dpc, outgrowth of a single secondary branch from both primary buds of *Hip1* mutant lungs (Fig. 1H) correlated with low levels of *Fgf10* expression (data not shown). The loss of secondary branching in *Hip1* mutant lungs was most likely a direct consequence of the failure of *Fgf10* expression at the prospective sites of secondary bud formation. Thus, whereas both *Shh* and *Hip1* mutants exhibit a deficiency in lung branching, the phenotype results from opposite actions of these factors. Loss of Shh-mediated inhibition of *Fgf10* results in a broad, mesenchymal up-regulation of *Fgf10* expression, leading to the loss of the focal sources of Fgf10 that normally trigger branching events. In contrast, enhanced Shh signaling in *Hip1* mutants leads to a nearly complete repression of normal *Fgf10* expression in the early lung, resulting in a failure of Fgf10-mediated initiation of secondary branching. Whereas *Fgf10* expression was clearly *Hip1* dependent, expression of *FgfR2*, the receptor for *Fgf10*, was unaltered (Fig. 3C,D), as was the expression of several genes that encode other signaling molecules that are implicated in the regulation of lung development, such as *Bmp4* (Fig. 3F,J), *Fgf9*, *Wnt7b*, and *Wnt2* (data not shown). Thus, modulating *Fgf10* expression may be the principal role of *Shh* signaling during lung development.

Hh signaling is not absolutely required for primary bud formation, as revealed by the presence of two stunted primary buds in *Shh* mutant lungs (Litingtung et al. 1998; Pepicelli et al. 1998). Primary buds also developed in *Hip1* mutants, a possible reflection of the time lag between Hh signaling and *Hip1* induction. In this model, any effects due to the lack of *Hip1* will not be apparent until after the outgrowth of the two primary buds when significant levels of *Hip1* transcripts have accumulated

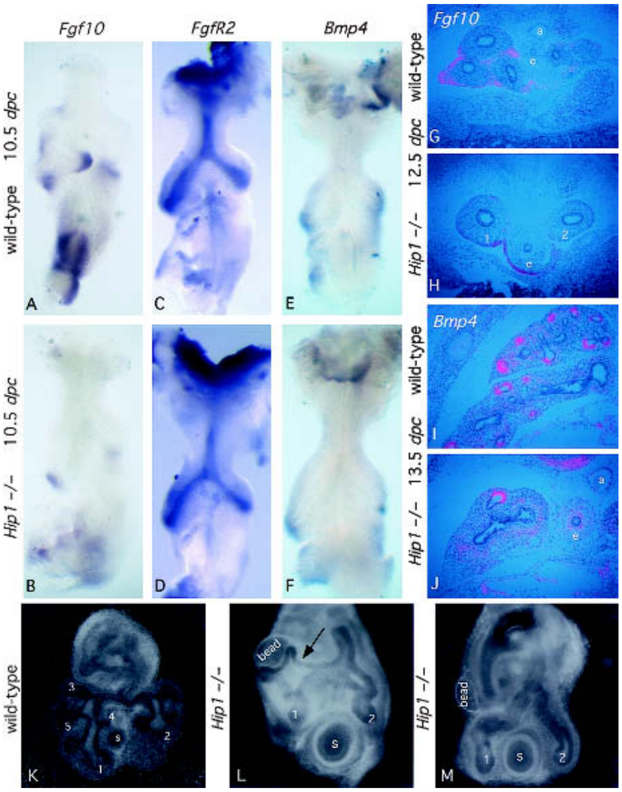

Figure 3. Analysis of major signaling pathways involved in lung branching in *Hip1* mutant lungs. (*A–F*) Whole-mount in situ hybridization, using digoxigenin-labeled ribo-probes, on wild-type (*A*,*C*,*E*) and *Hip1* mutant (*B*,*D*,*F*) lungs (ventral view) at 10.5 dpc. *Fgf10* expression in the mesenchyme at the prospective sites of secondary bud formation in wild-type lungs (*A*) is lost in the *Hip1* mutant lung (*B*). *FgfR2* is expressed in the epithelium of both the wild-type (*C*) and *Hip1* mutant (*D*) lungs with no apparent differences. The dynamic pattern of *Bmp4* expression in the distal epithelial cells of the terminal buds and in the adjacent mesenchyme is indistinguishable between wild-type (*E*) and *Hip1* mutant (*F*) lungs. (*G–J*) Isotopic section in situ using ^{33}P-UTP-labeled ribo-probes performed on sections of wild-type (*G*,*I*) and *Hip1* mutant (*H*,*J*) thoracic cavities at 12.5 and 13.5 dpc. Dorsal side is down. These images focus on the entire lung in both wild-type and *Hip1* mutant sections at 12.5 dpc. However, at 13.5 dpc, only images of the right lung are shown. The expression pattern of *Fgf10* (*G*,*H*) and *Bmp4* (*I*,*J*) correspond to the patterns described in the whole-mount in situ earlier, in which *Fgf10* expression is decreased at the distal tips of the *Hip1* mutant (*H*) primary buds (numbered 1 and 2) and lost from the future sites of secondary bud formation, but there are no changes in *Bmp4* expression between wild-type (*I*) and *Hip1* mutant (*J*) lungs. e, esophagus; a, aorta. (*K–M*) Induction of epithelial branching in *Hip1* mutant lungs by FGF10. (*L*) Dissected *Hip1* mutant lungs at 10.5 dpc in culture (primary buds are numbered 1 and 2), with beads soaked in recombinant FGF10 protein (labeled bead) implanted in the mesenchyme, show significant epithelial budding toward the bead at 84 h of culture. (*M*) In contrast, *Hip1* mutant lungs implanted with control (PBS) beads (labeled bead) show no sign of budding toward the bead at the corresponding time point. Wild-type lungs (buds are numbered 1–5, as seen in Fig. 1E), at 48 h (*K*) and 80 h (data not shown) of culture, show significantly more budding compared with those of the *Hip1* mutant. The right primary bud is to the left. Note that the branch (*L*, arrow) that grows toward the FGF10 bead represents a novel bud that arises at a more proximal position within the primary lung bud than the wild-type branches. s, stomach.

in response to Shh signaling such that Hip1 levels can now effectively modulate Shh action.

To test the hypothesis that the loss of branching in *Hip1* mutants reflects the absence of Fgf10 signaling, we implanted FGF10-soaked beads into the mesenchyme of *Hip1* mutant lungs at 10.5 dpc. FGF10 induced considerable epithelial budding toward the FGF10 bead (arrow in Fig. 3L), whereas PBS soaked beads failed to induce epithelial budding (Fig. 3M). Thus, loss of *Fgf10* expression, as a result of elevated Hh signaling, most likely explains the lack of secondary bud formation in *Hip1* mutant lungs.

Hip1 and Ptch1 share redundant roles in lung branching

The fact that *Hip1* mutant mice do not display overt phenotypes in multiple tissues that require Hh signaling for proper patterning may reflect a functional redundancy between *Ptch1* and *Hip1* because the expression of *Ptch1* overlaps with that of *Hip1* and both appear to function as negative regulators of Hh signaling. Preliminary studies support this model because attenuating *Ptch1* activity (*Ptch1*+/−) in a *Hip1* mutant background leads to an accelerated lethality around 11.5–12.5 dpc (data not shown). Lungs from *Hip1*−/−; *Ptch1*+/− animals (Fig. 4C) (n = 5) at this stage are consistently smaller in size (although the mesenchyme appears to be thicker) than *Hip1* mutant lungs (Fig. 4B) and also exhibit more severe branching defects from the two primary buds (Fig. 4, cf. B and C). If *Hip1* and *Ptch1* play a similar role in modulating Hh signaling, overexpression of *Ptch1* might rescue the lung defects in *Hip1* mutants. To test this hypothesis, we introduced a *Ptch1* transgene (*MTPtch1*; Milenkovic et al. 1999), in which *Ptch1* is expressed at a basal level under the control of the metallothionein promoter, into the *Hip1*−/− mutant background. *Hip1* mutant animals carrying *MTPtch1* (n = 3) exhibit a slight increase in lung size (Fig. 4, cf. E and F), whereas lungs from *Hip1*+/− animals carrying *MTPtch1* (Fig. 4D) cannot be distinguished from those of wild-type animals. In addition, a third right lobe (arrow in Fig. 4F), which is partially fused to the main right lobe, can also be observed in *Hip1*−/−; *MTPtch1* animals. These results indicate a modest rescue of lung defects in the *Hip1* mutant by *MTPtch1*. Taken together, these genetic studies reveal a functional redundancy between *Hip1* and *Ptch1* in lung branching and also suggest that similar functional interactions exist between *Hip1* and *Ptch1* in other tissues.

A model of Hip1's role in lung branching morphogenesis

The analysis of *Hip1* mutant lungs provides further insights into the interactions between Hh and Fgf signaling in lung branching morphogenesis. The mechanism by which *Fgf10* expression is initiated, thereby triggering lung bud outgrowth, is not clear, but analysis of both *Shh* and *Hip1* mutants indicates that neither is essential for this process. Localized *Fgf10* expression is essential for secondary bud formation. Hh signaling is not responsible for the activation of *Fgf10* expression associated with secondary bud formation but facilitates the correct spatial localization of *Fgf10* in the lung mesenchyme. This could be achieved by Hh inhibition of *Fgf10* expression in the interbud regions after *Fgf10* expression has been initiated (Fig. 5). However, *Shh* expression is up-regulated at sites of secondary bud formation where *Fgf10*

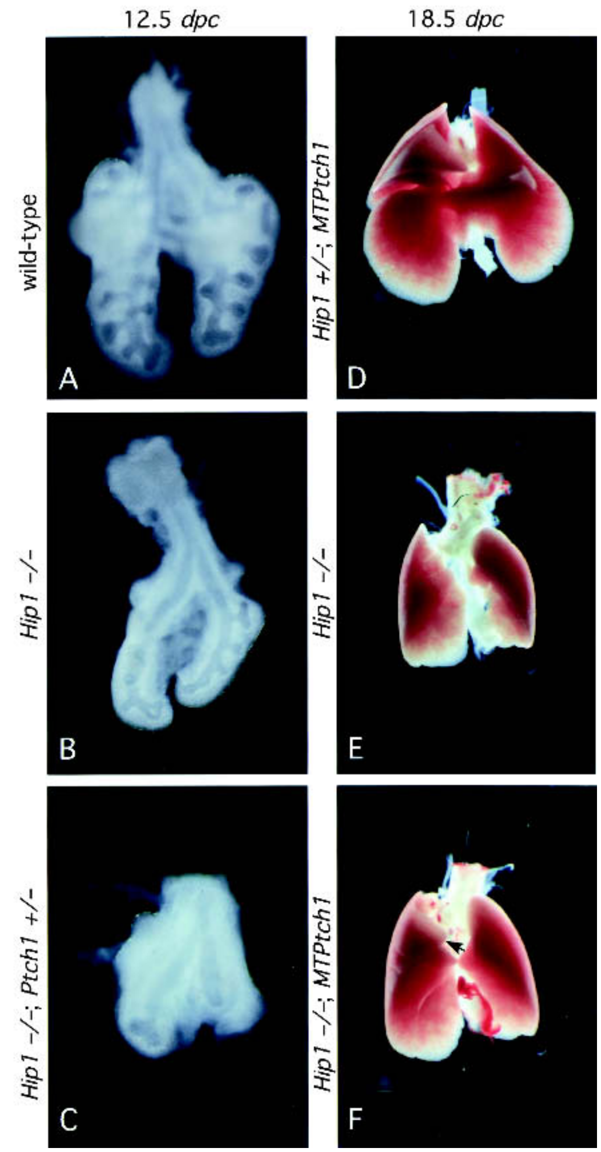

Figure 4. Hip1 and Ptch1 share redundant roles in lung branching. Ventral view of lungs dissected at 12.5 dpc (*A*–*C*) and 18.5 dpc (*D*–*F*). At 12.5 dpc, five distinct lobes are apparent in wild-type lungs (*A*), whereas only two lobes are generated in lungs from *Hip1* mutants (*B*). (*C*) Lungs from *Hip1*–/–; *Ptch1*+/– animals at this stage are smaller in size (although the mesenchyme appears to be thicker) than *Hip1* mutant lungs and also exhibit more severe branching defects from the two primary buds. (*D*) Animals carrying a *Ptch1* transgene (*MTPtch1*), in which *Ptch1* is under the control of the metallothionein promoter, do not exhibit any lung defect. When *MTPtch1* is expressed in *Hip1*–/– mutants, lungs from these animals (*F*) appear to be slightly larger in size, compared with lungs from *Hip1*–/– embryos (*E*). In addition, a third lobe (*F*, arrow), which is partially fused to the right lobe, can also be observed.

expression is actually maintained. How then is *Fgf10* expression maintained in the presence of high levels of *Shh* expression (and presumably high levels of Hh signaling) if Hh signaling also inhibits *Fgf10* expression at the sites of secondary buds?

We envisage two possible mechanisms. In the first, higher levels of *Shh* at the bud tips may not actually correspond to enhanced levels of Shh signaling, because negative feedback control on Shh signaling might effectively inhibit the signaling pathway. Once the amount of Ptch1 exceeds that required for Hh signaling, Ptch1, together with Hip1, sequester the Shh protein. This may quickly result in decreased Shh signaling, and as a result, less Shh signaling may result in regions of branching compared with the interbud regions. Thus, at the tips of the lung buds, *Fgf10* expression can be maintained where *Ptch1* and *Hip1* are up-regulated, but *Fgf10* expression is inhibited in the interbud region where initial signaling is not sufficient to maximally activate *Ptc1* and *Hip1* transcription. Although this type of mechanism is consistent with other feedback systems, it should be noted that in Shh-mediated induction of ventral cell identities in the neural tube and anterior-posterior digit identities in the limb, the highest threshold requirement for Shh signaling correlates with cells that appear to undergo maximal activation of *Ptc1* expression.

A second model supposes that broad Shh signaling from the distal epithelium establishes a general proximal zone of *Fgf10* repression in adjacent mesenchyme. As a result, *Fgf10* activation can only occur at some distance from the underlying epithelium, thereby providing a distant source of ligand to trigger local, directed epithelial outgrowth. Up-regulation of *Shh* in the branching epithelium may eventually inhibit the focal sources of

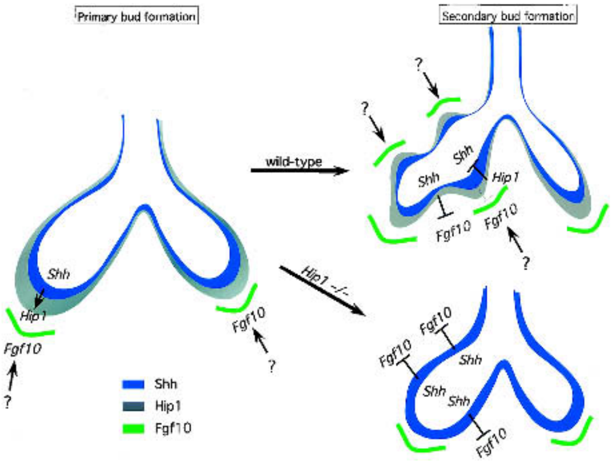

Figure 5 A model of Hip1's role in lung branching morphogenesis. During lung branching morphogenesis, the two primary buds initially elongate and later bulge to generate the secondary buds. Localized *Fgf10* (green) expression in the distal mesenchyme induces primary bud formation. The mechanisms by which *Fgf10* expression is initiated and localized are not clear. *Shh* (blue) is expressed in the epithelium and is up-regulated at the distal tips of the primary buds. In this model, in the wild-type lung, secondary bud formation is also induced by localized *Fgf10* expression in the mesenchyme corresponding to the future sites of secondary bud formation. It is likely that similar mechanisms (indicated by a question mark in the diagram) are used to localize *Fgf10* to these sites. Epithelial expression of Hh may help restrict *Fgf10* expression to the sites of secondary bud formation by inhibiting *Fgf10* expression. The Hh pathway transcriptionally activates *Hip1* (gray) and *Ptch1* (not shown), antagonists of Hh signaling, to down-regulate the Hh pathway at the sites of bud formation. As a result, the signals that activate *Fgf10* expression overcome the mild inhibition (broken line), if any, by Hh signaling. In contrast, in the interbud regions, the Hh pathway effectively inhibits *Fgf10* expression. In the *Hip1* mutant lung, there is a uniform up-regulation of the Hh pathway along the entire epithelium due to the absence of negative regulation by *Hip1*. Consequently, high levels of Hh inhibit *Fgf10* expression and disrupt secondary bud formation. Thus, *Hip1*'s negative regulation of the Hh pathway is necessary to ensure proper secondary bud formation.

Fgf10 expression as the branching tips approach these groups of cells. Whatever the exact mechanism, the dynamic interaction between Shh and *Fgf10* most likely extends beyond the initiation of secondary, lateral branches into later stages of branching morphogenesis because there is a marked reduction of airways in the single left lobe of the *Hip1* mutant lung. Interestingly, *Fgf4* expression in the limb is induced by *Shh* (Laufer et al. 1994; Niswander et al. 1994), and recent work suggests a link between Fgf and Hh signaling in expansion of the diencephalic primordium of the mouse brain (Ishibashi and McMahon 2002). Thus, the interaction between these two signaling pathways plays an important regulatory role in the morphogenesis of several distinct structures in the developing vertebrate embryo.

Materials and methods

Standard molecular biology techniques were performed as described (Sambrook and Russell 2001).

Generation of Hip1 null mice

A complete description of the targeting vector construct, chimera production, and allele identification is provided in the Supplemental Material.

Skeletal preparations, histology, and in situ hybridization

Skeletal preparations were performed as described (Hogan et al. 1994). Histological analysis, whole-mount in situ hybridization using digoxigenin-labeled probes, and section in situ hybridization using ^{33}P-labeled ribo-probes were performed as described (Wilkinson and Nieto 1993). For whole mount in situ hybridization, at least five mutant embryos were examined for each probe and consistent results were observed. For section in situ hybridization, at least two mutant embryos were examined for each probe and consistent results were obtained.

Lung organ culture

Heparin beads (Sigma) were cut with tungsten needles, washed three times in phosphate-buffered saline (PBS), soaked in recombinant human FGF10 (R&D Systems, Inc.) for 4–5 h at room temperature, and stored at 4°C. Prior to use, the FGF10 beads were washed three times in PBS (Park et al. 1998), then implanted into the mesenchyme of lungs isolated from 10.5 dpc mouse embryos dissected in L15 medium supplemented with 1% serum. The lungs were placed on nucleopore polycarbonate filters (Whatman) and cultured in DMEM:F12 (1:1) supplemented with 10% fetal calf serum (Hyclone), 1× penicillin/streptomycin, and 1× L-glutamine for up to 90 h.

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References

Bellusci, S., Grindley, J., Emoto, H., Itoh, N., and Hogan, B.L. 1997. Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development* **124:** 4867–4878.

Chuang, P.-T. and McMahon, A.P. 1999. Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. *Nature* **397:** 617–621.

Goodrich, L.V., Johnson, R.V., Milenkovic, L., McMahon, J.A., and Scott, M.P. 1996. Conservation of the hedgehog/patched signaling pathway from flies to mice: Induction of a mouse patched gene by Hedgehog. *Genes & Dev.* **10:** 301–312.

Hogan, B.L. 1999. Morphogenesis. *Cell* **96:** 225–233.

Hogan, B., Beddington, R., Costantini, F., and Lacy, E. 1994. *Manipulating the mouse embryo: A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ingham, P.W. and McMahon, A.P. 2001. Hedgehog signaling in animal development: paradigms and principles. *Genes & Dev.* **15:** 3059–3087.

Ishibashi, M. and McMahon, A.P. 2002. A Sonic hedgehog-dependent signaling relay regulates growth of diencephalic and mesencephalic primordial in the early mouse embryo. *Development* **129:** 4807–4819.

Laufer, E., Nelson, C.E., Johnson, R.L., Morgan, B.A., and Tabin, C. 1994. Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79:** 993–1003.

Litingtung, Y., Lei, L., Westphal, H., and Chiang, C. 1998. Sonic hedgehog is essential to foregut development. *Nat. Genet.* **20:** 58–61.

McMahon, A.P., Ingham, P.W., and Tabin, C.J. 2003. The developmental roles and clinical significance of Hedgehog signaling. *Curr. Top. Dev. Biol.* (In press).

Milenkovic, L., Goodrich, L.V., Higgins, K.M., and Scott, M.P. 1999. Mouse patched1 controls body size determination and limb patterning. *Development* **126:** 4431–4440.

Min, H., Danilenko, D.M., Scully, S.A., Bolon, B., Ring, B.D., Tarpley, J.E., DeRose, M., and Simonet, W.S. 1998. Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless. *Genes & Dev.* **12:** 3156–3161.

Niswander, L., Jeffrey, S., Martin, G.R., and Tickle, C. 1994. A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* **371:** 609–612.

Park, W.Y., Miranda, B., Lebeche, D., Hashimoto, G., and Cardoso, W.V. 1998. FGF-10 is a chemotactic factor for distal epithelial buds during lung development. *Dev. Biol.* **201:** 125–134.

Pepicelli, C.V., Lewis, P.M., and McMahon, A.P. 1998. Sonic hedgehog regulates branching morphogenesis in the mammalian lung. *Curr. Biol.* **8:** 1083–1086.

Sambrook, J. and Russell, D.W. 2001. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N., et al. 1999. Fgf10 is essential for limb and lung formation. *Nat. Genet.* **21:** 138–141.

Warburton, D., Schwarz, M., Tefft, D., Flores-Delgado, G., Anderson, K.D., and Cardoso, W.V. 2000. The molecular basis of lung morphogenesis. *Mech. Dev.* **92:** 55–81.

Wilkinson, D.G. and Nieto, M.A. 1993. Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225:** 361–373.