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Expression of *ptc* and *gli* genes in *talpid*^{3} suggests bifurcation in Shh pathway*

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*This paper is dedicated to Peter Thorogood who tragically died last summer. Peter studied limb development in *talpid*^{3} for his PhD

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SUMMARY

***talpid*^{3} is an embryonic-lethal chicken mutation in a molecularly un-characterised autosomal gene. The recessive, pleiotropic phenotype includes polydactylous limbs with morphologically similar digits. Previous analysis established that *hox-D* and *bmp* genes, that are normally expressed posteriorly in the limb bud in response to a localised, posterior source of Sonic Hedgehog (Shh) are expressed symmetrically across the entire anteroposterior axis in *talpid*^{3} limb buds. In contrast, Shh expression itself is unaffected. Here we examine expression of *patched* (*ptc*), which encodes a component of the Shh receptor, and is probably itself a direct target of Shh signalling, to establish whether *talpid*^{3} acts in the Shh pathway. We find that *ptc* expression is significantly reduced in *talpid*^{3} embryos. We also demonstrate that *talpid*^{3} function is not required for Shh signal production but is required for normal response to Shh signals, implicating *talpid*^{3} in transduction of Shh signals in responding cells. Our analysis of expression of putative components of the Shh pathway, *gli1*, *gli3* and *coupTFII* shows that genes regulated by Shh are either ectopically expressed or no longer responsive to Shh signals in *talpid*^{3} limbs, suggesting possible bifurcation in the Shh pathway. We also describe genetic mapping of *gli1*, *ptc*, *shh* and *smoothened* in chickens and confirm by co-segregation analysis that none of these genes correspond to *talpid*^{3}.**

Key words: *talpid*, Hedgehog, Signalling, *patched*, *gli*, *coupTFII*, *gli3*

INTRODUCTION

The developing chick limb bud is a well-established model for investigating cellular and molecular basis of embryonic pattern formation. Extensive experimental analysis has elucidated aspects of patterning of all three limb axes, anteroposterior, proximodistal and dorsoventral, and shown that they are interdependent and act coordinately. In addition, limb signalling pathways are also used elsewhere and therefore investigations of limb development may illuminate understanding of patterning of other regions of the embryo (reviewed Johnson and Tabin, 1997; Cohn and Tickle, 1996).

Anteroposterior limb patterning is controlled by a group of mesenchymal cells in the posterior limb bud known as the polarising region. Polarising region grafts to the anterior of another limb bud produce mirror-image digit duplications (Saunders and Gasseling, 1968; Tickle et al., 1975). The polarising region expresses *shh*, a signalling molecule that has been implicated in many patterning processes in developing embryos (reviewed Hammerschmidt et al., 1997). Ectopic expression of *shh* at the anterior of a developing limb bud can also induce mirror-image duplications suggesting that Shh mediates patterning activity of the polarising region (Riddle et al., 1993). Several other potential components of anteroposterior patterning have been identified, either because of their asymmetrical expression across the anteroposterior axis of the limb bud or because of their involvement in the Shh signalling pathway. These include members of *bmp* and *hox* gene families (Izpisua-Belmonte et al., 1991; Francis et al., 1994; Francis-West et al., 1995). For example, *bmp2* and 5′ members of the *hoxD* complex (*hoxD-11*, *hoxD-12* and *hoxD-13*), normally expressed in posterior limb bud mesoderm, are ectopically expressed in anterior limb buds after *shh* mis-expression or polarising region grafts. However, this induction of *bmp* and *hox-D* gene expression by Shh also requires the apical ectodermal ridge (AER), which runs along the distal limb bud rim. This apical ridge requirement can be substituted for by FGF4 protein, which is encoded by a gene that is normally expressed in posterior ridge and is itself induced in response to *shh* (Laufer et al., 1994; Niswander et al., 1994). Therefore expression of these *bmp* and *hox* genes probably requires convergence of Shh and FGF signalling pathways.

Several components of the Hh pathway have been identified in both vertebrates and *Drosophila*. Gli proteins have been implicated as possible transcription factors for Hh pathways and Ptc proteins as receptors (reviewed Ingham, 1998a; Johnson and

Scott, 1998). In chick, two *gli* genes (*gli1* and *gli3*) and one *ptc* gene have been described (Marigo et al., 1996a,b). Note *gli3* may be more closely related to *gli2/4* of other vertebrates (Borycki et al., 1998); *gli1* was originally referred to as *gli* (Marigo et al., 1996b). Ptc is an unusual receptor as it is both a target and a repressor of the Hh pathway. Basal levels of *ptc* are expressed throughout domains competent to respond to Hh signals, where they repress transcription of Hh target genes. Hh signals antagonise this repression by binding to Ptc, one result of which is increased *ptc* expression (reviewed Ingham, 1998b). The *Drosophila* homologue of *gli* genes, *ci*, has been postulated to have both repressor and activator functions. In the absence of Hh signals, it represses expression of *dpp*, a *Drosophila bmp* homologue, and *hh* itself. However, in presence of Hh signals, Ci activates transcription of at least some targets of the Hh pathway including *ptc* and *dpp*. These different functions are mediated by different forms of Ci protein (Aza-Blanc et al., 1997; Dominguez et al., 1996; Alexandre et al., 1996). In vertebrates, different Gli proteins may fulfil these different functions. Analysis of mouse mutations suggests that Gli3 represses *shh* expression (Masuya et al., 1995; Buscher et al., 1997). Gli1 has been implicated as a transcriptional activator for the Shh pathway (Hynes et al., 1997; Marigo et al., 1996b; Lee et al., 1997; Sasaki et al., 1997). In addition to being components of the Shh pathway, all three genes (*gli1*, *gli3* and *ptc*) in vertebrates are targets for transcriptional control by Shh: *ptc* and *gli1* expression increases in response to Shh; *gli3* expression decreases (Marigo et al., 1996a,b). However, in contrast to induction of *bmp2* and 5′ *hoxD* genes, induction of *ptc* and *gli1* expression in the chick limb bud does not require apical ridge.

talpid^3 (*ta*^{3}) is an embryonic lethal chicken mutation in a molecularly uncharacterised autosomal gene. Homozygous embryos have a pleiotropic phenotype, but *ta*^{3} limbs buds are initially shorter and broader than normal and eventually about 7 digits form, all of which are fused and look morphologically similar (Ede and Kelly, 1964b; Hinchliffe and Ede, 1967). In *ta*^{3} the relationship between *shh* expression and expression of 5′ *hoxD* genes, *fgf-4*, *bmp2* and *bmp7* is uncoupled: normally posteriorly restricted *fgf-4*, *bmp* and *hoxD* genes are expressed symmetrically across the entire anteroposterior axis but *shh* expression is still restricted to posterior limb (Izpisua-Belmonte et al., 1992; Francis-West et al., 1995). *bmp4* expression, which is normally strong anteriorly, is reduced in *ta*^{3} anterior limb buds and symmetrical around the bud rim (Francis-West et al., 1995). This suggests that *ta*^{3} may act between *shh* transcription and expression of *bmp* and *hoxD* genes. This is consistent with the *ta*^{3} phenotype, which includes abnormalities in many other patterning processes where Shh signalling has been implicated, such as dorsal/ventral patterning of neural tube and somites, correct spacing of eyes and development of face and feathers (Ede and Kelly, 1964a,b). However, since it is not known whether *bmp* and 5′ *hoxD* genes are direct targets of Shh signalling or further downstream, it is unclear at which point in these developmental signalling cascades *ta*^{3} normally acts.

Here we investigate *ptc* expression in *ta*^{3}. *ptc* is a direct target of Hh signalling in *Drosophila* (Ingham, 1993; Forbes et al., 1993; Alexandre et al., 1996; Struhl et al., 1997; Strigini and Cohen, 1997) and recent analysis suggests that this is also the case in vertebrates (Goodrich et al., 1996, 1997; Marigo et al., 1996a; Concordet et al., 1996; Lewis et al., 1998). *ptc* expression in *ta*^{3} should show whether *ta*^{3} acts in the Shh pathway, or whether it acts downstream of, or in parallel to, Shh signalling. *ptc* is also a possible candidate for *ta*^{3}: since Ptc represses Hh pathways in absence of Hh signals, mutations in *ptc* usually result in ectopic activation of Hh pathways throughout embryonic fields that are competent to respond to Hh (Ingham et al., 1991; Ingham and Hidalgo, 1993; Capdevila et al., 1994; Goodrich et al., 1997). Therefore, a *ptc* mutation in chicks would be expected to have a similar phenotype to *ta*^{3}: normal *shh* expression but expanded expression of Shh targets, such as *bmp* and 5′ *hoxD* genes.

Our examination of *ptc* expression in *ta*^{3} suggested that *ta*^{3} acts between *shh* and transcription of *ptc*. Therefore, we examined expression of other components of the Shh pathway in *ta*^{3} embryos. We also investigated whether the *ta*^{3} defect was in signalling cells or responding cells by determining whether *ta*^{3} limbs could respond to Shh protein and whether *ta*^{3} mesoderm was capable of signalling to normal mesoderm. Finally, we genetically mapped several components of the Shh pathway and investigated whether *ta*^{3} corresponded to any of these.

MATERIALS AND METHODS

Embryos

Embryos were obtained from fertilised Needle Farm White Leghorn chicken eggs or from *talpid*^{3} (*ta*^{3}) stock maintained at Roslin Institute, UK. Eggs were incubated at 38°C: wild-type chick embryos were staged according to Hamburger and Hamilton (1951); and *ta*^{3} embryos according to Hinchliffe and Ede (1967). Homozygous *ta*^{3} embryos were identified by characteristic limb morphology and/or by reduced spacing of eyes. If there was any ambiguity, then identification was confirmed by grafting a wing onto the wing stump of a wild-type host to allow it to develop further so that the *ta*^{3} shape became more obvious, or by in situ hybridisation on one limb with a gene that has an altered pattern of expression in *ta*^{3}.

Embryo manipulations

Stock solutions of bacterially expressed aminoterminal Shh protein (N-Shh), a kind gift from A. McMahon (Yang et al., 1997), were stored at −70°C and dilutions made in Tris chloride/sodium chloride buffer. To prepare Shh beads, 2 μl of N-Shh solution (14 mg/ml) was placed on a bacteriological grade Petri dish. 15-20 Affigel CM beads (200-250 μm in diameter) were rinsed in Tris chloride/sodium chloride buffer, then transferred to the drop of N-Shh solution and allowed to soak for between 1 and 2 hours at room temperature.

Wing buds of stage 20 wild-type chicken hosts were removed using tungsten needles and replaced by manipulated or non-manipulated *ta*^{3} limb buds, which were kept in place with platinum staples. In the case of manipulated *ta*^{3} limb buds, a Shh bead was placed under a loop of apical ectodermal ridge at the anterior margin of each bud before grafting to its new host. For control experiments, the same procedures were repeated with wild-type chick limb buds. In another series of experiments, posterior, apical and anterior mesenchyme was removed from *ta*^{3} limb buds and grafted to a host wing under the anterior apical ridge (Fig. 1). In all these cases, limb bud tissue was first soaked for 1 hour in trypsin at 4°C and the ectoderm removed. Host embryos were fixed in 4% paraformaldehyde (PFA) at different time intervals after operations for whole-mount in situ hybridisation.

In situ hybridisation

Embryos were processed according to standard methods (Riddle et al., 1993; Francis-West et al., 1995); precise protocol available on request. Probes were synthesised using the following templates: *ptc* (Marigo et al., 1996a), *gli1* (Marigo et al., 1996b), *gli3* (Marigo et al.,

1996b), *coupTFII* (Lutz et al., 1994), *bmp-2* (Francis et al., 1994) and *shh* (Cohn et al., 1995).

Genetic mapping

Genetic loci were mapped in the East Lansing reference back cross (Burt and Cheng, 1998; Jones et al., 1997). Mapping information has been submitted to chicken genome database at Roslin Institute, UK (http://www.ri.bbsrc.ac.uk/).

PCR-SSCP analysis

The following PCR primer pairs were designed for chicken *shh*, *ptc*, *gli1* and *smo* genes using the MIT PRIMER program.

shh (ROS0101)

553 bp product (GenBank L28099, nucleotides 879-1431); digested with *Pvu*II (at position 1171) to produce 293 bp and 260 bp products, which were verified by DNA sequencing.

#338 5′-TGAAGGACCTGAGCCCTG-3′ (879-906)
#355 5′-AGGAGCCGTGAGTACCAATG-3′ (1412-1431)

Position of intron-exon boundaries predicted from structure of *Drosophila hh* gene (L02793).

ptc (ROS0103)

218 bp (GenBank U40074, exon 22, nucleotides 4096-4313) product verified by digestion with internal *Ava*II site (at position 4233) to produce 138 bp and 80 bp DNA fragments.

#385 5′-TGCCAGCCTATCACTACTGTG-3′ (4096-4116)
#386 5′-CATTCGACATCCTGAAGCTC-3′ (4294-4313)

Position of intron-exon boundaries predicted from structure of human *ptc* (U43148) gene.

gli1 (ROS0109)

144 bp product (GenBank U60762, nucleotides 670-813) verified by digestion with internal *Pvu*II site (at position 708) to produce 104 bp and 40 bp DNA fragments.

#457 5′-GTACCAGAACCCTCCGGG -3′ (670-687)
#458 5′-TGTTTGATGGTCCCACGAG-3′ (795-813)

smo (ROS0114)

223 bp product (GenBank AF019977, 3′-untranslated region, nucleotides 2817-3039) verified by digestion with internal *Hin*dIII site (at position 2867) to produce 51 bp and 172 bp DNA fragments.

#467 5′-ATCCCAAAGTGCCTTCCAG-3′ (2817-2835)
#468 5′-GAGGAAGCCGTGAGCCTAC-3′ (3021-3039)

50 ng of chicken genomic DNA was amplified in 50 μl containing 200 mM dNTPs, 500 nM primers, 2 units Taq Gold DNA polymerase, 20 mM Tris-HCl (pH 8.3), 50 mM KCl and 0.75 mM $MgCl_2$. Thermal cycling conditions: initial denaturation step at 94°C for 1 minute, then 30 cycles of 94°C for 30 seconds, 65°C for 1 minute, 72°C for 3 minutes, followed by 72°C for 10 minutes. Aliquots (15 μl) of PCR products were mixed with equal volumes of formamide loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanolFF), heated to 99°C for 3 minutes and placed on ice prior to electrophoresis. Samples were run on 16 cm native 15% polyacrylamide gels (37.5:1) for 16 hours at 12 mA at 15°C and gels were silver stained as described previously (Jones et al., 1997).

RESULTS

ptc expression in *ta*^{3} embryos

As previously described (Francis-West et al., 1995), *shh* is expressed normally in *ta*^{3} limbs (confirmed here for 7 legs, 3 wings; Fig. 2B). In contrast, *ptc* expression is clearly altered in *ta*^{3} limb buds: in wild-type chick limbs, *ptc* is expressed at high levels posteriorly with expression gradually decreasing anteriorly (Fig. 2C; strongly stained wing), whereas in *ta*^{3} limbs, high levels of *ptc* expression are never detected (0/19, 11 wings; 8 legs). At stages 19-21, *ptc* is expressed uniformly at very low levels throughout *ta*^{3} limb mesenchyme except immediately under the AER (Fig. 2D). This resembles the level of expression in the very anterior of wild-type limbs (compare Fig. 2C and D). In *ta*^{3} limbs at stage 27, *ptc* expression is still very weak but has resolved into a wide distal band and more medial proximal band that resemble the fused mesenchymal condensations that form in *ta*^{3} limbs (Hinchliffe and Ede, 1967; 2 wings; 2 legs; Fig. 2F). In contrast, wild-type limbs still have strong posterior expression of *ptc* at this stage, but are also beginning to express *ptc* more anteriorly, around developing skeletal elements where *Ihh* is beginning to be expressed (Marigo et al., 1996a; Fig. 2E).

In wild-type chick embryos, high levels of *ptc* expression are also clearly visible in branchial arches and mouth. Branchial arches are misshapen in *ta*^{3} embryos (Fig. 2H) and there is no high-level expression of *ptc* in either branchial arches or mouth (5 embryos; Fig. 2G,H). Thus, high-level *ptc* expression, normally associated with *shh* expression, is absent in several different regions in *ta*^{3} embryos.

ta^3 is required for normal response to Shh signals

shh RNA is expressed normally in *ta*^{3} but it is possible that processing of Shh protein is defective. Therefore lack of high-level *ptc* expression in *ta*^{3} embryos could be due to either reduction in activity of the Shh signal itself, or a defect in ability of *ta*^{3} tissue to respond to Shh signals.

To determine whether the endogenous Shh signal is active in *ta*^{3} limbs, mesodermal tissue from the posterior of *ta*^{3} limbs was grafted to anterior of wild-type limbs and *ptc* expression assayed after 18 hours. Posterior *ta*^{3} mesenchyme can induce high-level expression of *ptc* in normal limb tissue (7/8: 4 grafts showed strong induction – Fig. 3A; 3 showed weaker induction). However, *ta*^{3} tissue itself still did not express high

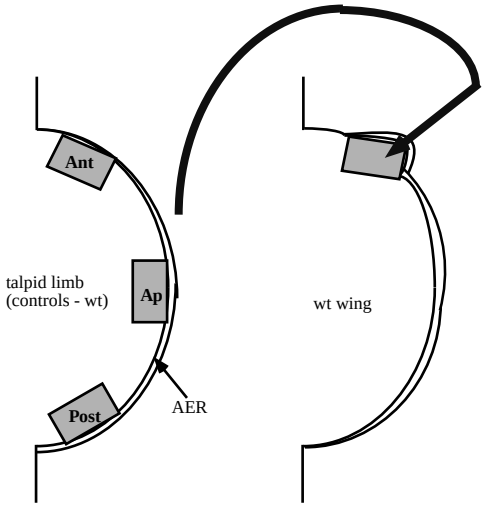

Fig. 1. Schematic representation of tissue graft experiments. Anterior (Ant), apical (Ap) and posterior (Post) mesodermal tissue removed from *ta*^{3} or wild-type (wt) limb buds and, after trypsinisation, grafted to anterior of wt host wing bud, underneath apical ectodermal ridge.

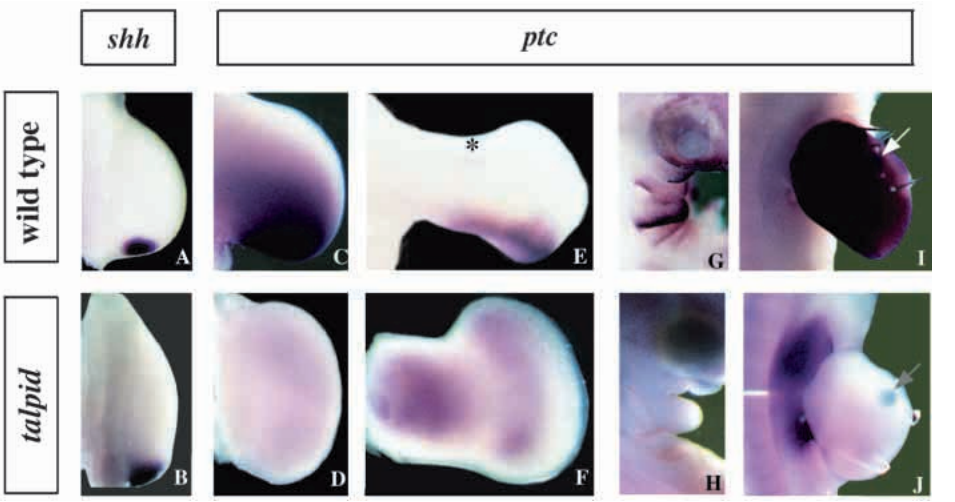

Fig. 2. *shh* and *ptc* expression in wild-type and *talpid*^{3} embryos. Transcripts revealed by in situ hybridisation with antisense RNA probes to *shh* (A,B) or *ptc* (C-J). (A,C,E,G,I) Expression in wild-type embryos; (B,D,F,H,J) expression in *ta*^{3} embryos. (A,B) Ventral views stage 20 wings: *shh* expression posteriorly restricted in *ta*^{3} limbs. (C,D) Dorsal views stage 20 wings, both stained for several hours. *ptc* expressed at very low levels in *ta*^{3} limbs, resembling basal levels of *ptc* expression in anterior of wild-type limbs. (E,F) Dorsal views stage 27 legs, *ta*^{3} leg stained much longer than wild-type leg. *ptc* just beginning to be expressed anteriorly (*) in proximal wild-type leg (E) where mesenchymal condensations are occurring, and in *ta*^{3} limb (F) low-level expression of *ptc* pre-figures fused condensations. (G,H) Branchial arches at stage 20/21; mis-shapen in *ta*^{3} (H) and lacking high-level *ptc* expression seen in wild-type arches (G). (I,J) Wings that had Shh bead implanted anteriorly (arrows), then grafted to wild-type host, fixed 18-23 hours later. Recombinant Shh induces high-level expression of *ptc* in wild-type (I) but not *ta*^{3} limbs (J).

levels of *ptc* (confirmed with sections: data not shown). Grafts of wild-type posterior tissue also induced high-level *ptc* expression in normal host tissue (3 grafts showed strong induction; 1 weaker; Fig. 3E).

ta^3 limb buds grafted to wild-type chicken embryos also induced *ptc* expression in host flank near the posterior of the grafted *ta*^{3} limb, despite there still being no high-level expression of *ptc* in the grafted *ta*^{3} limbs themselves (2/2, assayed 25-26 hours after grafting; Fig. 3I). However, *ptc* was never induced in host flank when wild-type limbs were grafted to wild-type hosts (0/4; assayed 19-24 hours after grafting; Fig. 3H). These results show that *ta*^{3} posterior limb mesenchyme can induce *ptc* expression in normal tissue, suggesting that the *ta*^{3} defect is not in Shh itself. In addition, the second result suggests that *ta*^{3} posterior limb mesenchyme can induce *ptc* at a greater distance than its wild-type counterpart.

At stages 20-24, polarising activity in *ta*^{3} limbs is not as posteriorly restricted as in wild-type limbs (Francis-West et al., 1995), so we tested whether apical or anterior *ta*^{3} tissue could also increase levels of *ptc* expression in wild-type tissue. Some, but not all, of these grafts induced *ptc* expression in wild-type host tissue around the graft (2/4 anterior grafts; 1/1 apical graft; Fig. 3B-D). In contrast, grafts of anterior and of apical wild-type tissue never induced *ptc* expression (0/3 apical; 0/1 anterior; Fig. 3F,G).

To determine whether ability of mesodermal cells to respond to Shh was affected in *ta*^{3} embryos, we investigated whether high-level expression of *ptc* could be induced in *ta*^{3} limbs with purified Shh protein. In one case, an Shh-bead was implanted to the wing of a *ta*^{3} embryo, which was fixed 24 hours later. Contralateral wing and wing with implanted Shh bead had identical low-level expression of *ptc*. In other cases, Shh beads were implanted anteriorly in *ta*^{3} limbs and then each manipulated *ta*^{3} limb grafted to a wild-type chicken embryo.

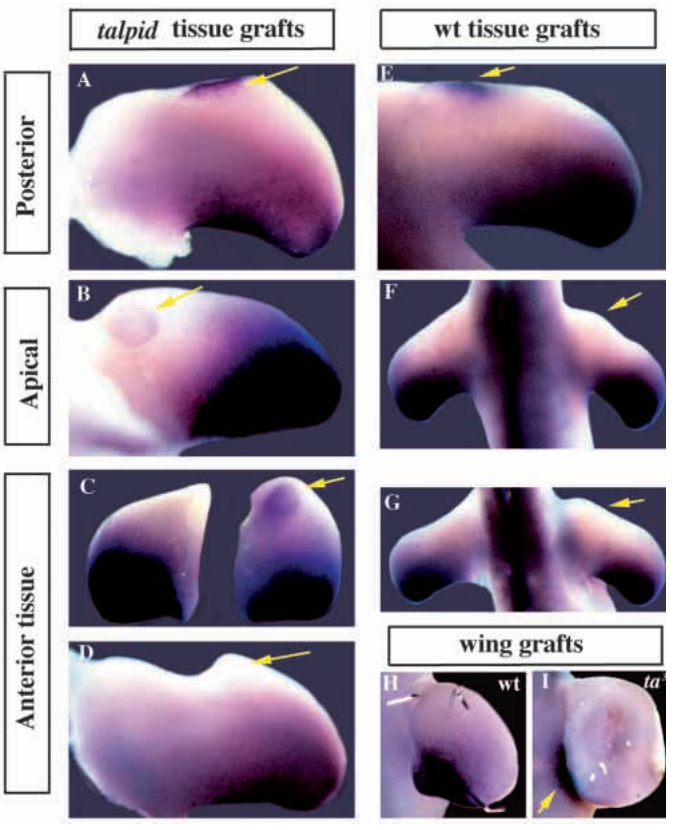

Fig. 3. *talpid*^{3} mesoderm can induce *ptc* expression in wild-type tissue. Transcripts revealed by in situ hybridisation with antisense RNA probes to *ptc*. (A-C) Grafts of *ta*^{3} posterior, apical and anterior mesoderm, respectively, to anterior of wild-type wings. In all cases, *ptc* expression is induced in wild-type host mesoderm overlying graft. (D) Graft of anterior *ta*^{3} mesoderm that did not induce *ptc* expression. Grafts of wild-type posterior mesoderm (E) induced high-level *ptc* expression; grafts of apical (F), anterior (G), wild-type mesoderm did not induce *ptc* expression. (F,G) Contralateral wing also shown for comparison. In all cases, grafts indicated with yellow arrows. (H,I) Wings grafted to wild-type host, fixed 24-26 hours later. *ta*^{3} wings induced high-level *ptc* expression in wild-type flank (I) indicated with yellow arrow; unlike wild-type wings (H).

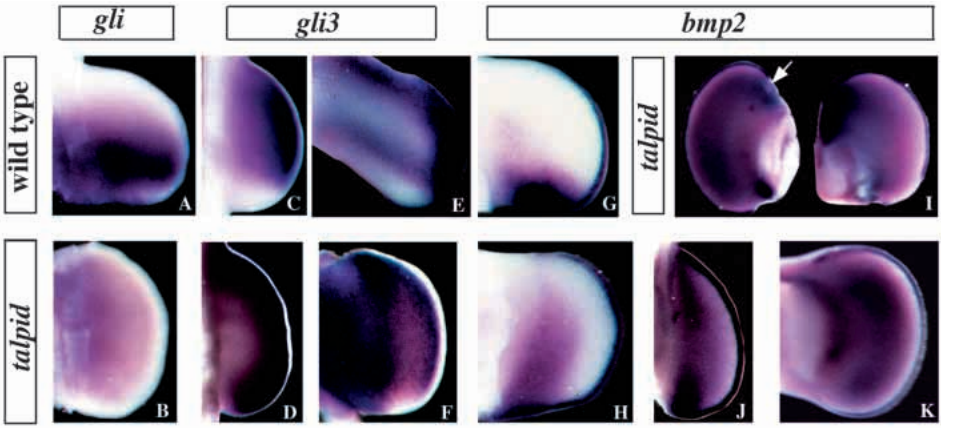

Fig. 4. Expression of *gli1*, *gli3* and *bmp2* in *talpid*^{3} limbs. Transcripts revealed by in situ hybridisation with antisense RNA probes to *gli1* (A,B), *gli3* (C-F) or *bmp2* (G-K). (A,C,E,G) wild-type limbs; (B,D,F,H,K) *ta*^{3} limbs. (A,B) Ventral views stage 21+ wings from wild-type and *ta*^{3} embryos, respectively, stained for several hours in same tube: *gli1* is expressed only very weakly in *ta*^{3} limbs, at similar level to anterior wild-type limbs. (C,D) ventral views stage 20 wings, note posterior expansion of *gli3* expression in *ta*^{3} limbs. (E,F) dorsal views stage 25 leg (E) stage 22 leg (F). *ta*^{3} leg (F) has very small posterior area with little *gli3* expression, but this area is smaller and less distinct that in wild-type limbs (compare F to C and E); (G,H) ventral views stage 21/22 wing (G) and leg (H). Note anterior expansion of wild-type mesodermal *bmp-2* expression domain in *ta*^{3} leg (H). (I) Wing with Shh bead inserted anteriorly (indicated with arrow) and contralateral wing. Both have identical expression of *bmp-2* but difficult to show due to different angles of view – wing with bead has more planar morphology. Fixed 23 hours after bead insertion at stage 24. (J,K) Ventral views stage 20 wing (J) and stage 24 leg (K), both with *bmp-2* expression throughout mesoderm. (J) Slightly higher levels of expression can be distinguished posteriorly.

ptc expression was not induced in any of these *ta*^{3} limbs (0/7 – 3 legs; 4 wings). However, there was clear induction of *ptc* in normal host flank, suggesting that Shh protein from the bead was active and had diffused across the *ta*^{3} limb (Fig. 2J). Beads were also inserted into wild-type wings and these wings were grafted to different wild-type hosts to check that grafting did not interfere with the ability of limb tissue to respond to ectopic Shh protein. In these cases, *ptc* was strongly induced in wild-type limbs (2/2). Weak expression of *ptc* was also induced in host flank (Fig. 2I). These experiments show that wild-type and *ta*^{3} limbs respond differently to Shh beads: unlike in wild-type limbs, purified Shh protein does not induce high-level *ptc* expression in *ta*^{3} limbs, suggesting a defect in response to Shh signals in *ta*^{3}.

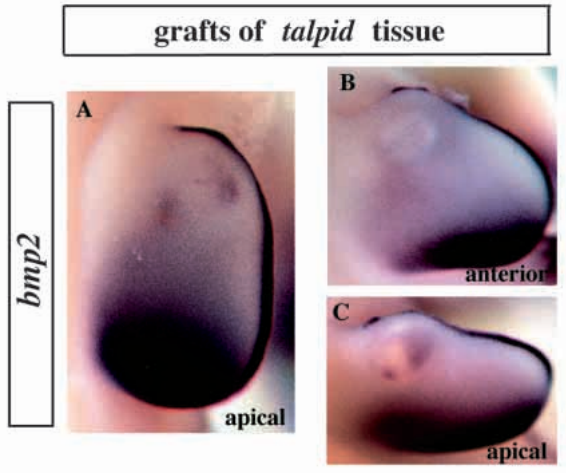

Fig. 5. *talpid*^{3} mesoderm can induce *bmp2* expression in wild-type tissue. Transcripts revealed by in situ hybridisation with antisense RNA probes to *bmp2*. Apical *ta*^{3} mesodermal tissue grafted to anterior of stage 19 (A) or stage 20/21 (C) wild-type wing. Anterior *ta*^{3} mesodermal tissue grafted to anterior of stage 20/21 wild-type wing (B). All limbs fixed 18-20 hours after grafting: (B,C) stage 24, (A) stage 22/23. In all cases, *bmp-2* is expressed strongly in anterior AER, weakly in graft edges.

Expression of *gli* genes in *ta*^{3} limbs

Drosophila Ci and vertebrate Gli-1 proteins are implicated as transcription factors for induction of high-level *ptc* expression (Alexandre et al., 1996; Marigo et al., 1996b; Hynes et al., 1997). In addition, expression of *gli* genes in vertebrates is regulated by Shh: expression of *gli1* increases in response to Shh and expression of *gli3* decreases (Marigo et al., 1996b; Lee et al., 1997).

In wild-type limbs, *gli1* is expressed posteriorly in a domain similar to *ptc* (Marigo et al., 1996b; Fig. 4A). However, in *ta*^{3} limbs, there is no high-level *gli1* expression: rather *gli1* is expressed very weakly throughout the limb except distally under the AER (14/14 – 10 legs; 4 wings, stages 20-24; Fig. 4B). Again, as for *ptc*, the level of *gli1* expression in *ta*^{3} limbs resembles the most anterior expression in wild-type limbs (compare Fig. 4B to 4A).

In contrast to *ptc* and *gli1*, *gli3* expression is expanded in *ta*^{3} limbs (7/7; 3 wings, 4 legs; stages 20-23; Fig. 4D,F). In wild-type limbs, there is a clear area posteriorly where *gli3* is not expressed (Fig. 4C,E). In some *ta*^{3} limbs (4/7), *gli3* expression is weaker posteriorly (Fig. 4F) but the area of weaker expression is always very small and much less distinct than the clear region in wild-type limbs. However, *gli3* expression extends to the very posterior of other *ta*^{3} limbs (3/7; Fig. 4D). These changes in *gli1* and *gli3* expression suggest that transcription of these genes, like that of *ptc*, is unable to respond to endogenous Shh signals in *ta*^{3} limbs.

bmp-2 expression is expanded in *ta*^{3} limbs

We reported previously that *bmp-2* expression is expanded in *ta*^{3} limbs and that the apical ridge shows very strong anterior expansion of *bmp-2* expression (Francis-West et al., 1995; here, 8/8, 4 legs, 4 wings, stages 21-24; Fig. 4H-K.) Our more recent analysis revealed further subtleties, in that expanded *bmp-2* expression in *ta*^{3} limb mesoderm is weaker than apical ridge expression and there is always a thin band where *bmp2*

is not expressed just under the ridge. Despite expansion of its domain, *bmp-2* is sometimes still expressed at slightly higher levels in posterior *ta*^{3} limb (6/8 limbs): in one leg, *bmp-2* was expressed in a wide, anteroposterior stripe that decreased anteriorly (stage 21/22; Fig. 4H) and, in 5 limbs, slightly higher posterior expression could be distinguished (Fig. 4J).

As *bmp-2* is expressed at high levels in *ta*^{3} limbs, unlike *ptc* and *gli1*, this suggests that even though transcription of all of these genes can be increased by Shh signals in wild-type limbs, these genes are regulated independently in *ta*^{3} limbs. We therefore tested whether *bmp-2* expression in *ta*^{3} limbs is altered by inserting a Shh bead into the anterior of the limb. However, we were unable to detect any changes in *bmp-2* expression (5/5; 22-23 hours after bead insertion; Fig 4I), suggesting that anterior *bmp2* expression may have already been maximal in these *ta*^{3} limbs.

Apical and anterior *ta*^{3} tissue can induce *bmp-2* in wild-type tissue

We also tested whether ectopic expression of *bmp-2* in *ta*^{3} tissue was maintained in a wild-type environment. We grafted mesenchyme from apex and anterior of *ta*^{3} limbs (Fig. 1) into anterior wild-type wings and examined expression of *bmp-2*, 18-20 hours later. In all cases, *bmp-2* was ectopically expressed in host AER near the *ta*^{3} graft and there was some persistent mesodermal expression of *bmp-2* in the graft edges. This residual expression was weaker than ectopic AER expression and wild-type *bmp-2* expression in posterior host limb mesoderm (3 apical grafts; 1 anterior graft; Fig. 5). In contrast, grafts of posterior wild-type tissue induced *bmp-2* expression (2/2), while a graft of apical wild-type tissue did not (data not shown).

Expression of *coupTFII* in wild-type and *ta*^{3} limbs

In *ta*^{3} limbs, expression of some target genes of Shh signalling is expanded (Francis-West et al., 1995; this paper), but transcription of *gli1*, *gli3* and *ptc* no longer seems to respond to Shh signalling. *coupTFII* is a direct target gene of Shh signalling in vitro but, unlike *ptc*, its transcription is probably not mediated by Gli-1 (Krishnan et al., 1997). We therefore determined whether expression of *coupTFII* in *ta*^{3} limbs followed either of these two patterns. It has been reported that *coupTFII* is expressed in chick limbs but no details were given (Lutz et al., 1994). At stages 21-24, *coupTFII* is expressed in the centre of normal limbs. Wing expression is broader proximally and extends slightly more distally than leg expression (Fig. 6A,B).

In *ta*^{3} wings, *coupTFII* is expressed throughout, except in a thin band of tissue just beneath the AER (3/3, stage 20-26; Fig. 6C,E). In younger *ta*^{3} legs, expression of *coupTFII* is similar (2/2, stage 20; Fig. 6D) but, in contrast, in older *ta*^{3} legs, *coupTFII* is not expressed in posterior mesoderm (3/3 stage 25-26; Fig. 6F) and the band of distal cells that does not express *coupTFII* is wider than at younger stages, or in *ta*^{3} wings of the same stage (compare Fig. 6F to E and D).

Expansion of *coupTFII* expression in *ta*^{3} limbs suggests that this gene is regulated in a similar way to normally posteriorly expressed *bmp* and *hox-D* genes (Izpisua-Belmonte et al., 1992; Francis-West et al., 1995). However, expression of *coupTFII* in wild-type limbs is more anterior than these other Shh targets and the experiments that suggest that *coupTFII* expression is induced by Shh have been conducted either in vitro or in neural tube (Lutz et al., 1994; Krishnan et al., 1997). Therefore, to establish if, and how, *coupTFII* can be regulated by Shh in limbs, we inserted Shh beads into anterior wild-type wings and examined *coupTFII* expression after 18-19.5 hours. In most wings, *coupTFII* expression increased (4/5; Fig. 6G), although in a few cases (2/5) there was less high-level expression adjacent to the bead than elsewhere (Fig. 6H). One wing (Fig. 6G) had an expression pattern very reminiscent of *coupTFII* expression in *ta*^{3} wings. Therefore *coupTFII* expression can respond to Shh signalling in the limb and expression of *coupTFII* in *ta*^{3} limbs is consistent with ectopic Shh signalling.

ta^3 is not *ptc*, *gli1*, *shh* or *smoothened*

Our analysis of gene expression in *ta*^{3} limb buds is consistent with *ta*^{3} encoding a component of the Shh pathway. We therefore investigated several genes in this pathway to see if they co-segregated with the *ta*^{3} phenotype. Polymorphisms were detected in *ptc* (Marigo et al., 1996a), *shh* (Riddle et al., 1993), *gli1* (Marigo et al., 1996b) and *smoothened* (*smo*) (Quirk et al., 1997) by SSCP analysis in the East Lansing reference mapping population (Burt and Cheng, 1998).The segregation patterns of parental alleles in the back cross progeny for all loci were compared with the inheritance of previously mapped markers.

The genetic locations of the genes are:

ptc, Chr. Z (137 cM) (LEI0254 – 0.0 cM, LOD 14.4 – PTCH – 0.0 cM, LOD 15.7 -MSU0352).

shh, Chr. 2 (61 cM) (MSU308 – 6.2 cM, LOD 9.8 – SHH – 10.4 cM, LOD 7.7 – ADL0270).

gli1, Chr. E22C19W28 (42 cM) (LEI0003 – 1.9, LOD 13.5 – GLI-1 – 20.3, LOD 4.6 -LEI0019).

smo, Chr. 1 (16 cM) (MSU0343 – 0.0 cM, LOD 15.7 – SMOH – 0.0 cM, LOD 12.0 -MSU0314).

The mutant allele in all *ta*^{3} carriers is derived from a single grand sire and is inherited as a single autosomal, recessive lethal mutation. Therefore a simple co-segregation test of *smo*, *shh*, *gli1* and *ta*^{3} in the *ta*^{3} carrier parents (n=13) and *ta*^{3} progeny (n=19) will either support or exclude the role of these genes in the *ta*^{3} gene defect. If the *ta*^{3} gene defect were in one of these genes, then all *ta*^{3} homozygous progeny should also be homozygous for one of the candidate gene polymorphisms. Conversely, if there is no association then all genotypes for the candidate gene will be found within *ta*^{3} homozygotes. We were unable to detect any polymorphisms in the *ptc* gene of any *ta*^{3} carriers, but genetic mapping (described above, Fig. 7A) showed that this gene maps to the Z sex chromosome. Since *ta*^{3} is inherited as an autosomal mutation, we can clearly exclude *ptc* as a potential candidate for the *ta*^{3} defective gene. SSCP alleles were detected in the other candidate genes (Fig. 7B). All classes of genotypes were found in *ta*^{3}/*ta*^{3} mutants, thus excluding *smo*, *shh* and *gli1* as the defective gene in *ta*^{3}. Unforunately, we were unable to extend this analysis to *gli3*, as we were unable to detect any polymorphisms in this gene in either East Lansing or *ta*^{3} crosses.

Gene expression in trunk of *ta*^{3} embryos

coupTFII is normally strongly expressed in two domains in ventral neural tube (Lutz et al., 1994; Fig. 8A), and has been implicated in induction of motoneurons by Shh. Therefore we

investigated whether *coupTFII* expression was altered in *ta*^{3} neural tubes. We found that, unlike *ta*^{3} limbs, in which *coupTFII* expression is expanded, *coupTFII* is not expressed in neural tubes of *ta*^{3} embryos though it is still expressed at high levels in lateral somites (compare Fig. 8B and A). However, the effect of the *ta*^{3} mutation on *shh* expression also differs between limb and neural tube. In *ta*^{3} limbs, *shh* is expressed normally, but *shh* expression in ventral neural tube is discontinuous and reduced in both trunk and head (compare Fig. 8C,E to D,F,G). However, *shh* is still expressed normally in *ta*^{3} notochords (Fig. 8G).

We also examined expression of *ptc*, *gli1* and *gli3* in trunks of *ta*^{3} embryos. *ptc* is still expressed in medial neural tube, though at lower levels than in wild-type embryos. However, compared to wild-type embryos, there is hardly any expression of *ptc* around the notochord in ventral somites (compare Fig. 8H to I). *gli1* is also still expressed in neural tubes of *ta*^{3} embryos but like *ptc* there is less expression of *gli1* in *ta*^{3} somites (compare Fig. 8J to K). In contrast *gli3* is strongly expressed in *ta*^{3} neural tube and somites. Neural tube expression of *gli3* is expanded ventrally and appears stronger than in wild-type embryos (compare Fig. 8M to L).

We also observed characteristic morphological abnormalities in *ta*^{3} trunks. *ta*^{3} neural tubes are usually ovoid and have larger lumens than in wild-type embryos (Ede and Kelly, 1964b; Fig. 8I,M). In addition, in two *ta*^{3} embryos, the neural tube bifurcated caudally.

DISCUSSION

talpid^3 is probably involved in response to Shh signalling

shh is expressed normally in *ta*^{3} limbs, but we show here that the high-level expression of *ptc* and *gli1 t*hat is normally induced in and around *shh*-expressing cells is absent in *ta*^{3} limbs, and that normal posterior repression of *gli3* expression does not occur. This suggests that the *ta*^{3} gene product acts between *shh* expression and Shh regulation of *ptc*, *gli1* and *gli3* transcription. It is unlikely that *ta*^{3} is required for correct processing of Shh protein as posterior *ta*^{3} tissue can induce *ptc* expression in wild-type tissue. Instead the *ta*^{3} gene product appears to be required for response to Shh signals because purified recombinant Shh protein does not induce ectopic *ptc* expression in *ta*^{3} limbs as it does in wild-type limbs. Furthermore, *ta*^{3} tissue grafted into wild-type limbs continues to express low levels of *ptc*, even when surrounding normal tissue is expressing higher levels. The pleiotropic morphological phenotype of *ta*^{3} embryos is also consistent with *ta*^{3} being required for correct Shh signalling as abnormalities have been observed in many embryonic regions in which Shh signalling has been implicated. In two of these regions (branchial arches and mouth), high-level *ptc* expression is also absent. However, gene mapping and co-segregation analyses of genetic markers for *ptc*, *smo*, *shh* and *gli1* genes exclude them as candidate genes for *ta*^{3}.

Comparing *talpid*^{3} phenotype to other mutations

The morphological limb phenotype of *ta*^{3}, especially polydactyly, resembles mutant phenotypes that arise because of increased Shh signalling (or a reduction in Ptc activity). For example, mice and humans heterozygous for a mutation in *ptc* have phenotypes that occasionally include polydactyly (Hahn et al., 1996; Johnson et al., 1996; Goodrich et al., 1997); transgenic mice that ectopically express *shh* specifically in their skin show polydactyly of both forelimbs and hindlimbs, with each limb forming about 8 morphologically similar digits (Oro et al., 1997), and most polydactylous mutations in mouse investigated so far (*Strong's luxoid, hemimelic extra toes, extra toes, recombinant induced mutant 4, luxate, X-linked polydactyly, Alx-4*) are associated with ectopic expression of *shh* in anterior of the limb bud (Buscher and Ruther, 1998; Buscher et al., 1997; Chan et al., 1995; Masuya et al., 1995, 1997; Qu et al., 1997). One exception is the mouse mutation *doublefoot*, which has normal *shh* expression, and, unlike the other mouse mutants, does not have mirror-image digit duplications but identical digits like *ta*^{3}. However, unlike *ta*^{3}, *doublefoot* has a dominant phenotype and ectopic expression of Shh target genes including *ptc* and *gli1* (Hayes et al., 1998; Yang et al., 1998). This suggests that its phenotype is also due to ectopic activation of the Shh pathway, either because of a mutation in an, as yet unknown, component of the pathway (Hayes et al., 1998) or due to ectopic expression of another *hh* gene, *Ihh* (Yang et al., 1998). *ta*^{3} is the only polydactylous mutation examined so far in which *ptc* and *gli1* are not ectopically expressed at high levels.

Other aspects of *ta*^{3} resemble mutations that have reduced Shh activity. For example, in *ta*^{3} embryos, the eyes are closer together and, in extreme cases, fuse, which is reminiscent of holoprosencephaly and cyclopia seen in humans and mice with *shh* mutations (Chiang et al., 1996; Roessler et al., 1996; Belloni et al., 1996). Our analysis of gene expression in *ta*^{3} neural tubes is also consistent with reduced Shh signalling: expression of *shh* is reduced in ventral neural tube, the motoneuron domain of *coupTFII* expression is lost and there is lower expression of *ptc* and *gli1*, and expanded expression of *gli3*. This is reminiscent of mouse embryos lacking Shh function, which do not form a morphologically distinct floor plate, but it contrasts with mice lacking Ptc function in which floor plate characteristics, including expression of *shh*, are expanded dorsally (Chiang et al., 1996; Goodrich et al., 1997). This paradox – that some aspects of the *ta*^{3} phenotype resemble a loss of Shh signalling, and some resemble a gain of Shh signalling – suggests a more complex basis for the *ta*^{3} phenotype than for any of the other polydactylous mutations.

talpid^3 phenotype suggests a bifurcation in the Shh signalling pathway

The *ta*^{3} gene product is required for high-level expression of *ptc* and *gli1* and for posterior repression of *gli3* expression in the limb, but is not required for expression of *fgf-4* or the normally posteriorly expressed *bmp* or 5′ *hoxD* genes (Izpisua-Belmonte et al., 1992; Francis-West et al., 1995). *ta*^{3} limb morphology most resembles what we would expect from gain of Shh signalling, but expression of *ptc* and *gli* genes suggests loss of Shh signalling. Recent experiments in cell culture and *Drosophila* have suggested that the Hh pathway is not necessarily linear and that Gli proteins are not the only transcription factors for Shh signalling (Ohlmeyer and

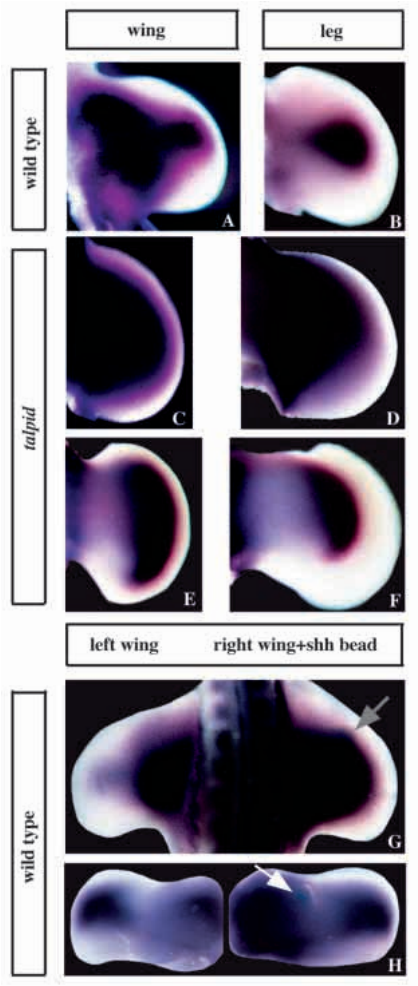

Fig. 6. *coupTFII* expression in wild-type and *talpid*^{3} embryos. Transcripts revealed by in situ hybridisation with antisense RNA probes to *coupTFII*. (A,C,E) Dorsal views wings; (B,D,F) dorsal views legs; (A,B) wild-type limbs; (C-F) *ta*^{3} limbs; (A-D) stage 20-21; (E,F) limbs from same stage 26 embryo. Expression of *coupTFII* expanded throughout almost all limb-bud mesoderm in *ta*^{3} embryos, except for distal and posterior mesoderm in older legs. (G,H) Wild-type wing buds with Shh bead inserted anteriorly, fixed 18-19.5 hours after bead insertion, together with contralateral wings. In both cases, arrow points to bead position. (G) Expression of *coupTFII* in wing exposed to Shh resembles expression of *coupTFII* in *ta*^{3} wing (C). (H) Wing exposed to Shh has ectopic expression of *coupTFII* but expression to right of bead weaker than elsewhere in limb.

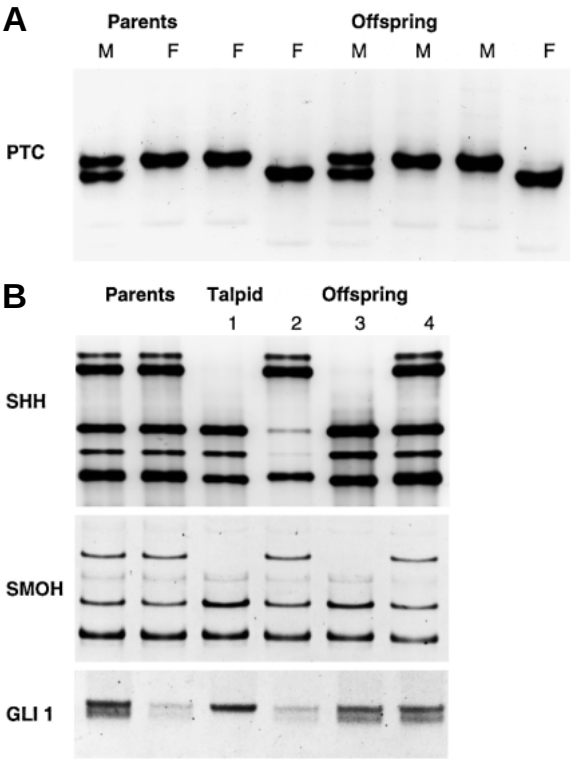

Fig. 7. Genetic mapping. (A) Silver-stained SSCP gel showing sex-linked inheritance of *ptc* in the progeny of the East Lansing backcross population. M, male; F, female; Jungle Fowl-specific allele is the lower band in the male parent. (B) Silver-stained SSCP gels showing independent segregation of the *ta*^{3} mutant phenotype and polymorphisms within the genes for *smo*, *shh* and *gli1* in the progeny of *ta*^{3} carriers.

Kalderon, 1997; Lessing and Nusse, 1998; Krishnan et al., 1997). This is also suggested by recent analysis of mice lacking Gli1 and Gli2 function (Matise et al., 1998). There is considerable evidence that Gli proteins are transcription factors that mediate Shh upregulation of *ptc* transcription (Marigo et al., 1996b; Hynes et al., 1997), but expression of *coupTFII* in vitro does not seem to be mediated by Gli, even though it is directly induced by Shh (Krishnan et al., 1997). The *ta*^{3} limb phenotype could thus be explained if Gli proteins regulate expression of *gli1*, *gli3* and *ptc*, but another transcription factor regulates expression of *bmp2* and 5′ *hoxD* genes, *fgf4* and *coupTFII*, either directly or indirectly, and *ta*^{3} is only required for the Gli branch of the pathway (Fig. 9A).

A bifurcation in the Shh pathway could also explain why some aspects of *ta*^{3} more resemble loss of Shh signalling and others gain of Shh signalling. This would be predicted if different branches are required to different extents in different tissues. However, lack of *coupTFII* expression in *ta*^{3} neural tubes is puzzling. Based on our observations in the limb, we would have expected loss of high-level *gli1* and *ptc* expression and an expansion of *gli3* and *coupTFII* expression in *ta*^{3} trunks but, while the other expression patterns are as expected, *coupTFII* expression is lost in *ta*^{3} neural tubes. This could be a secondary effect of reduced floor plate in *ta*^{3} embryos. The vertebrate trunk is an unusual site of Shh activity in that very high levels are required to induce floor plate, which then itself expresses *shh* (Roelink et al., 1995; Marti et al., 1995). Floor plate development and associated *shh* expression is disturbed in *ta*^{3} embryos, probably because the Gli pathway branch is explicitly needed for floor plate development (Sasaki et al., 1997; Ruiz i Altaba, 1998; Ding et al., 1998; Matise et al., 1998). This could result in an overall level of Shh signal too low for *coupTFII* expression to be induced in ventral neural tube. However, in *gli2* mouse mutants, motoneuron development can occur even in absence of floorplate, although, in these embryos, notochord remains in close association with neural tube for longer than in normal mouse development (Ding et al., 1998; Matise et al., 1998).

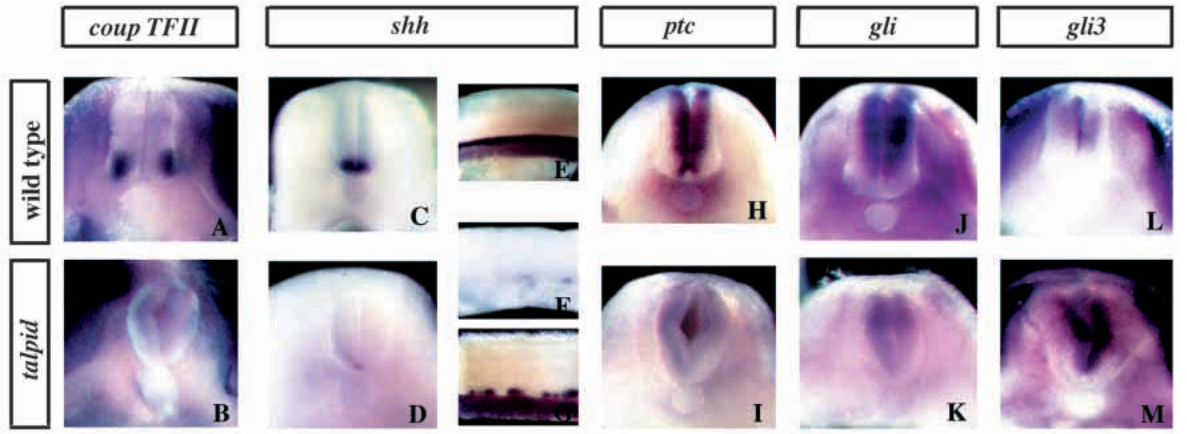

Fig. 8. Gene expression in wild-type and *talpid*^{3} trunks. Transcripts revealed by whole-mount in situ hybridisation with antisense RNA probes to *coupTFII* (A,B), *shh* (C-G), *ptc* (H,I), *gli1* (J,K), and *gli3* (L,M): transverse sections cut by hand. (A,C,E,H,J,L) wild-type embryos and (B,D,F,G,I,K,M) *ta*^{3} embryos. *coupTFII* not expressed in ventral neural tube of *ta*^{3} embryos (compare B to A). *shh* expressed normally in notochord in younger *ta*^{3} embryos (G and compare E) but reduced and discontinuous in ventral neural tube (compare F,G to E and D to C). (C,D,F) Stages when Shh no longer expressed in notochord in either *ta*^{3} or wild-type embryos but still expressed in floor plate. *ptc* and *gli1* no longer expressed at high levels in ventral somites around notochord in *ta*^{3} embryos (compare I,K to H,J). *gli3* expression expanded ventrally in neural tube (compare M to L).

Absence of high-level *ptc* expression in *ta*^{3} may produce secondary phenotypic effects

High-level *ptc* expression, at least in *Drosophila*, sequesters Hh and prevents its diffusion (Chen and Struhl, 1996; Taylor et al., 1993; Fig. 9B). Therefore, in absence of high-level *ptc* expression in *ta*^{3} limbs, it is possible that Shh protein is diffusing farther and can act over a longer distance than normal. This possibility is suggested by induction of *ptc* expression in wild-type hosts when we grafted *ta*^{3} limbs, but not when we grafted wild-type limbs, and by induction of *ptc* in wild-type tissue by grafts of anterior and apical *ta*^{3}, but not wild-type tissue. As *ptc* expression can be induced by Shh, these results suggest that there may be more widespread distribution of Shh protein in *ta*^{3} limbs. This would also explain anterior expansion of normally posterior gene expression and polarising activity in *ta*^{3} limbs (Francis-West et al., 1995). Consistent with this hypothesis, reaggregates of disaggregated mesoderm from a whole leg or just the posterior third, which have polarising region cells, and hence *shh* expression and protein distributed randomly throughout the limb, show many similarities to *ta*^{3} limbs: identical digits and *hox-D*, *bmp2* and *fgf-4* genes expressed across the entire anteroposterior axis (Hardy et al., 1995).

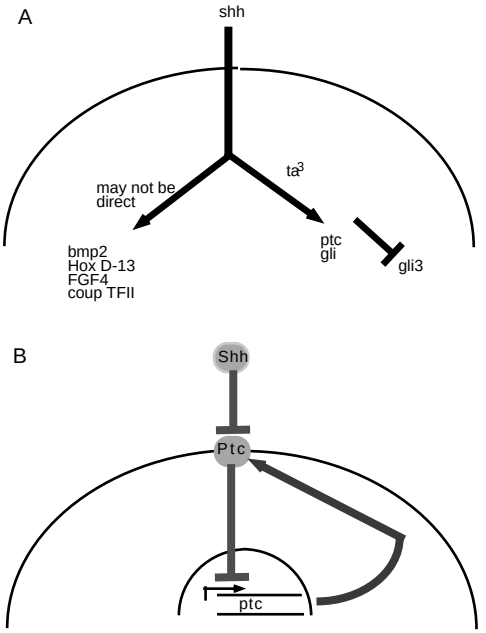

Fig. 9. Model of the Shh pathway. (A) Possible model of Shh regulation of different target genes, based on *ta*^{3} phenotype. Shh pathway bifurcates, with Gli proteins controlling transcription of *gli* genes and *ptc*, and unknown transcription factor(s) controlling expression of *bmp*, *hox-D* and *coupTFII* genes: *ta*^{3} only required for Gli branch of pathway. Transcriptional regulation of second group of genes need not be direct result of Shh signalling. (B) Basal levels of Ptc repress Shh pathway in absence of Shh signals: this repression is inhibited when Shh binds Ptc. Ptc transcription increased by Shh signalling, (or loss of Ptc function): increased levels of Ptc may normally localise Shh protein and/or dampen Shh signalling.

Sensitivity of cells to Hh signals may also increase in the absence of high-level *ptc* expression. Ptc normally represses Hh signalling pathways in the absence of Hh, but high levels of *ptc*, normally induced by Shh, may also dampen the effect of Shh signals. Evidence that a precise balance between Hh and Ptc is required for correct development comes from the fact that overexpression of *ptc* can reduce Hh signalling (Johnson et al., 1995; unpublished results K. E. L., P. Currie and P. W. I.), and from analyses of Gorlin's syndrome and human holoprosencephaly, which are caused by heterozygosity for loss-of-function *PTCH* or *SHH* mutations, respectively (Johnson et al., 1996; Hahn et al., 1996; Belloni et al., 1996; Roessler et al., 1996). If this 'dampening' effect is lacking, i.e. there is no *ptc* upregulation, a lower exogenous Shh signal may be sufficient to activate posterior genes, and therefore enough Shh may be diffusing even to anterior regions of the limb to produce uniform expression of genes such as *bmp-2*.

The combination of Ptc sequestering Shh protein and/or dampening Shh signals could also explain the difference between *ta*^{3} and the phenotype that is obtained from placing beads soaked in aminoterminal Shh (N-Shh) in the anterior of the limb. N-Shh diffuses a long way from the bead in these experiments, yet this results in symmetrical digit duplications rather than posterior identity across the limb bud (Yang et al., 1997). However, N-Shh induces *ptc* (Yang et al., 1997; Porter et al., 1996) and it seems likely that Ptc protein in cells around the bead begins to sequester N-Shh. Therefore, cells far away from the bead initially receive high levels of Shh activity but then receive lower levels. In contrast, Shh activity in *ta*^{3} limbs is not localised or dampened by *ptc* expression.

Interaction between Hh and Ptc may also explain why, in contrast to the *ta*^{3} polydactylous phenotype where all digits are morphologically similar, most mouse polydactylous mutations specify a sequence of different ectopic digits (Buscher and Ruther, 1998; Buscher et al., 1997; Chan et al., 1995; Masuya et al., 1997; Qu et al., 1997). One prediction would be that these mutants all ectopically express *ptc*, which localises any ectopic Shh signalling. Indeed, *ptc* is expressed ectopically in *extra-toes* and *Strong's luxoid* (Goodrich et al., 1996; Platt et al., 1997; Buscher et al., 1997).

In conclusion, genes that are regulated by Shh in chicken limbs behave in one of two abnormal ways in *ta*^{3}: transcription of *ptc*, *gli1* and *gli3* no longer responds to Shh signalling; in contrast expression of *bmp*, *hox-D* and *coupTFII* genes expands. Since the *ta*^{3} defect is in responding cells and not production of Shh signal, we propose a bifurcation in the Shh pathway and suggest that the *ta*^{3} gene product is a component of the Shh signal transduction pathway, required after the branch point, for regulation of the first group of genes. In addition, we propose that expanded expression of the second group of genes, and expansion of polarising activity in *ta*^{3} limbs, is due to a wider distribution of Shh protein, caused by lack of high-level *ptc* expression. This hypothesis can also account for different aspects of the pleiotropic *ta*^{3} phenotype.

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