Mouse hitchhiker mutants have spina bifida, dorso-ventral patterning defects and polydactyly: identification of Tulp3 as a novel negative regulator of the Sonic hedgehog pathway

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The mammalian Sonic hedgehog (Shh) signalling pathway is essential for embryonic development and the patterning of multiple organs. Disruption or activation of Shh signalling leads to multiple birth defects, including holoprosencephaly, neural tube defects and polydactyly, and in adults results in tumours of the skin or central nervous system. Genetic approaches with model organisms continue to identify novel components of the pathway, including key molecules that function as positive or negative regulators of Shh signalling. Data presented here define Tulp3 as a novel negative regulator of the Shh pathway. We have identified a new mouse mutant that is a strongly hypomorphic allele of Tulp3 and which exhibits expansion of ventral markers in the caudal spinal cord, as well as neural tube defects and preaxial polydactyly, consistent with increased Shh signalling. We demonstrate that Tulp3 acts genetically downstream of Shh and Smoothened (Smo) in neural tube patterning and exhibits a genetic interaction with Gli3 in limb development. We show that Tulp3 does not appear to alter expression or processing of Gli3, and we demonstrate that transcriptional regulation of other negative regulators (Rab23, Fkbp8, Thm1, Sufu and PKA) is not affected. We discuss the possible mechanism of action of Tulp3 in Shh-mediated signalling in light of these new data.

INTRODUCTION

Birth defects remain the greatest cause of infant mortality in the Western world. These developmental anomalies can affect any organ in the body and are often severely disabling or fatal. In the UK, over 90 000 pregnancies per year (~2.3%) are affected by a major congenital malformation, and amongst the most common anomalies are defects of the nervous system and limbs, which are observed in 10 and 16% of affected fetuses, respectively (1). Reducing the clinical burden from birth defects requires a more complete understanding of the causative factors, including knowledge of the genetic, cellular and molecular mechanisms involved, in order to permit the development of appropriate preventative therapies. Studies with model organisms are essential in helping to unravel the complex network of processes involved in normal development, and the identification of mutant animals provides key insights into the pathways that are involved in these disorders. Mutagenesis of mice with ethylnitrosourea (ENU) has proven to be a powerful method for the generation of new mutations that provide good models of human developmental disorders (2–4). Investigation of these mutants promises to shed new light on the causes of these defects as well as identifying novel components of known signalling pathways.

Development of the mammalian nervous system involves the initial formation of the neural tube, the precursor of the brain and
spinal cord, and the subsequent differentiation of the neural tissue into spatially appropriate neuronal cell types. The rolling up and closure of the neural plate to form the neural tube constitutes the process of neurulation, and this occurs with a characteristic sequence of events and varying neural fold morphology in different regions of the embryo (5–7). Initiation of neural tube closure occurs at the level of the cervical/hindbrain boundary (Closure 1) at E8.5 in mouse, and continues from here both cranially into the hindbrain and caudally along the developing spine. Two further sites of closure initiation are observed in the cranial region, at the forebrain–midbrain boundary (Closure 2) and at the most rostral extent of the forebrain (Closure 3). Neurulation in the head is completed with closure of the intervening regions, termed the anterior neuropore and hindbrain neuropore, by E9.5. Neural tube closure continues until E10.5 in a cranio-caudal direction from the site of Closure 1 along the developing spine. The region immediately caudal to the closed neural tube, in which the neural folds are elevating and about to close, is termed the posterior neuropore (PNP). The morphology of the neural folds within the PNP varies along the spinal axis, and this has led to the characterization of three modes of spinal neurulation (6). In mode 1 (E8.5–E9), the neural plate exhibits pronounced bending at the ventral midline, the median hinge point (MHP), but straight lateral folds, whereas in mode 2 (E9–E9.75) the neural folds bend both at the MHP and also at paired dorso-lateral hinge points (DLHPs). Closure of the upper sacral level involves mode 3 neurulation (E9.75–E10.5), in which the neural plate bends solely at the DLHPs.

The Sonic hedgehog (Shh) signalling pathway is essential for the normal growth and patterning of many organs including the nervous system and limbs (8,9). Disruption of Shh signalling results in a range of congenital defects, including holoprosencephaly, craniofacial abnormalities and skeletal malformations (8,9). Conversely, inappropriate activation of the pathway leads to neural tube defects, polydactyly, basal cell carcinomas and tumours of the central nervous system (7,10,11). Shh plays a key role in regulating closure of the neural tube since Shh expression in the notochord is necessary and sufficient to inhibit formation of DLHPs, through a mechanism involving antagonism of Bmp signalling (12,13). Shh signalling from the notochord and, subsequently, the floor plate is also important for correct dorso-ventral (DV) patterning of the nervous system (14–18). The spatially restricted specification of neuronal subtypes is essential for the subsequent assembly of functional neuronal circuits. High concentrations of Shh induce floor plate markers, whereas lower concentrations specify increasingly more dorsal interneuron subtypes within the ventral neural tube. Absence of Shh signalling results in loss of ventral markers and complete dorsalization of the spinal cord, whereas ectopic Shh signalling leads to expansion of ventral markers at the expense of more dorsal marker domains. Patterning along the anterior–posterior (AP) axis of the limb is also dependent on the Shh pathway (19–21). Shh is expressed in the zone of polarizing activity (ZPA) in the posterior domain of the limb buds, and the spatial and temporal gradients of Shh activity across the limb determine digit number and identity. Increased or ectopic Shh activity leads to polydactyly, whereas decreased Shh activity results in narrowed limbs and reduced number of digits.

The vertebrate Shh pathway shares fundamental similarities with that of Drosophila (22–24). Hh ligand binds to Patched (Ptc) receptor, releasing the repressive activity of Ptc on Smoothened (Smo) and, thereby, allowing Smo to initiate intracellular signalling. Downstream of Smo the signal culminates with the activation of the Ci/Gli transcriptional activators and removal of the Ci/Gli repressors. Vertebrates express three Gli family members, Gli1–3; Gli1 functions as a transcriptional activator, whereas Gli2 and Gli3 can act both as transcriptional activators and, following proteolytic removal of the C-terminal region, as transcriptional repressors. The relative importance of the Gli activator and repressor forms differs in the patterning of the neural tube and limb. Neural tube patterning is mostly dependent on ventral Gli1 and Gli2 activators, with less important roles of Gli3 repressor dorsally (15,25–29). In contrast, limb patterning is generated predominantly by Gli3, with opposing gradients of Gli3 activator and Gli3 repressor (30–32). Although vertebrate and Drosophila hedgehog pathways are grossly similar, the intermediate steps between the release of Smoothened and the activation or processing of Ci/Gli are distinct between species and, indeed, are not yet fully understood (9,22,33,34).

Forward genetic screens in mouse have successfully identified additional components of the vertebrate Shh pathway, with the advantage of no prior bias on the type of molecule that may be involved (35,36). A number of positive regulators of Shh signalling have recently been discovered, based on loss of ventral cell types in the neural tube, including proteins involved in the formation/maintenance of the primary cilia or intraflagellar transport (37). Several Shh pathway components localize within these subcellular organelles (38–42), and the general consensus is that some aspects of vertebrate Shh-mediated Gli processing occur within cilia. Specific proteins have been revealed as negative regulators of the pathway, since disruption of their function leads to activation of Shh signalling, as observed by the expansion of ventral marker domains within the neural tube. The known negative regulators include Ptc1, Sufu, protein kinase A (PKA), Rab23, Fkbp8 and Thm1 (42–51). PKA phosphorylates Gli3 prior to processing by the ubiquitin-proteasome pathway (52), whereas Sufu inhibits the transcriptional activity of Gli activators in the nucleus and may also sequester Gli in the cytoplasm (49,50,53,54). Thm1 is involved in intraflagellar transport (42). The functions of Rab23 and Fkbp8 are not clear, although they may have roles in vesicular trafficking (55,56). Clearly, the vertebrate Shh pathway is complex, and our incomplete understanding of the processing and regulation of intermediate steps suggests that additional components remain to be discovered.

Here, we report a new ENU-induced mouse mutant that we have named hitchhiker (hhkr), which exhibits a range of developmental abnormalities including neural tube defects and polydactyly. We reveal that hitchhiker carries a strongly hypomorphic mutation in Tulip3, and we show that mutants have ventralization of the caudal spinal cord. We demonstrate that Tulip3 functions genetically downstream of Shh and Smo, to give activation of Shh pathway targets in the absence of Shh and Smo; complementary results are reported in an accompanying paper (Norman et al., submitted for publication). In addition, we show that the hitchhiker mutant exhibits a genetic interaction with Gli3 in limb development, but does not substantially affect Gli3 processing or expression.
We demonstrate that transcriptional regulation of other negative regulators (Rab23, Fkbp8, Thm1, Sufu and PKA) is not affected in hitchhiker mutants. We propose that Tulp3 functions as a novel negative regulator of the Shh pathway and we discuss the possible mechanism of action of Tulp3 in Shh-mediated signalling in light of these new data.

RESULTS

hitchhiker is a novel mutant with defects in neural tube and limb development

hitchhiker (hhkr) arose during an on-going screen for recessive ENU-induced mutations that affect the morphology of midgestation embryos. The mutant was initially identified through the presence of neural tube defects, with exencephaly and/or lumbosacral spina bifida aperta, and mutants also commonly exhibited oedema (Fig. 1A–D). Histological sections revealed abnormal morphology of the lower spinal neural tube in mutants in which the neural tube had closed, with an expanded lumen and reduced thickness of the neuroepithelium, particularly in the ventral half of the spinal cord (Fig. 1F, compare with E). Mutants also demonstrated oedema in the surrounding tissue, and small, dorsally misplaced dorsal root ganglia (Fig. 1F). Sections through other mutants demonstrated the widely splayed neural folds characteristic of spina bifida (Fig. 1G). Some hitchhiker fetuses exhibited a closed thoracolumbar neural tube but which was not enclosed within the bony vertebral column, either with or without lumbosacral spina bifida (Fig. 1I, compare with H), and this phenotype may be similar to the human defect of spina bifida occulta. Histological sections through E15.5 hitchhiker embryos with this phenotype confirmed the presence of a closed neural tube, but with a grossly enlarged spinal canal, a thin dorsal covering of surface ectoderm and flanked by the widely spaced cartilage primordia of the developing vertebrae (Fig. 1K, compare with J). Skeletal preparations of E18.5 fetuses with the exposed thoracolumbar neural tube (without spina bifida) clearly demonstrated splayed vertebrae in the thoracolumbar region (Fig. 1M, compare with L) and also revealed abnormal morphology of the ribs, commonly with bifurcations (Fig. 1O, compare with N). Since the ribs, vertebrae and dorsal dermis all derive from the somites (57), the observation of defects in these tissues might suggest possible abnormalities in somitic development in hitchhiker.

The hitchhiker line was named after a limb defect, in which the first digit exhibited an abnormal backward-bent position (Fig. 1P and Q). More often, mutants exhibited preaxial polydactyly on both fore- and hindlimbs, with six or occasionally seven digits (Fig. 1R and S). Skeletal preparations revealed a spectrum of defects, including apparent bifurcation or duplication of digit one, or full or partial duplication of another digit (Fig. 1T–W and data not shown).

The observed phenotypes were all partially penetrant and occurred in any combination: 68% of fetuses exhibited polydactyly (n = 73; ≥E12.5); 65%, spina bifida aperta; 58%, oedema; 37%, exencephaly; 8%, spina bifida occulta and 5% no detectable defect (n = 126, ≥E10.5). Approximately 6% of heterozygotes exhibited a mild head misshaping or slight caudal oedema.

hitchhiker carries a splice site mutation in Tulp3 causing a strongly hypomorphic function

Genetic mapping using a genome-wide marker panel and nine affected individuals revealed linkage of hitchhiker to Chromosome 6 (Fig. 2A), and analysis with additional markers and individuals refined the interval to 12 Mb (Fig. 2B). Within this region lies Tulp3; since a targeted allele of Tulp3 exhibits neural tube defects similar to hitchhiker (58), we examined Tulp3 in our mutant, RT–PCR analysis revealed a reduced size for one Tulp3 amplicon (Fig. 2C), and sequencing revealed a deletion of 52 bp corresponding precisely to exon 2 (data not shown). Genomic DNA analysis identified a single nucleotide substitution at the highly invariant position 2 of the splice donor site immediately 3’ to exon 2 (Fig. 2D and E) (59).

Deletion of exon 2 creates a frameshift that is predicted to cause premature truncation of the protein (Fig. 2F) and a null phenotype. Western blots confirmed a dramatic reduction in Tulp3 protein in hitchhiker homozygotes, although long exposures detected Tulp3 at ~4% of the wild-type level (Fig. 2G and H), suggesting that hitchhiker is a strong hypomorph of Tulp3 rather than a complete null. Tulp3 protein was reduced to 60% wild-type level in heterozygotes (Fig. 2G and H). To further verify that Tulp3 mutation is responsible for the hitchhiker phenotype, we intercrossed hitchhiker heterozygotes with mice heterozygous for the Tulp3-targeted allele, Tulp3<sup>tm1Jng</sup>, with both strains on a C3H background. This yielded embryos exhibiting the range of phenotypes displayed by either homozygous mutant (Fig. 2I and J; n = 8), indicating a failure of complementation. The phenotype of both the targeted allele homozygotes and the double heterozygous embryos appears to be slightly more severe than that of hitchhiker homozygotes, with more extensive oedema and defects in eye development. Together, these data indicate that hitchhiker is a strong hypomorph of Tulp3.

Neural tube defects in hitchhiker result from a failure of brain and PNP closure

Following identification of the mutant gene, we conducted detailed morphological and histological examination of mutant embryos during the stages of neurulation in order to determine the cause of the neural tube defects. In the cranial region, mutant embryos exhibited normal de novo closure at the future cervical region (Closure 1) and the rostral extent of the forebrain (Closure 3) (data not shown). In contrast, the occurrence of Closure 2, at the forebrain–midbrain boundary, was delayed or failed: 22% of hitchhiker embryos exhibited failure of Closure 2 even at the 24–27 somite stage, while a further 15% had achieved Closure 2 but retained an open hindbrain neuropore at this stage, in contrast to the complete head closure seen in wild-type embryos by the 21 somite stage (Fig. 3A and B). The exencephalic phenotype observed in 37% of fetuses at later stages, therefore, corresponds precisely to the earlier failure of either Closure 2 or hindbrain neuropore closure.
In the spinal region, embryos displayed a defect in PNP closure. From around the 17-somite stage, hitchhiker mutants exhibited a significantly enlarged PNP, compared with stage-matched wild-type and heterozygous littermates (Fig. 3C–E). In wild-types and heterozygotes, the PNP remained open in only 25% of embryos by the 27 somite stage ($n = 4$ of 20), whereas 72% of hitchhiker mutants exhibited an open PNP at this stage ($n = 13$ of 18). These figures
correspond closely to the spina bifida observed in later embryos, both in the incidence (65%) and the region affected; at later stages, hitchhiker embryos exhibited an open neural tube corresponding to the region between somites 24 and 34. More caudally, the spinal neural tube appeared normal, suggesting that secondary neurulation proceeds unhindered.
Thus, in the spinal region as in the cranial region, we have demonstrated that neural tube defects occur as a primary defect in neural tube closure.

Previous detailed analysis of spinal neural tube closure has documented three ‘modes’ of closure, characterized by the precise morphology of the neural folds (6). Histological sections through the PNP revealed abnormal morphology in *hitchhiker* embryos. At the 24 somite stage, wild-type and heterozygous embryos exhibited prominent DLHPs, characteristic of mode 2 neurulation (Fig. 3F–H). In contrast,
**hitc**h**h**iker mutants exhibited a reduction in this dorso-lateral bending, with small DLHP seen only in the sections immediately adjacent to the closed neural tube and straight neural folds observed more caudally (Fig. 3I–K). The rates of cell proliferation and cell death in the neuroepithelium of the caudal neural tube at E9.5 were not significantly different between hitc**h**h**i**ker mutants and wild-type littermates (Fig. 3L–N and data not shown). These results suggest that the abnormal neural fold morphology may be causative for the delay in PNP closure in hitc**h**h**i**ker mutants.

hitc**h**h**i**ker mutants exhibit increased Shh pathway activity during spinal neural tube closure

To investigate the cause of the neuroepithelial defects, we examined the expression of Shh, a key molecule involved in the regulation of neural tube closure. Strong expression of Shh in the notochord represses DLHP formation during mode 1 neurulation, whereas weaker Shh activity occurs during mode 2 neurulation permits DLHPs to form (13). By whole mount in situ hybridization, Shh expression appeared comparable between hitc**h**h**i**ker mutants and wild-type embryos at E9.5 (Fig. 4A and B), and transverse sections through the caudal region revealed similar intensities of expression in the notochord (Fig. 4C and D). At this stage and axial level, Shh expression is not yet induced in the wild-type floor plate, and Shh is similarly absent from the hitc**h**h**i**ker floor plate (Fig. 4C and D). To investigate the activity of the Shh pathway, as opposed to the expression of Shh ligand, we examined the expression of the transcriptional target of Shh signalling, Ptc**h**1. Whole mount in situ hybridization revealed a dramatic up-regulation of Ptc**h**1 expression in the caudal region of the neural tube in hitc**h**h**i**ker mutants (Fig. 4E and F). Transverse sections through these embryos demonstrated an expanded domain of Ptc**h**1 expression throughout the DV extent of the caudal neural tube in hitc**h**h**i**ker, compared with wild-type (Fig. 4G and H). Quantitative analysis by real-time RT–PCR (qRT–PCR) detected a 1.65-fold increase in Ptc**h**1 expression in the caudal third of E9.5 embryos, compared with wild-type (Supplementary Material, Fig. S1A). In addition, a similar qRT–PCR analysis of Gli1, another transcriptional target of the Shh pathway, detected a 1.5-fold increase in Gli1 expression in hitc**h**h**i**ker (Supplementary Material, Fig. S1B; P < 0.05). These data suggest that the activity of the Shh pathway is increased in hitc**h**h**i**ker mutants, at E9.5, independently of a change in Shh expression. We suggest that this may inhibit the formation of DLHPs.

Further analysis at E10.5 revealed continued overexpression of Ptc**h**1 in hitc**h**h**i**ker mutants, compared with wild-type littermates. At this stage as at E9.5, the most dramatic up-regulation of Ptc**h**1 expression was observed in the caudal neural tube, but ectopic expression was also evident in the caudal somites (Fig. 4I–L). Although Shh expression looked normal at E9.5, by E10.5 Shh is ectopically expressed in hitc**h**h**i**ker mutants, in approximately one-third of the spinal cord anterior to the hindlimb bud (Fig. 4M and N; asterisks). Transverse sections through the lumbar neural tube demonstrated a dramatic increase in the Shh expression domain. Shh expression extends throughout the ventral half of the neural tube in hitc**h**h**i**ker mutants, whereas it is restricted to the floor plate in wild-type embryos (Fig. 4O and P). The spatial and temporal relationship between the onset of Ptc**h**1 and Shh overexpression suggest that in hitc**h**h**i**ker mutants, activation of the Shh pathway occurs independently of increased Shh ligand, which then subsequently (and indirectly) induces the ectopic expression of Shh within the neural tube.

hitc**h**h**i**ker mutants exhibit ventralization of the caudal spinal cord

Activity of the Shh pathway is a key regulator of DV patterning within the neural tube, and the observed changes in Ptc**h**1 and Shh expression in hitc**h**h**i**ker mutants prompted us to examine the expression of markers of DV patterning at E10.5. As anticipated from the expansion of Shh mRNA expression, we detected dorsal expansion of both Shh and Foxa2 protein expression, markers of floor plate, in the lumbar neural tube of hitc**h**h**i**ker mutants (Fig. 5A–D), although the region that exhibits the columnar morphology characteristic of floor plate appears to be unaltered. In addition, Nkx2.2, which marks the progenitors of the V3 ventral interneurons adjacent to the floor plate, and Nkx6.1, and Olig2 which mark other ventral and ventro-lateral neurons, are all expanded dorsally in hitc**h**h**i**ker mutant embryos (Fig. 5E–J). HB9/MNR2 and Isl1/2, markers of differentiating motorneurons, were also ectopically expressed in dorsally extended domains in hitc**h**h**i**ker mutants (Fig. 5K–N). Correspondingly, markers of dorso-lateral and dorsal neurons, Pax6, Pax7 and Msx, exhibited smaller expression domains in hitc**h**h**i**ker mutants, with a dorsal shift in their ventral limit of expression (Fig. 5O–T). Patterning of the neural tube at more cranial spinal levels was largely unaffected in hitc**h**h**i**ker mutants (Supplementary Material, Fig. S2). Thus, our data reveal ventralization of the caudal neural tube in hitc**h**h**i**ker mutants, consistent with Tulp3 acting as a negative regulator of the Shh pathway.

Genetic analysis reveals Shh pathway activation in hitc**h**h**i**ker occurs independently of Shh ligand

The expression changes observed at E9.5 suggested that up-regulation of the Shh pathway in hitc**h**h**i**ker mutants occurs independently of a change in Shh expression. We hypothesized that mutation of Tulp3 leads to loss of repression of the Shh pathway, downstream of Shh ligand. To test this genetically, we intercrossed hitc**h**h**i**ker with the Shh-targeted null allele (60). Doubly heterozygous (Shh/+; hhkr/+)) mice were viable and fertile, and intercrosses yielded E10.5 embryos with the expected Mendelian ratio of genotypes. Although hhkr homozygotes often exhibited exencephaly and caudal oedema (Fig. 6B), Shh null mutants were characterized by holoprosencephaly and reduced growth (Fig. 6C, compare with A), as described previously (60). Compound homozygous Shh/Shh;hhkr/hhkr embryos exhibited exencephaly, caudal oedema and spina bifida (Fig. 6D). These phenotypes are characteristic of hitc**h**h**i**ker and distinct from those of the Shh null, demonstrating that hitc**h**h**i**ker is epistatic to Shh.
ventralized phenotype of hhkr embryos (Fig. 6I–L). In Shh mutants, Nkx2.2, Nkx6.1, Olig2, HB9 and Islet1 are absent while Pax6, Pax7 and Msx are expanded ventrally (Fig. 6M–P and data not shown), as reported previously (45). In the Shh/Shh;hhkr/hhkr double mutants, Nkx2.2, Nkx6.1, Olig2, HB9 and Islet1 were expressed throughout a dorsally extended domain in double mutants, in a pattern similar to hhkr (Fig. 6Q–S and data not shown). The ventral limits of Pax6, Pax7 and Msx expression domains were shifted dorsally (Fig. 6T and data not shown), similar to hitchhiker. Thus, doubly homozygous Shh/Shh;hhkr/hhkr embryos revealed ventralization of the neural tube similar to that in hitchhiker mutants, and dissimilar to the dorsalization seen in Shh mutants. In addition, analysis of Ptch1 and Gli1 expression demonstrated increased expression in Shh/Shh;hhkr/hhkr embryos, similar to that observed in hhkr (Supplementary Material, Fig. S3), and in striking contrast to the absence of Ptch1 expression in Shh mutant embryos. These data further support our conclusion that Tulp3 acts downstream of Shh, and indicates that mutation of Tulp3 leads to activation of Shh pathway targets in the absence of Shh ligand.

**hitchhiker is genetically epistatic to Smoothened**

Since the hedgehog pathway can be activated by ligands other than Shh, we sought to test whether the pathway activation observed in hitchhiker mutants could be caused by increased expression of another ligand, such as Indian hedgehog or Desert hedgehog. Immunostaining with the 5E1 antibody that was raised against Shh (61), but which detects all hedgehog ligands (62), detected no observable signal in the notochord, neural tube or adjacent tissue of hitchhiker embryos, and transverse sections reveal expression is confined to the notochord (C and D). (E–H) Ptch1 expression at E9.5 is increased in hitchhiker mutants (F) compared with wild-type (E), in the caudal half of the embryo. Transverse sections demonstrate up-regulated and expanded expression in the caudal neural tube of mutant (H) compared with wild-type (G). (I–L) At E9.5, Shh expression is comparable in wild-type (I and K). (M–P) At E10.5, ectopic Shh expression is evident in hitchhiker embryos along approximately one-third of the spinal cord, anterior to the hindlimb (N, asterisks, compare with M). Transverse sections reveal greatly expanded Shh expression domain within the caudal neural tube of hitchhiker (P) compared with wild-type (O). Scale bars: A, B, E, F, I, J, M and N: 1 mm; C, D, G, H, K, L, O, P: 100 μm.

To investigate this further, we assessed the DV patterning in the caudal neural tube of double mutant embryos. Immunostaining on transverse sections through the caudal spinal cord of Shh mutants revealed a dorsalized phenotype, distinct from both the wild-type expression pattern (Fig. 6E–H) and
**hitchhiker** limb buds exhibit ectopic anterior activation of Shh signalling and a genetic interaction with Shh and Gli3

The **hitchhiker** mutants exhibit preaxial polydactyly, and we hypothesized that this might be caused by ectopic activation of the Shh pathway, similar to that observed in the neural tube. To address this, we examined the expression of targets of the Shh pathway in the developing limb buds. *Shh* expression appeared normal in **hitchhiker** limb buds, with a single domain of expression in the ZPA at E11.0 (Fig. 8A). In contrast, ectopic expression of *Ptch1* was observed in the anterior region of mutant limb buds at E11.0, whereas expression appeared normal in the posterior region (Fig. 8B). We also observed ectopic expression of *Gli1* in the anterior domain (Fig. 8C), although this was less robust than for *Ptch1*, and *Hoxd13* (Fig. 8D) another target of Shh pathway activation. Thus, **hitchhiker** mutants show normal expression patterns of markers in the posterior region of the limb buds but appear to have ectopic activation of Shh pathway targets in the anterior region of the limb, in the absence of ectopic Shh ligand.

To determine whether Tulp3 acts downstream of Shh in the limb (as well as in the neural tube), we examined limb morphology in *Shh/Shh;hhkr/hhkr* double mutants. Although **hitchhiker** mutants exhibited preaxial polydactyly with six digits on both forelimbs and hindlimbs (Fig. 9B, compare with A), *Shh* mutants are characterized by shortened and narrowed limbs bearing only a single digit (Fig. 9C), as described previously (64). The *Shh/Shh;hhkr/hhkr* double homozygotes reproducibly demonstrated an intermediate phenotype, with two digits on the forelimbs and four digits on hindlimbs (Fig. 9D). Although the limbs of double homozygotes do not resemble either single mutant, the mutation of Tulp3 can partially rescue the severe limb defects of *Shh* mutants, consistent with Tulp3 acting genetically downstream of Shh.
The downstream effectors of the Shh pathway are the three Gli-family transcription factors, with Gli3 playing the major role in limb patterning (30–32). To investigate the relationship between Gli3 and Tulp3 genetically, we intercrossed hitchhiker mutants with mice heterozygous for the Gli3 null allele, Gli3<sup>−/−</sup> (65). Doubly heterozygous hhkr/+;Gli3/+ mice were viable and fertile, and intercrosses yielded embryos of all the expected genotypes except for surviving doubly homozygous mutants, at E15.5. Analysis of digit number in the different genotypic classes revealed an additive genetic interaction between hhkr and Gli3 (Table 1). Heterozygous hitchhiker mice exhibited normal limbs (Fig. 9F, compare with E), whereas Gli3/+ fetuses exhibited an extra preaxial digit (Fig. 9G). The hhkr/+;Gli3/+ double heterozygotes appeared almost phenotypically indistinguishable to single Gli3/+ heterozygotes, with most limbs exhibiting a single extra digit (Fig. 9H). The hhkr/hhkr single homozygotes exhibited preaxial polydactyly with six or seven digits (Fig. 9I). Introduction of one Gli3 mutant allele onto the homozygous hhkr/hhkr genotype appears to exacerbate the hitchhiker phenotype, with more severe polydactyly than

Figure 6. Genetic analysis of epistasis places Tulp3 downstream of Shh. (A–D) E10.5 embryos generated from intercrossing Shh/+;hhkr/+ doubly heterozygotes, demonstrating phenotypes of wild-type (A), hitchhiker (B), Shh (C) and Shh/Shh;hhkr/hhkr doubly homozygous (D) embryos. Double mutants grossly resemble hitchhiker. (E–T) Immunostaining of transverse sections through the neural tube immediately anterior to the hindlimb bud with antibodies against Nkx2.2 (E, I, M and Q); Nkx6.1 (F, J, N and R); HB9/MNR2 (G, K, O and S) and Pax6 (H, L, P and T) in wild-type (E–H), hitchhiker (I–L), Shh (M–P) and Shh/Shh;hhkr/hhkr doubly homozygous (Q–T) embryos. Immunostaining of double mutants is similar to hitchhiker. Scale bar: A–D: 2.5 mm; E–T: 300 μm.
observed in either hhkr/hhkr single mutants or Gli3/+ heterozygotes, with often seven or eight digits (Fig. 9J; compare with I and G; Table 1). Homozygous Gli3/Gli3 embryos exhibited severe polysyndactyly with often seven to nine digits (Fig. 9K), as described previously (65). Introduction of one hhkr mutant allele onto the Gli3/Gli3 genotype (hhkr/+:Gli3/Gli3) appears to make no difference to the limb defect, which is almost indistinguishable to that observed in Gli3/Gli3 single mutants, with seven to nine digits formed (Fig. 9L, compare with K; Table 1). Doubly homozygous hhkr/hhkr;Gli3/Gli3 embryos did not survive to E15.5. Attempts to collect embryos at E12.5 yielded only one double mutant in which limb morphology was abnormal, but retarded development meant digit number could not be reliably ascertained. The absence of doubly homozygous mutant data precludes a formal assessment of epistasis. However, the analysis of intermediate genotypes demonstrates that there is a genetic interaction between the two loci, and suggests that Tulp3 and Gli3 may act in concert during limb morphogenesis.

Figure 7. Ventralization of the spinal cord in hitchhiker occurs independently of Smo. (A and B) Immunostaining of wild-type and Shh/Shh;hhkr/hhkr double homozygous embryos with 5E1 antibody to detect all Hedgehog ligands. (C–F) E10.5 embryos generated from intercrossing Smo/+;hhkr/+ double heterozygotes, demonstrating phenotypes of wild-type (C), hitchhiker (D), Smo (E) and Smo/Smo;hhkr/hhkr doubly homozygous (F) embryos. Double mutants more closely resemble hitchhiker than Smo. (G–V) Immunostaining of transverse sections through the neural tube immediately anterior to the hindlimb bud with antibodies against Nkx2.2 (G, K, O and S), Islet1 (H, L, P and T), Pax6 (I, M, Q and U) and Pax7 (J, N, R and V) in wild-type (G–J), hitchhiker (K–N), Smo (O–R) and Smo/Smo;hhkr/hhkr (S–V) doubly homozygous embryos. Scale bar: A and B: 500 μm, C–F: 2.5 mm; G–V: 300 μm.
Shh pathway activity is mediated by the stimulation of Gli activators and inhibition of Gli repressors. Since we have demonstrated that Tulp3 acts genetically downstream of Shh and Smo, and genetically interacts with Gli3, we sought to determine whether Gli2 and Gli3 expression are altered in **hitchhiker** mutants. By qRT–PCR, the expression of Gli2 and Gli3 was not detectably altered in the caudal end of E9.5 mutant embryos, compared with wild-type (Fig. 10A). Other mutants that affect Shh signalling demonstrate dramatic differences in Gli3 processing, as seen, for example, in the *ift52*, *ift57*, *ift88/polaris*, *ift172* and *kf3a* mutants (66–69), at least one mutant that affects retrograde intraflagellar transport results in activation of the Shh pathway (42). This prompted us to examine cilial morphology in **hitchhiker** mutants. Scanning electron microscopy (SEM) on limb buds from E11.5 embryos revealed the presence of cilia on both wild-type and **hitchhiker** mutant cells, with no overt difference in their length or morphology (Fig. 11D–I). Immunofluorescence with anti-acetylated tubulin and anti-polaris on cultures of mouse embryonic fibroblasts or limb bud cells confirmed the presence of cilia on both **hitchhiker** mutants and wild-types (Fig. 11C). We conclude that Tulp3 does not regulate the expression of *rab23*, *fkbp8*, *thm1* or Sufu, nor the activity of PKA.

Recent evidence indicates that cilia play a major role in Shh pathway activity. Although most mutants affecting cilial morphology or function lead to a decrease in Shh pathway function, such as seen in the *ift52*, *ift57*, *ift88/polaris*, *ift172* and *kf3a* mutants (66–69,72,73), at least one mutant that affects retrograde intraflagellar transport results in activation of the Shh pathway (42). This prompted us to examine cilial morphology in **hitchhiker** mutants. Scanning electron microscopy (SEM) on limb buds from E11.5 embryos revealed the presence of cilia on both wild-type and **hitchhiker** mutant cells, with no overt difference in their length or morphology (Fig. 11D–I). Immunofluorescence with anti-acetylated tubulin and anti-polaris on cultures of mouse embryonic fibroblasts or limb bud cells confirmed the presence of cilia on both **hitchhiker** mutants and wild-type cells with no detectable difference in polaris distribution (Fig. 11J–M and data not shown). Thus, no gross defect in cilial morphology was observed in **hitchhiker** homozygotes.

**DISCUSSION**

In this study, we have identified a novel ENU-induced strongly hypomorphic allele of Tulp3 which exhibits ventralization of the caudal neural tube, neural tube defects and polydactyly. We demonstrate ectopic expression of Shh pathway targets, independently of a change in Shh expression, and show...
Shh signalling in the observed increased/ectopic expression of genes activated by novel components of the Shh pathway (45,47,74). First, we results, which are the established criteria for identifying this conclusion is based on three sets of experimental demonstrated that Tulp3 functions as a negative regulator of requirement for Tulp3 in neural tube closure (58); however, Tulp3 has not previously been associated with DV patterning hitchhiker mutants. Neural tube patterning involves both the roof plate and surface ectoderm, with modulation of patterning ventralizing signal of Shh from the notochord and floor plate, and the dorsalizing signal of BMPs and Wnts from the expression patterns are not identical between double mutants expressing independently of Shh and Smo. However, the expression patterns are not identical between double mutants and hitchhiker homoyzogotes. Nkx2.2 and Islet1 are expressed throughout the most ventral (floor plate) domain in double mutants, whereas they are excluded from this region in wild-type and hitchhiker homoyzogotes. Foxa2 is expressed in an expanded domain in hitchhiker homoyzogotes, but appears to be absent from double mutants (Supplementary Material, Fig. S3). In addition, low-intensity Pax6 expression was evident throughout the ventral neural tube of Shh/Shh;hhkr/hhkr mutants, while it is excluded from this region in wild-types and hitchhiker homoyzogotes. These results indicate that the activation of Foxa2 expression and the repression of Nkx2.2, Islet1 and Pax6 from the ventral domains remain
dependent on functional Shh and Smo activity. Foxa2 induction and Nkx2.2, Islet1 and Pax6 repression require very high levels of Shh activity (84–86), suggesting that down-regulation of Tulp3 in hitchhiker mutants is not sufficient to induce high-level activation of the pathway. A similar situation seems to occur in the developing limbs, whereby the intermediate phenotype of double mutants suggests that, in the absence of Shh, mutation of Tulp3 is not sufficient to induce full activation of the Shh pathway. Intriguingly, Shh+/−;Gli3+/+ mice typically form limbs with two to four digits (31), similar to the phenotype observed in Shh/−/−;hhkr/hkr double homozygotes. Although Tulp3 mutation can lead to activation of some markers in a Shh-independent manner, the level of Shh pathway activation does not reach the highest levels that normally occur and, therefore, some residual Shh-dependent patterning remains.

Some regulators of Shh activity are also transcriptional targets of the Shh pathway. By western blotting, we found no substantial change in the abundance of Tulp3 protein in Shh, Smo or Gli3 mutants (data not shown), suggesting that Tulp3 is not itself a transcriptional target of the Shh pathway. Involvement of Tulp3 as a negative regulator of the Shh pathway suggests that down-regulation of Tulp3 activity might be required for Shh signalling. Tulp3 has several putative sites for phosphorylation, myristoylation and glycosylation (87). It is possible that regulation of Tulp3 activity might occur through effects on post-translational modification.

Possible functions of Tulp3 in the Shh pathway

The tubby family consists of four proteins, Tubby and Tulp1–3, which are characterized by the C-terminal ‘tubby’ domain (88,89). Disruption of other tubby proteins has been associated with a range of phenotypes, including retinal and cochlear degeneration following progressive cell apoptosis, and maturity-onset obesity (90–94). No tubby member has previously been linked to Shh signalling.

We have demonstrated that hitchhiker mutants exhibit a phenotype consistent with Tulp3 acting as a negative regulator of Shh signalling, and we have positioned Tulp3 genetically downstream of Shh and Smo. Many regulators of the Shh pathway have recently been discovered, and several have been shown to act genetically downstream of Shh and Smo. These include proteins involved in the formation or maintenance of cilia and intraflagellar transport, such as Arl13b/hennin, IFT88/polaris/flexo, IFT172/wimple, Kif3a, Rpgrip1l/Fantom and Thm1 (38,42,66,69,72,95). Disruption of IFT or cilia usually results in reduced Shh pathway activity in the neural tube, evidenced by the absence of Shh-dependent ventral cell types, as seen in mutants for IFT88/polaris/flexo, IFT172/wimple, Kif3a, Rpgrip1l/Fantom, Dyn2h1/Dnchc2, IFT52/Ngd5, IFT57/hippi and Ofd1 (67,68,72,96,97). However, mutation of Arl13b/hennin results in constitutive activation of the Shh pathway at an intermediate level (95), whereas loss of Thm1 causes increased Shh activity and expansion of ventral markers (42). The Gli-mediated transcriptional response to Shh and the effects of the negative regulators Sufu and PKA are abrogated in cells mutant for IFT components (98). The negative regulators Rab23 and Fkbp8 function genetically between Shh/Smo and Gli2 (45,47,99,100), and their activity is dependent on functional IFT (66,100). Rab23 is a member of the Rab-GTPase family of vesicular transport proteins and may regulate vesicular transport within (or dependent on) cilia, although the targets of Rab23 trafficking have not been identified (55,99,101). Fkbp8 is a member of the FK506-binding protein family (immunophilins), and may also play a role in membrane-associated proteasome function and trafficking (56,102).

The importance of cilia is further underlined by the finding that several Shh pathway components are localized within the cilia, including Smo, Ptc1, Sufu and Gli proteins (38–41,67,98). Smo becomes enriched in cilia following treatment of cells with Shh (39,40,98), and this ciliary localization is necessary (39) but not sufficient (98) for pathway activation. Tulp3 protein can be detected at the tips of cilia (37), and evidence from other members of the tubby family suggests that Tulp3 might play a role in vesicular trafficking. Tulp1 interacts with several proteins implicated in the formation and movement of vesicles including F-actin, dynamin-1, clathrin heavy chain, dynein intermediate chain and tubulin (103,104). Tulp1 mutants exhibit accumulation of rhodopsin-containing vesicles in the interphotoreceptor matrix, consistent with loss of polarized trafficking between photoreceptor segments (91,105). However, examination of ciliary morphology in hitchhiker mutants has detected no overt cilial defect, whereas immunofluorescence studies revealed no apparent difference in localization of polaris/IFT88. Thus, there is no evidence to suggest that ciliary morphology or transport is grossly disrupted in hitchhiker mutants, although more subtle structural or functional defects would not have been detected.

An alternative hypothesis for the role of Tulp3 is as a transcriptional regulator. The tubby proteins are characterized by the C-terminal ‘tubby’ domain that binds to double-stranded DNA, while the N-terminal region can function as a
Figure 10. Gli2 and Gli3 expression and Gli3 processing are not altered in hitchhiker mutants. (A) Quantitative RT–PCR analysis with primers for Gli2 and Gli3 on RNA from caudal ends of E9.5 wild-type, heterozygous and hitchhiker mutant embryos, showing no change in expression. (B) Western blot analysis on total cell lysates from E9.5 limb buds demonstrate no apparent change in abundance of the full length 190 kDa Gli3 isoform (Gli3FL) nor the 83 kDa cleaved repressor isoform (Gli3R), in hitchhiker embryos compared with wild-type or heterozygous littermates. Western blots with extracts from Gli3+/+ and Gli3/Gli3 mutants confirm correct Gli3 band identity; β-Tubulin was used as a loading control. (C) Western blots with nuclear extracts from E10.5 caudal ends demonstrate similar abundance of the 190 and 83 kDa Gli3 isoforms in hitchhiker embryos compared with wild-type or heterozygous littermates; histone H1 was used as a loading control.

Figure 11. Evaluation of changes in downstream molecules and cilia structure. (A) Quantitative RT–PCR analysis with primers for Fkbp8, Rab23 and Thm1 (Ttc21b) on RNA from caudal ends of E9.5 wild-type, heterozygous and hitchhiker mutant embryos, showing no significant change in expression. (B) Western blot analysis on total cell lysates from E10.5 limb buds for Sufu expression revealed no change in hitchhiker mutants; fatty acid synthase (Fas) was used as a loading control. (C) PKA activity with or without cAMP addition, in protein extracts from E9.5 caudal ends of wild-type and hitchhiker embryos; no significant difference is detected. (D–I) SEM of E11.5 forelimb buds from wild-type (D–F) and hitchhiker mutant (G–I) embryos revealing cilia of normal length and morphology. (J–M) Immunostaining of MEFs from wild-type (J and K) and hitchhiker embryos (L and M) with anti-acetylated tubulin (green), anti-polaris (red) antibodies and DAPI (blue) demonstrate similar staining in mutants and wild-type cilia. Scale bars: 2 µm.
Kif3a mutants for sing and degradation of Gli2 (111). Many mutants that negative modulators of Shh signalling.

tor, we have excluded transcriptional modulation of the known Gli1 and Gli2, independently of cytoplasmic sequestration tors. Sufu can inhibit the transcriptional activator function of activity or may affect the interaction of Gli with other regular

nucleus, suggesting that Tulp3 does not act to regulate entry we observed no alteration in abundance of Gli3 within the not associated with Gli expression or processing. In addition, we have shown that expression of Rab23, Fkhp8, Thml, Gli3 and Sufu and activity of PKA are not detectably altered in hitchhiker mutants. Thus, although we cannot rule out a transcriptional role for Tulp3 on some other yet undiscovered negative regulator, we have excluded transcriptional modulation of the known negative modulators of Sh signalling.

Modulation of Gli protein activity is the downstream consequence of the Shh pathway, since the presence of Shh ligand acts to inhibit cleavage of Gli3 (30) and suppress the processing and degradation of Gli2 (111). Many mutants that exhibit down-regulation of the Shh pathway show impaired Gli3 processing, with a decrease in the amount of Gli3 repressor relative to the amount of full-length Gli3 protein, as seen in mutants for Dyn2h1/Dnchc2, IFT88/polaris, IFT172/wimple, Kif3a, Rgrip11/fantom and the chicken talpid3 mutant (38,67–69,72,73,112). Gli3 processing is also disrupted in the Thml mutant, which has activation of Sh signalling (42). The negative regulator Sufu binds to the Gli proteins (113,114) and may act in part by sequestering Gli1 and Gli2 in the cytoplasm (49,53,114,115), a function that involves CRM1-dependent nuclear export of Gli1 but not Gli2 (54). We have shown that Tulp3 genetically interacts with Gli3 in regulating limb development. However, we observed no change in the expression levels of Gli2 or Gli3, and we detected no substantial change in the abundance of the Gli3 isoforms in hitchhiker. This suggests that Tulp3 function is not associated with Gli expression or processing. In addition, we observed no alteration in abundance of Gli3 within the nucleus, suggesting that Tulp3 does not act to regulate entry into the nucleus.

It is possible that Tulp3 may function as a co-factor in Gli activity or may affect the interaction of Gli with other regulators. Sufu can inhibit the transcriptional activator function of Gli1 and Gli2, independently of cytoplasmic sequestration (54). Sufu mutant MEFs exhibit constitutive Gli activity yet do not show accumulation of Gli1 in the nucleus (50). Sufu interacts with SAP18, Galectin3 and other nuclear proteins (116) as well as with Gli1 bound to DNA (53), and Sufu represses Gli-mediated transcription by recruiting the histone deacetylation machinery through interaction with SAP18 (117). Although we detected no change in Sufu expression level, it is possible that loss of Tulp3 might affect the interaction of Sufu with Gli; recent data have revealed that Cdc21 can act as a pathway activator, through binding to Sufu and relieving its inhibition on Gli (118). Alternatively, Tulp3 might act to regulate the expression or activity of other factors that regulate Gli protein function, such as MED12 (119), MIM/BEG4 (120), CBP (81), Ski (121), Dyrk1 (122), Dyrk2 or MAP3K10 (123). The precise functional and molecular relationship between Gli3 and Tulp3 requires further analysis.

It is intriguing that the hitchhiker limb buds exhibit a localized domain of ectopic gene expression, at the anterior limit of the limb buds. This is distinct from the ectopic expression patterns observed in other mutants, in which there is an anterior expansion of the normal expression domains. Many other mutants with polydactyly have impaired processing of Gli3, and this may cause the limb defect. It is possible that Tulp3 acts in regulation of specific targets of Gli3 function, which then subsequently results in the region-specific activation of other molecules. Microarray analysis of transcriptional changes in Gli3 mutant limb buds identified a number of targets, including some that are expressed in localized anterior domains and which are down-regulated in Gli3 mutants; these include Pax9, Cdo and Id2 and novel transcript BG074838 (124). We might hypothesize that Tulp3 has a role in co-regulation of specific Gli3 targets, resulting in loss of an anterior-specific factor, rather than a general change in Gli3 effects in the limb.

hitchhiker mutants exhibit spina bifida as a result of a failure of neural tube closure

We have demonstrated that spina bifida in hitchhiker results from a primary failure of spinal neural tube closure. Closure of the PNP occurs with three ‘modes’ of closure, at different gestational ages and, therefore, at different axial levels, typified by the formation of MHPs (mode 1), DLHPs (mode 3) or both (mode 2) (6). The regulation of DLHP formation is modulated by Sh signalling, with strong Sh expression from the notochord during mode 1 neurulation inhibiting DLHPs (12), through the noggin-mediated antagonism of Bmp signalling (13). Formation of DLHPs is critical for spinal closure, and failure to form DLHPs results in severe spina bifida as seen in the Zic2 mutant (13). In hitchhiker mutants, the formation of DLHPs appears to be partially suppressed, although they are not inhibited completely, and correspondingly, the spina bifida in hitchhiker is less severe than observed in Zic2 mutants. We have demonstrated increased activation of the Shh pathway at the caudal end of hitchhiker mutants, and this would be anticipated to repress DLHP formation. Indeed, the loss of other negative regulators of the Shh pathway can also result in spina bifida, including Sufu, Rab23, PKA and Ptc1 (45,46,48,109). However, we also observed defects in DV patterning of the neuroepithelium at E9.5 (data not shown). It is likely that PNP closure is inhibited by a combination of these effects.

Regional differences in the role of Tulp3

Our data reveal a role for Tulp3 in DV patterning of the neural tube, with a more important role in caudal than cranial regions of the spinal cord. Tulp3 is expressed ubiquitously (58),
suggesting that the AP difference is not attributable to differential expression. It is notable that the role of other molecules in DV patterning also varies along the AP axis. For example, mutation of Rab23, Fkbp8 or PKA-deficiency affect only caudal spinal cord patterning (45–47,110), whereas the Dyn2h1/Dnchc2 mutant exhibits a more severe patterning disruption in rostral than caudal spinal cord (72) and retinoic acid modulates patterning in the rostral spinal cord of quail embryos (125). Smo−/−;Gli3−/− embryos show reduced rescue of the ventral markers more caudally, and this is taken to indicate an AP difference in the requirement for Hh/Gli3 interactions in the specification of similar cell identities (75). The molecular basis of the regional differences and the relationship between the pathway regulators has yet to be fully elucidated.

The data presented here led to similar conclusions as a study performed with the Tulp3-targeted allele (Norman et al., submitted). However, there are minor variations between the phenotypes, such as the absence of Foxa2 expression in the (72) and retinoic acid modulates patterning in the rostral spinal cord of quail embryos (125). Smo−/−;Gli3−/− embryos show reduced rescue of the ventral markers more caudally, and this is taken to indicate an AP difference in the requirement for Hh/Gli3 interactions in the specification of similar cell identities (75). The molecular basis of the regional differences and the relationship between the pathway regulators has yet to be fully elucidated.

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The data presented here led to similar conclusions as a study performed with the Tulp3-targeted allele (Norman et al., submitted). However, there are minor variations between the phenotypes, such as the absence of Foxa2 expression in the Shh/Shh;hhkr/hhkr double mutant, compared with expression of this floor plate marker in double mutants with the Tulp3 null allele, and no significant change in proliferation in the caudal neural tube of hitchhiker, compared with decreased proliferation in the Tulp3 knockout. These qualitative differences likely reflect the difference in abundance of Tulp3 protein, in the two alleles, since the targeted allele is likely to be a true null, while we have demonstrated in hitchhiker that a small amount (~4%) of wild-type protein remains.

In conclusion, correct regulation of the activity of the Shh pathway is essential for normal development and continued health in adults. Misregulation during embryonic development generates a spectrum of congenital abnormalities, including holoprosencephaly, neural tube defects and polydactyly. The pathway is essential for normal development and continued identification of new components of the Shh pathway, such as Tulp3, coupled with further research to understand their molecular function, holds significant promise for the diagnosis and treatment of human disease.

**MATERIALS AND METHODS**

**Mice and embryos**

*hitchhiker* was identified during a three-generation (G3) recessive mutagenesis screen (details to be described elsewhere). Briefly, C57BL/6 males were injected with ENU and out-crossed to C3H/HeH; male F1 offspring were mated to C3H/HeH and F2 females backcrossed to their father. G3 embryos were examined at E13.5 for developmental abnormalities. Affected fetuses were used for genetic mapping with a 55-marker genome-wide SNP panel (sequences available on request), and additional microsatellite markers. The mutant line was maintained by backcrossing to C3H/HeH and, following gene identification, genotyped for the mutation by pyrosequencing. The MGI accession ID for this allele is MGI:3662473. Mice heterozygous for *Shh*^<sup>tm1Chg</sup> (60) or *Smo*^<sup>tm1Ame</sup> (63) were maintained on 129S6/SvEv and genotyped by PCR (N. Warr, personal communication). Mice heterozygous for the Tulp3<sup>tm1Jng</sup>-targeted allele (58) on C3H/HeJ were obtained from J. Eggenschwiler (Princeton University). Mice heterozygous for *Gli3* deletion (*Gli3<sup>ChJ/+</sup>) on a mixed C57BL/6/C3H background were obtained from T. Theil (Edinburgh University) and genotyped as described (126). Animals were maintained following guidelines of the Medical Research Council and in accordance with the Animals (Scientific Procedures) Act, 1986.

Mice were maintained routinely on a 12 h light–dark cycle (dark from 19:00 to 07:00 h). Embryos were generated by overnight matings, with the day of finding a copulation plug designated as embryonic day (E) 0.5. Some mice were maintained on a reverse light–dark cycle (dark from 10:00 to 22:00 h), and litters from these animals were designated as E1.0 on the day of plugging. Embryos were dissected in PBS with 10% newborn calf serum and processed according to downstream application.

**Sequencing**

RNA was extracted from E13.5 fetuses using GenElute (Sigma) and reverse transcribed with MMLV-RT (Invitrogen). DNA and cDNA were amplified with intron or exon-specific *Tulp3* primers (sequences available on request), purified using Qiaquick (Qiagen) then sequenced with BigDye reagent (ABI) and ABI3700.

**Histology and in situ hybridization**

Histology and skeletal preparations used standard protocols. Wholomount *in situ* hybridization was performed essentially as described (127), using digoxigenin-labelled riboprobes for *Shh*, *Ptc1*, *Gli1*, and *Hoxd13*. At least three embryos of each genotype were analysed with each probe and processed under identical conditions. Embryos were photographed on a Leica MZ16 stereomicroscope and vibratome sectioned at 35–50 μm as described (128). Slide *in situ* hybridization was performed using 10 μm cryosections as described elsewhere (129), with digoxigenin-labelled riboprobes for *Ptc1* and *Gli1*.

**Protein extraction, western blots and PKA assay**

Total cell lysates were generated in RIPA buffer (PBS with 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS). Nuclear fractions were generated using the Proteoextract Subcellular Fractionation kit (Calbiochem). Proteins were quantitated using the DC assay (Biorad). Westerns used 1–5 μg protein per lane on 7% or 3–8% Tris-Acetate NuPAGE gels with SeeBluePlus2 or HiMark ladders (Invitrogen). Proteins were transferred onto Hybond ECL (GE Healthcare) and detected with antibodies against Tulp3 (1:300; gift from J. Eggenschwiler), Gli3 (1:1000; Santa Cruz sc-20688), Sufu (1:200; sc-10933), β-tubulin (1:5000; sc9104), histone H1 (1:1000, sc-8030) or fatty acid synthase (1:1000; sc-55580) with HRP-conjugated secondary antibodies (1:12 000; DAKO) and detection with ECL Advance (GE Healthcare). Extracts from Gli3<sup>ChJ/XJ</sup> (65) and *Sufu*/−/− (50) mutants were used to verify Gli3 and Sufu band identity, respectively, and the antibodies used have been published previously (70,130). Bands were quantitated from scanned images using Adobe Photoshop CS2; data are expressed as means ± standard errors, and significance tested using two-sample equal
variance, two-tailed distribution Student’s t-test. PKA activity was measured on extracts from E9.5 caudal thirds using a non-radioactive assay (Calbiochem), following Bradford assay quantitation.

SEM, cell culture and immunofluorescence
Embryos were processed for SEM as described (38) and imaged on an Hitachi S-530 scanning electron microscope. Mouse embryonic fibroblast primary cultures were immunostained for N-acetylated tubulin (1:10 000; Sigma) or Polaris (1:1000; gift from B. Yoder) as described (38). Immunofluorescence on 10 μm cryosections was performed as described (131), using antibodies against Nkx2.2, Shh, Islet1, Msx1/2, Foxa2, HB9/MNR2, Nkx6.1, Pax6 and Pax7 (1:10; Developmental Studies Hybridoma Bank) and Olig2 (1:500, Abcam ab33427). Cell proliferation rates were determined by staining with antibody against phospho-Histone H3 (pHH3, Upstate Cell Signalling). Primary antibody staining was detected with appropriate secondary antibodies conjugated with AlexaFluor-488 or AlexaFluor-594 (1:250 dilution; Invitrogen), mounted in Vectashield with DAPI (Vector Laboratories) and imaged with a Zeiss Axiophot microscope. Quantitation of proliferation rates was performed by counting pHH3+ cells and total (DAPI+) cells within the caudal neuroepithelium of E9.5 (17–19 somite) embryos; the mitotic index was calculated as the average from 12 sections from each of six embryos of each genotype, with analysis from the region immediately posterior to the caudal extremity of the vitelline artery thus within the PNP.

Quantitative real-time RT–PCR
Total RNA was extracted from caudal thirds of E9.5 embryos and DNase treated using RNAeasy plus mini kit (Qiagen), quantified with a Nanodrop ND1000 and the integrity assessed by gel electrophoresis. Double-stranded cDNA was then synthesized from 1 μg RNA using High Capacity cDNA Archive kit (Applied Biosystems). Quantitative real-time PCR was performed with 10 ng cDNA using TaqMan® gene expression assays on a 7500 Fast Real-Time PCR System (Applied Biosystems). The assays used were: Mm00494645_m1 (Gli1); Mm00487418_m1 (Fkbp8); Mm01293116_m1 (Gli2); Mm00492333_m1 (Gli3); Mm01545399_m1 (Hprt); Mm00436209 (Rab23); Mm00436026 (Pch1) and Mm01270405 (Ttc21b). Samples were analysed in quadruplicate, using at least three embryos of each genotype, and calculations performed using the comparative C_T method. The values were normalized to the endogenous reference gene (Hprt) and the transcript levels are presented as fold change relative to the wild-type sample in relative quantification (RQ) units. Error bars indicate the calculated maximum (RQ_MAX) and minimum (RQ_MIN) expression levels, with a 95% confidence level. Statistical significance was tested using two-sample equal variance, two-tailed distribution Student’s t-test and is also represented as non-overlapping error bars if the samples are significantly different (P < 0.05).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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