Development 128, 733-742 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV7857



# An absolute requirement for Cubitus interruptus in Hedgehog signaling

**Nathalie Méthot and Konrad Basler**

Institut für Molekularbiologie, Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland
*Author for correspondence (e-mail: basler@molbio.unizh.ch)

*Accepted 20 December 2000; published on WWW 7 February 2001*

## SUMMARY

**Hedgehog (Hh) proteins play diverse organizing roles in animal development by regulating gene expression in responding cells. Several components of the Hh signal transduction pathway have been identified, yet their precise role in mediating the various outputs of the pathway is still poorly understood. The Gli homolog Cubitus interruptus (Ci) is involved in controling the transcription of *Drosophila* Hh target genes and thus represents the most downstream component known in this pathway. We address the question of whether the Hh pathway is distally branched or, in other words, whether the regulation of Ci activity is the sole output of Hh signaling. Putative Ci-independent branches of Hh signaling are explored by analyzing the behavior of cells that lack Ci but have undergone maximal activation of the Hh transduction pathway due to the removal of Patched (Ptc). The analysis of target gene expression and morphogenetic read-outs of Hh in embryonic, larval and adult stages indicates that Ci is absolutely required for all examined aspects of Hh outputs. We interpret this as evidence against the existence of Ci-independent branches in the Hh signal transduction pathway and propose that most cases of apparent Ci/Gli-independent Hh output can be attributed to the derepression of target gene expression in the absence of Ci/Gli repressor function.**

Key words: Hedgehog, signal transduction, Gli proteins, Cubitus interruptus, *Drosophila*

## INTRODUCTION

The development of multicellular organisms is governed by cell-cell communication. During all stages of animal development, cells influence each others fate and behavior by sending and receiving extracellular signals. Despite the countless and diverse instances of cell signaling, animals use a surprisingly small repertoire of signaling molecules. One of the most remarkable discoveries of the past decade is the finding that members of the EGF, FGF, Wnt, Hedgehog (Hh) and TGFβ families of secreted peptides account for innumerable cases of embryonic cell communication events (reviewed by Kingsley, 1994; Mason, 1994; Hammerschmidt et al., 1997; Schweitzer and Shilo, 1997; Wodarz and Nusse, 1998). Hh signaling, for example, has been shown to pattern *Drosophila* segments, wing, leg, eye, and regions of the brain. In vertebrates, Hh is involved in the specification of left-right asymmetry, and it patterns the ventral neural tube, adjacent somites, limbs, eyes, hair and lungs (reviewed by Lawrence and Struhl, 1996; Neumann and Cohen, 1997; Ingham, 1998; Ruiz i Altaba, 1999; McMahon, 2000).

An intriguing question raised by these findings is how a handful of signals can elicit such a large repertoire of cellular responses. Among many conceivable explanations, we analyze the possibility that the pathways transducing these signals are bifurcated and hence contain multiple cytoplasmic and nuclear endpoints. We focus on the Hh pathway for which this possibility has been proposed in several instances. Below we briefly outline the mechanics of Hh transduction and summarize the experimental findings that have been taken as evidence for a distal bifurcation in this pathway. We then describe our strategy to scrutinize this conception.

The most upstream events in Hh signal transduction are mediated by two multi-transmembrane-domain proteins, Patched (Ptc) and Smoothened (Smo). In the absence of Hh, Ptc inhibits the latent signaling activity of Smo. The binding of Hh to Ptc reduces this inhibition and allows Smo to be active (reviewed by Alcedo and Noll, 1997). In a poorly understood manner, Smo activity is transduced intracellularly by several components, among them Cos2, Protein kinase A, Fused, Su(fu) and Slimb (reviewed by Ingham, 1998). The distal most element known in the Hh pathway is the zinc-finger protein Cubitus interruptus (Ci), the *Drosophila* homolog of the vertebrate Gli proteins. Ci appears to mediate most of the Hh signal from the cytoplasm to the nucleus where it directly regulates the transcription of target genes (reviewed by Aza-Blanc and Kornberg, 1999). In the absence of Hh signal, Ci is proteolytically cleaved to a 75 kD repressor form (here referred to as Ci[rep]). In the presence of Hh signal this cleavage is prevented and Ci instead functions as a transcriptional activator (Ci[act]; Aza-Blanc et al., 1997; Méthot and Basler, 1999). However, five reports suggest that there are Ci/Gli-independent mechanisms of Hh signaling:

(1) Proper response of chick embryonic tissue to Sonic hedgehog (Shh) requires the activity of the *talpid*$^3$ gene (Lewis et al., 1999). In *talpid*$^3$ mutant limb cells, expression of the Shh

target genes *ptc* and *gli1* is reduced, mimicking a 'loss of Hh signaling' situation. The polydactily and ectopic expression of other Hh target genes associated with the *talpid*$^{3}$ genotype, however, resemble a 'gain of Hh signaling'. These opposite phenotypes have been interpreted as evidence for a bifurcation in the Shh signaling pathway, in which Talpid$^{3}$ would function in one of two branches (Lewis et al., 1999).

(2) A Shh-response element was identified in the COUP-TFII promoter (Krishnan et al., 1997a). This element is Shh responsive, even in the presence of protein synthesis inhibitors (Krishnan et al., 1997b). No DNA-binding site, however, was present for Gli proteins. Hence it was concluded that the response to Shh is channeled through a protein with DNA-binding activity unrelated to that of the Ci/Gli family of transcription factors (Krishnan et al., 1997b).

(3) Similarly, a 150 bp element was identified in the *wingless* (*wg*) promoter, which is highly conserved in other *Drosophila* species (Lessing and Nusse, 1998). The activity of this element depends on Ptc activity, but it contains no consensus Ci-binding sites. Likewise, it was concluded therefore that Ci cannot be the sole endpoint of the Hh pathway.

(4) The formation of the larval light-sensing organ in *Drosophila* (Bolwig's organ) and the expression of *atonal* in its precursor cells are dependent on the Hh signal and on Smo, but apparently not on Ci (Suzuki and Saigo, 2000). These findings were taken as evidence of Ci-independent Hh signaling and it was concluded that a considerable diversity must exist in the downstream pathway of Hh signaling in *Drosophila* (Suzuki and Saigo, 2000).

(5) A very recent study by Gallet et al. suggests that also some aspects of the embryonic transcription of *wg* and *rhomboid* are regulated by Hh in a Ci-independent manner (Gallet et al., 2000).

(6) Finally, embryos mutant for *ci* have a much milder segment polarity phenotype than embryos mutant for *hh* (Nüsslein-Volhard et al., 1984; Slusarski et al., 1995; our own observations, see Results and Fig. 6B,C). The weak phenotype of *ci* mutant embryos can be taken as evidence that a gene product other than Ci is able to transduce part of the embryonic Hh signal.

Evidently, a number of findings argue for a branching in the Hh pathway and the existence of Ci/Gli-independent outputs of Hh signaling. Moreover, some responses to Hh may require no transcriptional output at all, and may have cytoplasmic endpoints instead. Questions about branching and crosstalk in signaling pathways are by no means unique for the Hh pathway (see for example Denhardt, 1996; Schamel and Dick, 1996; Selbie and Hill, 1998; Zhang and Derynck, 1999; Zwick et al., 1999). We have chosen the following genetic approach to address them for the *Drosophila* Hh pathway. We wanted to maximally activate the pathway, while at the same time closing its main outlet valve. To activate the Hh pathway, Ptc is removed by genetic means. This is, to our knowledge, the most powerful and most upstream means by which to activate the Hh pathway. If at the same time Ci is also removed, we can ask what, if any, aspect of the Hh signal can still be detected at various stages of development.

Surprisingly, but unambiguously, we find that Hh acts obligatorily through Ci, in each situation analyzed. We interpret this as strong evidence against a distal branching in the pathway and conclude that any parallel pathway downstream of Smo must converge at Ci. While our results show that Hh does not have any effect on target gene expression in the absence of Ci, they also indicate that the opposite is not the case. Ci can exert effects in the absence of Hh signaling and we attribute this activity of Ci to its default function as a transcriptional repressor. The presence and absence of this repressor function in *hh* versus *ci* null mutant animals, respectively, may be the main source for the numerous reports claiming Ci-independent Hh outputs.

## MATERIALS AND METHODS

### Alleles

Alleles used in this study were as follows: *ci*$^{94}$, a null allele of *ci* (Slusarski et al., 1995; Méthot and Basler, 1999); *ptc*$^{IIW}$, a null allele of *ptc* that does not produce detectable protein (Capdevila et al., 1994; Chen and Struhl, 1996); *ptc*$^{S2}$, which genetically behaves as a null allele, but can still make Ptc protein (Phillips et al., 1990; Chen and Struhl, 1996); *hh*$^{AC}$, a null allele (Lee et al., 1992); and *Df(2R)en*$^{E}$, a deficiency that removes *engrailed* (*en*) and the closely related *invected* gene (Tabata et al., 1995).

### Transgenes and reporters

Transgenes and reporters were P[*ci*$^{+}$], a 16 kb genomic *ci* fragment that rescues *ci*$^{94}$ animals (Méthot and Basler, 1999); P[*hsp70-GFP*] (Méthot and Basler, 1999); P[*ubi-nlsGFP*], GFP under the control of the *ubiquitin* promoter (Luschnig et al., 2000); *dpp*$^{P10638}$, referred to as *dpp-lacZ* (Zecca et al., 1995); and *hh*$^{P30}$, referred to as *hh-lacZ* (Lee et al., 1992).

### Antibodies

Clones of mutant alleles were generated by Flp-mediated mitotic recombination (Xu and Rubin, 1993). For analysis in wing imaginal discs, larvae were heat-shocked in late first instar for 30 minutes at 35°C, and heat-shocked again in late 3$^{rd}$ instar phase to induce marker gene *(hsp70-GFP)* expression. Following a 1 hour recovery period, larvae were dissected, discs were fixed and stained with the appropriate antibodies. The following antibodies were used: mouse monoclonal anti-Ptc (gift from I. Guerrero); mouse monoclonal and rabbit polyclonal anti-GFP (Clontech); mouse monoclonal (Promega) and rabbit polyclonal (Cappel) anti-β-gal; mouse monoclonal anti-En 4D9; and Alexa 488 and 594 secondary antibodies (Molecular Probes).

### Genotypes

The genotypes of larvae were as follows.

*ci*$^{94}$ clones, anti-Ptc or anti-En

*y w hsp70-flp*; *FRT42 P[ci*$^{+}$*] hsp70-GFP/FRT42*; *ci*$^{94}$/*ci*$^{94}$

*en*$^{E}$ *ci*$^{94}$ clones, *dpp-lacZ*

*y w hsp70-flp*; *dpp*$^{P10638}$ *FRT42 P[ci*$^{+}$*] hsp70-GFP/FRT42 en*$^{E}$; *ci*$^{94}$/*ci*$^{94}$

*ptc*$^{S2}$ clones, anti-Ptc

*y w hsp70-flp*; *FRT42 ptc*$^{S2}$*/FRT42 P[ci+] hsp70-GFP*; *ci*$^{94}$*/Dp(1;4)1021[y*$^{+}$*]*

*ptc*$^{IIW}$ clones, anti-En

*y w hsp70-flp*; *FRT42 ptc*$^{IIW}$*/FRT42 P[ci+] hsp70-GFP*; *ci*$^{94}$*/Dp(1;4)1021[y*$^{+}$*]*

*en*$^{E}$ *ptc*$^{IIW}$ clones, *dpp-lacZ*

*y w hsp70-flp*; *FRT42 ptc*$^{IIW}$ *en*$^{E}$*/dpp*$^{P10638}$ *FRT42 P[ci*$^{+}$*] hsp70-GFP*; *ci*$^{94}$*/Dp(1;4)1021[y*$^{+}$*]*

*ci*$^{94}$ *ptc*$^{S2}$ clones, anti-Ptc, anti-En

*y w hsp70-flp*; *FRT42 ptc*$^{S2}$*/FRT42 P[ci*$^{+}$*] hsp70-GFP*; *ci*$^{94}$*/ci*$^{94}$

*en*$^{E}$ *ptc*$^{IIW}$ *ci*$^{94}$ clones, *dpp-lacZ*

*y w hsp70-flp*; *FRT42 ptc*$^{IIW}$ *en*$^{E}$*/dpp*$^{P10638}$ *FRT42 P[ci*$^{+}$*] hsp70-GFP*; *ci*$^{94}$*/ci*$^{94}$

### Germline clones

Germline clones were generated by applying a heat shock (1 hour at 37°C) to third instar larvae of the following genotypes.

*ci*$^{94}$ germline clones

*y w hsp70-flp*; *FRT42 P[ci*$^{+}$*] P[ubi-nlsGFP]/FRT42*; *ci*$^{94}$*/ci*$^{94}$

*ci*$^{94}$ *ptc*$^{IIW}$ germline clones

*y w hsp70-flp*; *FRT42 P[ci*$^{+}$*] P[ubi-nlsGFP]/FRT42 ptc*$^{IIW}$; *ci*$^{94}$*/ci*$^{94}$

*ci*$^{94}$ *hh*$^{AC}$ germline clones

*y w hsp70-flp*; *FRT 82 P[ci*$^{+}$*] P[hsp70-GFP] P[ubi-nlsGFP]/FRT82 hh*$^{AC}$; *ci*$^{94}$*/ci*$^{94}$

### Embryos

Adult females were recovered and crossed to *y w; ci*$^{94}$*/Dp(1;4)1021[y*$^{+}$*]* males. Eggs were collected for 3 hour periods before examination under UV light for GFP fluorescence. Embryos that lacked GFP fluorescence were selected, aged for 24 hours and processed for cuticle preparation. *ci*$^{94}$*/ci*$^{94}$ embryos could be distinguished from their *ci*$^{94}$*/Dp(1;4)1021[y*$^{+}$*]* siblings by the pale coloration of their denticles.

Zygotic mutant embryos for *ci*$^{94}$, *ci*$^{94}$ *ptc*$^{IIW}$, *ci*$^{94}$ *hh*$^{AC}$ and *ci*$^{94}$ *wg*$^{CX4}$ were obtained from *y w*; *ci*$^{94}$*/Dp(1;4)1021[y*$^{+}$*]*, *y w hsp70-flp*; *FRT42 ptc*$^{IIW}$*/CyO[y*$^{+}$*]*; *ci*$^{94}$*/Dp(1;4)1021[y*$^{+}$*]*, *y w hsp70-flp*; *FRT82 hh*$^{AC}$*/TM6b[y*$^{+}$*]*; *ci*$^{94}$*/Dp(1;4)1021[y*$^{+}$*]*, and *y w hsp70-flp*; *wg*$^{CX4}$*/CyO[y*$^{+}$*]*; *ci*$^{94}$*/Dp(1;4)1021[y*$^{+}$*]* parents, respectively. Embryos of the correct genotype could unambiguously be identified by selecting against *y*$^{+}$ marked balancer chomosomes.

## RESULTS

### Genetic set up

To achieve our goal of activating the Hh pathway proximally while simultaneously closing it distally, we set out to generate *ptc ci* double mutant cells by somatic recombination (Fig. 1). We introduced a genomic *ci*$^{+}$ transgene on the right arm of chromosome 2. This transgene contains all regulatory and coding elements for Ci and fully rescues the null *ci*$^{94}$ mutation homozygously present on the fourth chromosome (Méthot and Basler, 1999; Slusarski et al., 1995). In trans to this *ci*$^{+}$ transgene we placed a null allele of *ptc*, *ptc*$^{IIW}$, which is unable to generate any Ptc protein (Chen and Struhl, 1996). Flp-mediated recombination at cytological position 42 of heterozygous animals (Xu and Rubin, 1993) causes the generation of cell clones that lack any functional copies of the *ptc* and *ci* genes (Fig. 1). Moreover, these mutant cells also lack functional copies of the marker genes *shavenoid* (*sha*, affecting trichomes) or *hsp70-GFP*, which allows the detection of these cells in adult or larval tissues, respectively (Fig. 1).

Below, we analyze the *ptc ci* double mutant genotype in three different situations. First Hh target gene expression is examined in imaginal discs. We then assess the phenotype of adult clones, and finally we look at pattern formation in the embryo.

### Induction of *engrailed* and *ptc*

Hh induces the expression of *engrailed* (*en*) at late stages of wing disc development (Blair, 1992; Sanchez-Herrero et al., 1996; Strigini and Cohen 1997; Alves et al., 1998). This induction takes place ectopically in *ptc* mutant cells located in the anterior compartment (Fig. 2B). By contrast, the induction of *en* expression is completely reverted in *ptc ci* double mutant cells (Fig. 2C).

Another target gene of Hh signaling is *ptc* itself. In all animal systems examined so far, *ptc* expression is highly upregulated in Hh-transducing cells (Forbes et al., 1993; Goodrich et al., 1996; Marigo et al., 1996). To monitor *ptc* transcription in *ptc* mutant cells, we used the *ptc*$^{S2}$ allele, which generates a signaling-inactive Ptc protein that can be visualized with an antibody against Ptc (Chen and Struhl, 1996). *ptc*$^{S2}$ mutant cells strongly upregulate the expression of Ptc (Fig. 3B). This is in contrast to *ci*$^{94}$ cells located near the anteroposterior compartment boundary, in which Ptc upregulation does not occur in response to Hh secreted from posterior compartment cells (Fig. 3A). Clones doubly mutant for *ci* and *ptc* were also incapable of upregulating Ptc$^{S2}$ protein (Fig. 3C). We conclude that Ci is absolutely required for the Hh-induced upregulation of *ptc* and *en* transcription in the wing imaginal disc.

### Regulation of *dpp* transcription

The third case of a Hh target gene examined, *dpp*, is complicated by three circumstances. First, *dpp* expression is regulated by two different transcriptional activities of Ci

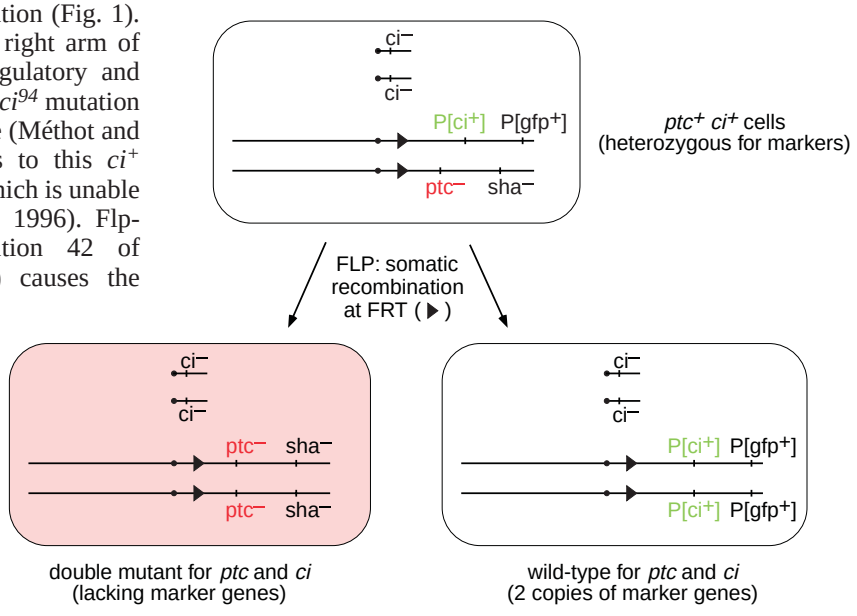


**Fig. 1.** Genetic set-up to test the role of Ci in the output of the Hh signal. The genotype of a somatic cell (top) that undergoes Flp-mediated recombination in G2 phase to give rise to two genetically different daughter cells (bottom), one of which is doubly mutant for *ci* and *ptc*, and is genetically labeled by the *shavenoid* (*sha*) mutation or the lack of green fluorescent protein (GFP) expression. See text for details.

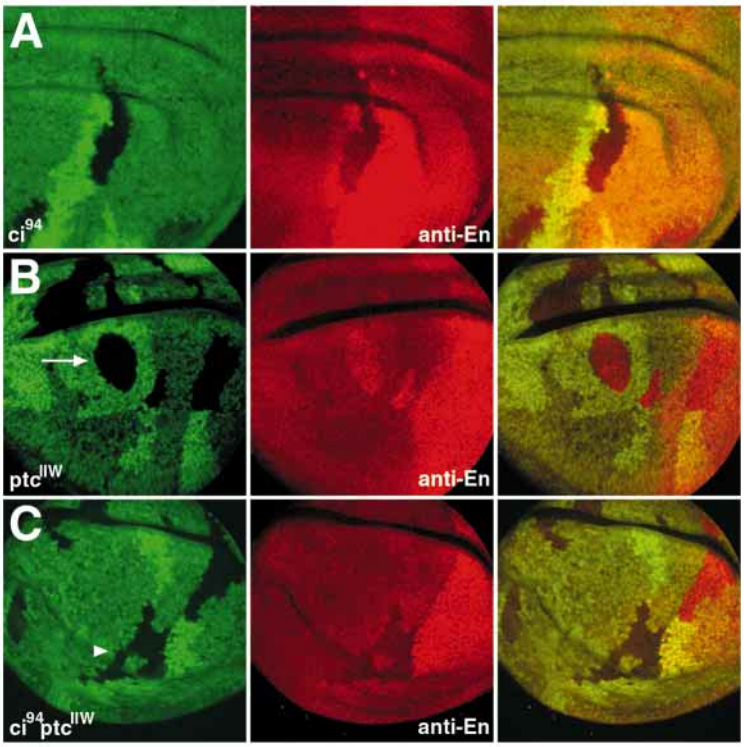


**Fig. 2.** Ci is required for the Hh-mediated induction of En expression. In this and the subsequent two figures, anterior is leftwards and dorsal is upwards. Clones lacking *ci* (A), *ptc* (B) and *ci ptc* (C) are marked by the absence of GFP staining (green). En protein (red) is expressed in all posterior cells and a few anterior cells abutting the compartment boundary of late 3rd instar wing imaginal discs (Blair, 1992). (A) En is not expressed in anterior cells lacking Ci. The anterior origin of this $ci^{94}$ clone is determined by the anterior location of the twin spot (bright green). Posterior $ci^{94}$ clones do not have altered En expression. (B) $ptc^{IIW}$ clones ectopically express En. Note the round, smooth border of these clone (arrow). En expression in posterior clones is unaffected. (C) Anterior cells lacking simultaneously *ptc* and *ci* do not express En. Note the 'wiggly' shape of the clone (arrowhead).

(Méthot and Basler, 1999; Müller and Basler, 2000). The repressor form of Ci prevents basal levels of *dpp* expression in anterior cells that do not receive the Hh signal. *ci* null mutant cells express low levels of *dpp*, irrespective of their position within the anterior compartment. In Hh-transducing cells, the generation of Ci[rep] is prevented and Ci[act] is formed instead, which upregulates the basal transcriptional activity of *dpp*. Hence Hh-receiving and non-Hh-receiving anterior cells react differently to the loss of *ci* activity by reduction or gain of *dpp* transcription, respectively. The second complication is that *dpp* is negatively regulated by En (Sanicola et al., 1995; Méthot et al., 1999). As described above, *ptc* mutant cells express En during late wing disc development, which results in the downregulation of *dpp* expression. To circumvent this problem we generated clones of cells that lacked *en* together with *ptc* or *ci*. Finally, *ci* mutant cells cause *dpp* expression also in neighboring wild-type cells, because anterior $ci^{-}$ cells secrete low levels of Hh (Dominguez et al., 1996; Méthot and Basler, 1999). Moreover, the extent of this non-autonomous *dpp* induction appears to depend on the levels of Ptc present in Hh-receiving cells (see below).

Anterior clones doubly mutant for $ci^{94}$ and $en^{E}$ behave in all respects like $ci^{94}$ single mutant clones (Méthot and Basler, 1999) and express low levels of *dpp* (Fig. 4A). Clones lacking $ptc^{IIW}$ and $en^{E}$ exhibit very high levels of *dpp* expression, regardless of their position within the wing imaginal disc (Fig. 4B). In *ptc en ci* triple mutant clones, the levels of *dpp* expression reverted to those found in *ci en* double mutant or *ci* single mutant anterior clones (Fig. 4C). We noted one difference between *ci en* and *ci ptc en* clones. Non-autonomous *dpp* expression is detected only in a few wild-type cells surrounding *ci en* clones. In contrast, widespread non-autonomous *dpp* transcription is often associated with *ci ptc*, or *ci ptc en* clones (Fig. 4 and data

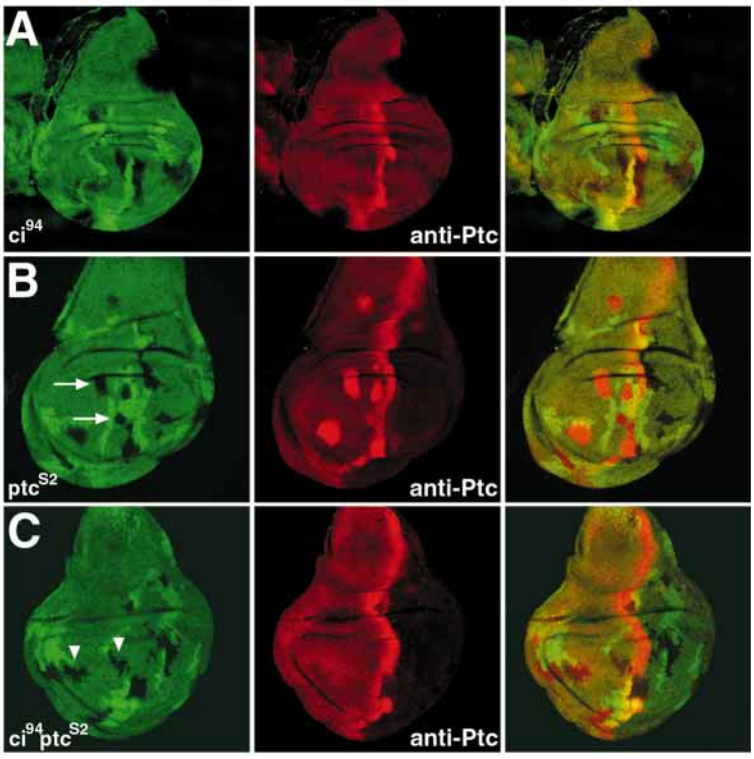


**Fig. 3.** Ci is required for the Hh-induced upregulation of Ptc expression. High levels of Ptc protein (red) are found in a stripe of three to five anterior cells abutting the compartment boundary, in response to Hh signaling. Elsewhere in the A compartment, Ptc is expressed at low levels that do not depend on the Hh signal. (A) Cells lacking Ci do not upregulate Ptc protein levels when located within the endogenous Ptc stripe. No ectopic Ptc upregulation is seen in $ci^{-}$ clones and the low, Hh-independent *ptc* transcription is unaffected. (B) Cells that lack functional Ptc protein (in $ptc^{S2}$ clones) ectopically express Ptc to levels similar to those found in the endogenous Ptc stripe. Note the round, smooth borders of these clones (arrows). (C) Simultaneous removal of Ptc and Ci function does not cause the induction of ectopic $Ptc^{S2}$ protein. Ptc also fails to be upregulated in cells located near the compartment boundary. Note the 'wiggly' shape of the clones (arrowheads).

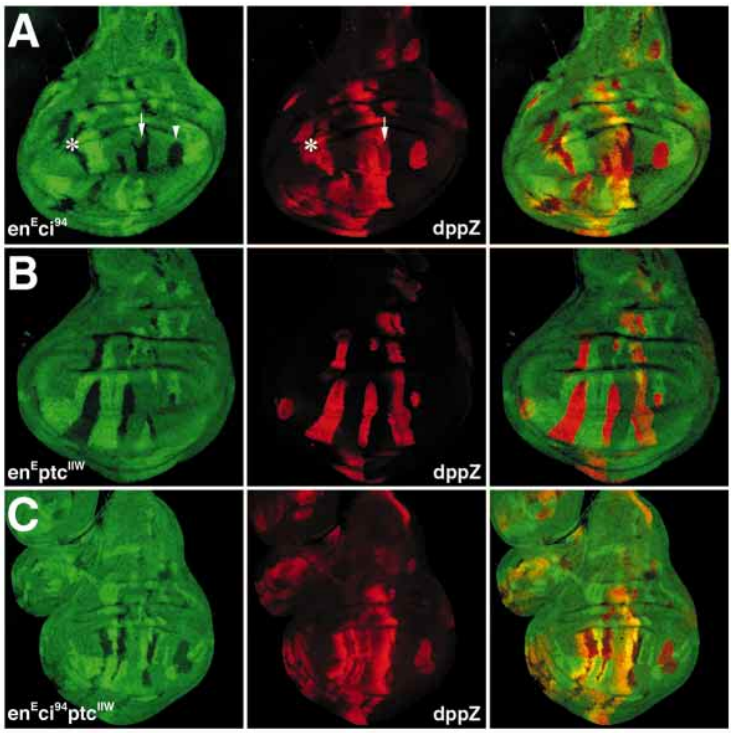


**Fig. 4.** Ci is absolutely required for the proper expression of *dpp*. *dpp* expression (*dpp-lacZ* in red) is controled by at least three transcriptional regulators. En represses *dpp* expression in posterior cells (Méthot and Basler, 1999; Sanicola et al., 1995). Ci[rep] prevents *dpp* expression in far anterior cells, and Ci[act] stimulates the basal *dpp* transcription in cells receiving the Hh signal (Méthot and Basler, 1999). The combined action of these regulators results in a *dpp* expression stripe of seven to ten anterior cells abutting the compartment boundary (Méthot and Basler, 1999). (A) Absence of *ci* and *en* leads to weak ectopic *dpp* expression in far anterior (asterisk) and posterior (arrowhead) cells, and a reduction of *dpp* expression in anterior cells exposed to Hh (arrow). (B) Removal of *ptc* and *en* results in strong ectopic *dpp* expression, irrespective of the clone position. (C) Simultaneous removal of *ptc*, *en* and *ci* recapitulates the effects of removing only *en* and *ci*. In this case however, non-autonomous *dpp* expression is enhanced (see text). Expression levels in A cannot be compared with those in B and C because different PMT settings were used to record the images in these two experiments.

not shown). This difference is due to the reduced $ptc^+$ dosage as these clones were generated in $ptc^{IIW}$/+ heterozygous animals. A twofold difference in levels can cause a discernible effect on the ability of Ptc to sequester Hh or inhibit the signaling activity of Smo (Chen et al., 1996; data not shown). No differences, however, were observed within *ptc en ci* versus *en ci* mutant clones. From this we conclude that maximal activation of the Hh pathway by removal of *ptc* has no effect on *dpp* transcription if Ci is lacking.

### Cell-adhesion states

An important function of Hh signaling during *Drosophila* development is the establishment of different cell adhesion states. Hh-transducing cells sort out from cells that do not transduce the Hh signal, a phenomenon underlying the formation and maintenance of the anteroposterior compartment boundaries (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Lawrence et al., 1999; Dahmann and Basler, 2000). This behavior is faithfully reproduced by *ptc* mutant cell clones. Anterior $ptc^-$ cells minimize contact with neighboring $ptc^+$ cells, causing mutant clones to be round with smooth borders (arrows in Figs 2, 3). The target genes for this behavior are unknown. However, we find that *ptc ci* double mutant cells mix well with surrounding cells, causing mutant clones to exhibit 'wiggly' boundaries (arrowheads in Figs 2, 3), indistinguishable from those of wild-type control clones (not shown). Hence the Hh-induced establishment of different cell adhesion states can not bypass Ci.

### Adult morphogenesis

For all Hh targets examined so far, Ci was absolutely required for their Ptc-mediated regulation. We are aware that we cannot score all Hh target genes individually. Many Hh targets have not yet been identified in *Drosophila*, a situation that challenges our intention to assess the existence of a Ci-independent branch in the Hh pathway. A close approximation to examine all Hh target genes, however, is to examine the complex phenotypes of adult differentiated clones. If the Hh pathway accommodates one or several Ci-independent branches, and if these can have an effect on the morphology or behavior of adult cells, they should be revealed in *ptc ci* double mutant clones.

Several characteristic defects are associated with *ci* mutant wing clones (summarized in Table 1). These include perturbations of veins L2, L3 and L4, as well as wing duplications (Fig. 5A,B). Vein L4, which is located in the posterior compartment, is affected only by *ci* mutant clones of anterior origin that migrate posteriorly, owing to a change in their cell sorting behavior (N. M. and K. B., unpublished observations; Dahmann and Basler, 2000). Vein L4, disrupted by the presence of a *ci* clone, will often bear an ectopic campaniform sensilla (Fig. 5D), a neuronal organ that is normally found in association with vein L3 (Cole and Palka, 1982). *ci* mutant tissue that comprises wing margin territory in the region of disrupted vein L4 can form socketed bristles (Fig. 5C). These structures are normally only found in the anterior compartment, and their formation is repressed by the action of anterior *en* activity (Hidalgo, 1994). This activity depends on Ci (see Fig. 2A) and is therefore not present in *ci* mutant clones that then form ectopic socketed bristles.

Clones mutant for *ptc*, on the other hand, behave differently.

**Table 1. $ptc^{IIW}$ $ci^{94}$ clones have properties similar to $ci^{94}$ clones in adult wings**

| | Structure affected (number of clones) | | | | |
|---|---|---|---|---|---|
| Clone type examined | Survival in region 1 | Vein 2 bifurcation | Vein 3 duplication | Vein 4 defects | Number of wings |
| $ci^{94}$ | 19 | 10 | 15 | 16 | 36 |
| $ptc^{IIW}$ | 1 | 1 | 18 | 0 | 44 |
| $ci^{94}$ $ptc^{IIW}$ | 12 | 8 | 17 | 19 | 67 |

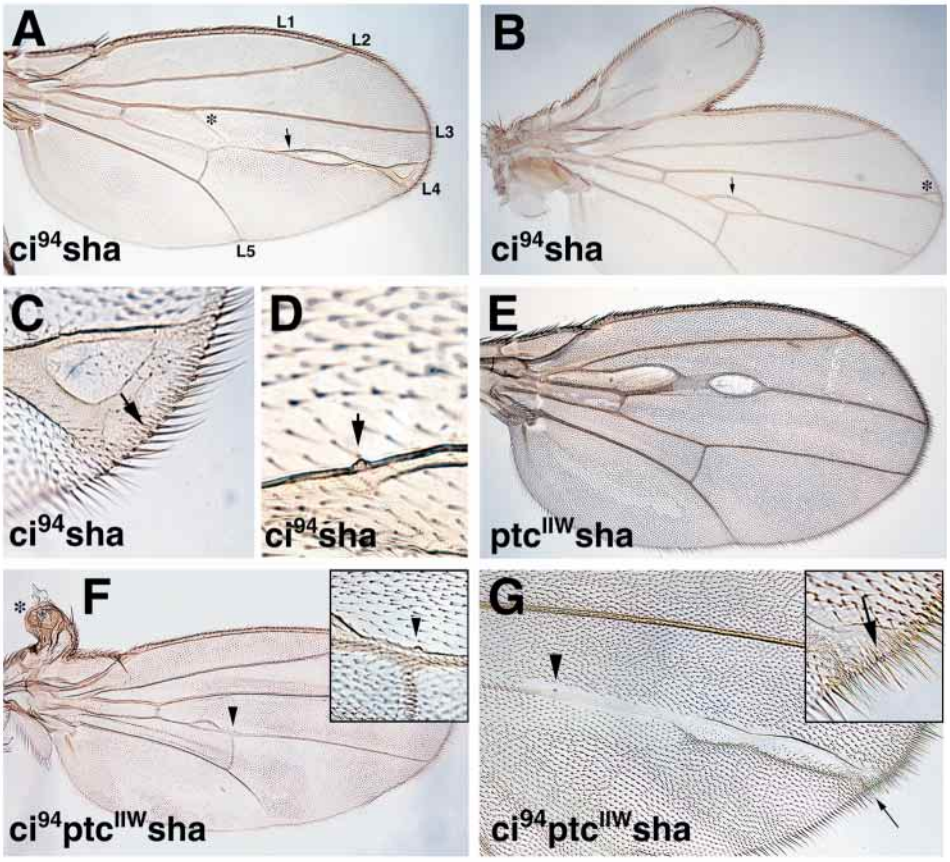


**Fig. 5.** *ptc ci* double mutant clones affect wing morphogenesis in the same manner as *ci* single mutant clones. Vein identities are indicated in A. Clones were identified via the *sha* phenotype. (A-D) Features of wings bearing *ci* clones. Lack of Ci results in forking of L3 (A,B, asterisks), defects in L4 (A,B, arrows) and the formation of ectopic wing tissue (B). (C) High magnification of the tip of L4 in A, showing a socketed bristle. (D) High magnification of L4 in A reveals the presence of a campaniform sensilla. (E) $ptc^{IIW}$ clones in adult wings often lead to the duplication of L3. (F,G) $ptc^{IIW}$ $ci^{94}$ double mutant clones in adult wings behave like $ci^{94}$ clones. Wing duplication (F, asterisk), L4 disruption with campaniform sensilla (F,G, arrowhead), socketed bristles at tip of L4 (G, arrow), and L3 forking (not shown).

Very few clones survive in the region between L1 and L2 ('region 1'), as opposed to *ci* clones (Table 1; Phillips et al., 1990). Duplications of L3 are often observed (Fig. 5E), but never disruption of L4 (Table 1). Clones doubly mutant for *ptc* and *ci* exhibit the same features as *ci* single mutant clones. They survive in region 1, give rise to wing duplications, L3 duplication and forking, and cause L4 disruptions with campaniform sensillae and socketed bristles (Fig. 5F,G; Table 1). Among the numerous clones analyzed, we failed to detect a property of *ptc ci* double mutant clones that could not be observed with *ci* single mutant clones.

Taken together, the molecular and morphological data presented here indicate that Ci is absolutely necessary for all aspects of Hh signaling during wing development. We could not uncover evidence for any output of Hh signaling that bypasses Ci. If parallel pathways exist downstream of Ptc, they must converge on Ci.

## Embryonic segmentation in the absence of Ci

Much of what is known about the Hh signaling pathway derives from studies of the *Drosophila* embryo where Hh plays a key role in the establishment of the segmented body pattern (Nüsslein-Volhard and Wieschaus, 1980). In particular, Hh signaling is critically involved in the subdivision of the ventral larval cuticle into alternating bands of naked and denticle-covered cuticle (Bejsovec and Wieschaus, 1993; Forbes et al., 1993). Naked cuticle is specified by *wg* activity in three to four cell rows anterior to the Wg source, and in one cell row posteriorly (Bejsovec and Martinez Arias, 1991; Bejsovec and Wieschaus, 1993; Gritzan et al., 1999; Sanson et al., 1999). *wg* itself is a Hh target gene, and is expressed in one row of cells anterior to the *en*- and *hh*-expressing cells (Baker, 1988; DiNardo et al., 1988; Hidalgo and Ingham, 1990; Ingham, 1993; Ingham et al., 1988). Lack of Hh results in absence of Wg, and hence absence of naked ventral cuticle (Hidalgo and Ingham, 1990; Ingham et al., 1991; Bejsovec and Wieschaus, 1993; Fig. 6A,B). Ectopic *hh* signaling results in an expansion of the *wg* expression domain and thus an expansion of naked cuticle (Ingham, 1993; Tabata and Kornberg, 1994).

If the Hh pathway obligatorily acts through Ci, as concluded above, it is puzzling that *ci* mutant embryos differ so obviously from *hh* mutant embryos. While cuticles of *hh* mutant embryos are short and show a severe 'lawn of denticle'-phenotype (Nüsslein-Volhard et al., 1984; Fig. 6B), $ci^{94}$ mutant embryos are almost of wild-type size and exhibit a considerable amount of naked cuticle between denticle belts (Slusarski et al., 1995; Fig. 6C). There are at least four possible explanations for the mild segmentation phenotype of *ci* mutant embryos. (1) The $ci^{94}$ allele could not be null in the embryo. (2) Some of the Hh signal may be transduced by maternally provided *ci* product. (3) Ci[rep] may repress some Hh target genes in *hh* but not $ci^{94}$ mutant animals. (4) Embryonic cells may be able to transduce some of the Hh signal by an alternative pathway which bypasses Ci. Below, we address these points individually.

We have carefully characterized the $ci^{94}$ allele (Slusarski et al., 1995) and found it to contain a 5 kb deletion within the *ci* locus. This deletion removes the promoter and the first exon of *ci* including the sites for transcriptional and translational initiation (Méthot and Basler, 1999). No Ci protein derived from this allele can be detected immunologically (Méthot and

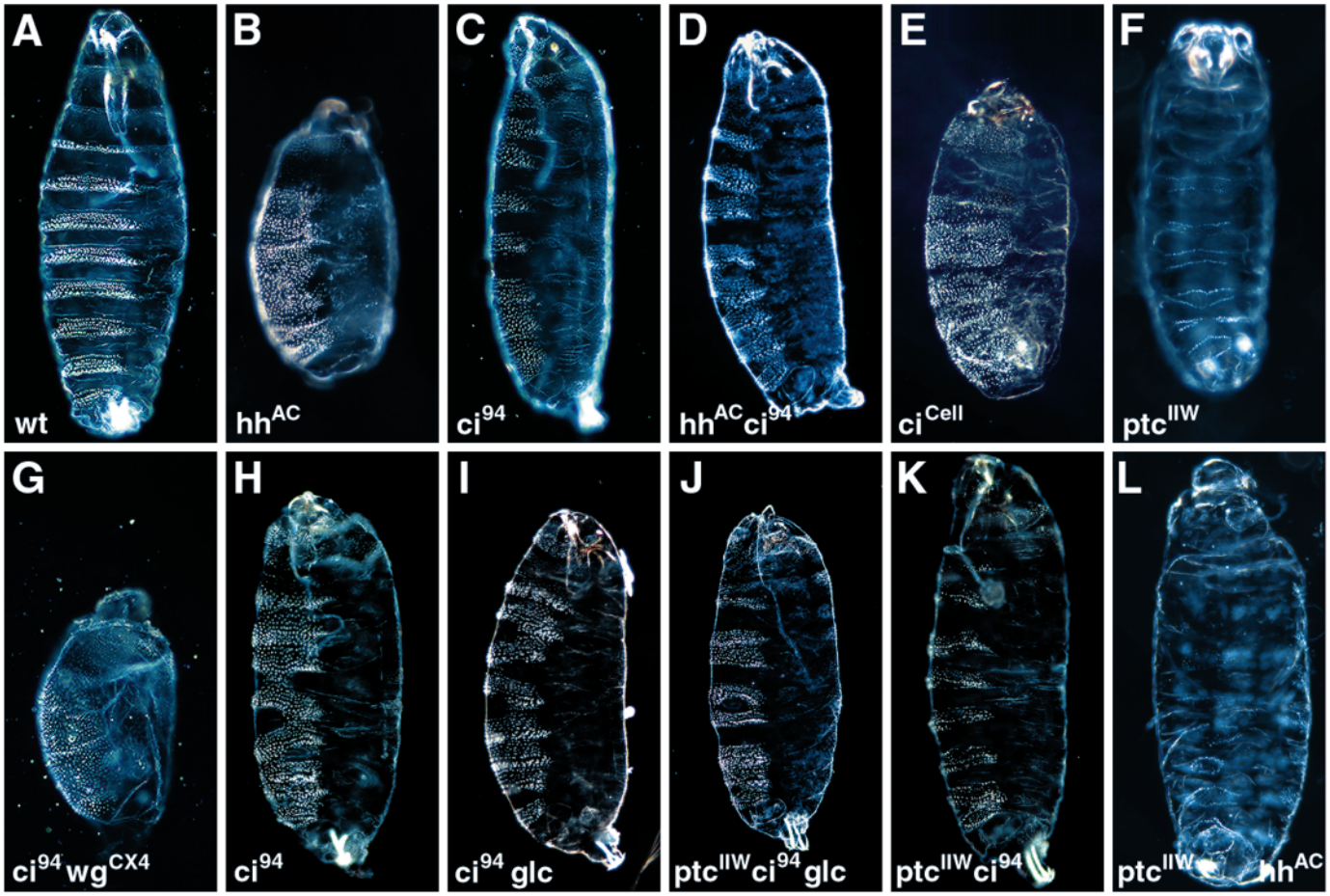


**Fig. 6.** Hh-mediated patterning of the embryonic epidermis is absolutely dependent on Ci activity. In all panels anterior is upwards. Genotypes are indicated at the bottom of each panel. Embryos derived from mutant germ line clones are labeled with 'glc' (I,J). (A-C) The alternation of naked and denticle-covered cuticle observed in wild-type embryos (A) is severely disrupted in *hh* mutant animals (B), which are short and exhibit a 'lawn-of denticle' phenotype, but it is only mildly affected in *ci* mutant embryos (C). (D) *hh ci* double mutant embryos look indistinguishable from *ci*, rather than *hh* single mutant embryos. (E) Cuticles from $ci^{Cell}$ homozygous animals resemble those from *hh* embryos. (H,I) No maternal contribution of Ci is required for epidermal patterning. However, in the absence of *ptc*, some maternal Ci product can transduce the abnormally early activation of the Hh pathway. (H, K) This causes the phenotype of zygotically *ptc ci* mutant embryos (K) to be different from that of *ci* single mutants (H). In contrast, embryos derived from *ptc ci* mutant germ line clones (J) look like *ci* mutants. The main difference between *ci* mutant and *hh* mutant phenotypes must stem from a derepression of *wg* expression in the *ci* versus the *hh* mutant background. *ci wg* double mutants (G) are short and exhibit a 'lawn-of-denticle phenotype', similar to that of *hh* mutants. Finally, the absence of a functional *hh* gene does not aggravate the *ptc* mutant phenotype (F,L).

Basler, 1999; Slusarski et al, 1995). We also investigated the possibility that maternally contributed *ci* product weakens the zygotic null phenotype by generating *ci* mutant germline clones. The location of the *ci* locus on the fourth chromosome precluded the use of classical mitotic recombination to generate germline clones. To circumvent this problem, we made use of a genetic constellation similar to that depicted in Fig. 1, except that we used as a marker a transgene giving rise to maternally contributed GFP (P[*ubi-nlsGFP*]; Luschnig et al., 2000). $ci^{-/-}$ embryos derived from $ci^{94}$ mutant germline clones (Fig. 6I) are indistinguishable from $ci^{-/-}$ embryos derived from heterozygous mothers (Fig. 6H). Thus, the Hh signal is not transduced by maternally provided *ci* product.

We next addressed the possibility that, as in the wing, there are Hh target genes that are repressed as well as activated by Ci. In support of this view, we find that the *hh* mutant phenotype becomes considerably weaker by the additional removal of *ci*. Such double mutant embryos resemble those that lack only *ci* (Fig. 6C,D). This argument can be turned around to deduce that if an embryo lacks *ci*, it makes no difference whether it expresses *hh* or not. This result is a strong indication that there is no Hh output bypassing Ci.

As a further confirmation that $hh^{-}$ embryos have a stronger phenotype than $ci^{-}$ embryos because they continuously generate Ci[rep] we analyzed the cuticles of $ci^{Cell}/ci^{Cell}$ embryos. $ci^{Cell}$ is an allele that can give rise only to the repressor form of Ci due to an 8 bp deletion that results in a truncation of the Ci protein at amino acid 975 (Méthot and Basler, 1999). $ci^{Cell}$ embryos indeed show a segment polarity phenotype that is stronger than that of $ci^{94}$ embryos and resembles more closely that of *hh* mutant embryos (Fig. 6E).

### *wg* as a target of Ci[rep] in *hh* mutant embryos

From the above experiments we conclude that *hh* mutant

embryos are more severely affected than *ci* null mutants, because some Hh target genes may – in the absence of Hh signaling – also be a target of Ci[rep]. In a *ci* null background no Ci[rep] is made, a situation that may lead to a de-repression of such genes. As Wg specifies naked cuticle fate, *wg* is a prime candidate target gene of Ci[rep] in *hh* mutant embryos. Late embryonic expression of *wg* may be repressed by Ci[rep] in a *hh* mutant background, but de-repressed in a *ci* null context. To test this hypothesis, we sought to prevent a potential de-repression of *wg* expression in the *ci* null mutant background. The most effective way to achieve this by genetic means, is to generate *ci wg* double mutant embryos. Indeed, while *ci* single mutant and *ci hh* double mutant embryos are close to wild-type size and show considerable naked cuticle ventrally, *ci wg* double mutants are small and exhibit a 'lawn-of-denticle' phenotype (Fig. 6G). From this we conclude that the de-repression of Ci target genes in *ci* null animals must occur at the level of *wg* transcription and not further downstream in the specification of naked cuticle.

### *ptc ci* double mutant versus *ci* single mutant embryos

If indeed no aspect of the Hh signal is able to bypass Ci, the prediction can be made that *ptc ci* double mutant and *ci* single mutant embryos exhibit identical phenotypes, a condition that we have shown to be met by wing cells. Surprisingly however, *ptc ci* embryos show a higher degree of naked cuticle compared with *ci* mutants (Fig. 6K). The double mutant phenotype clearly differs from that of *ptc* single mutants that retain only two isolated denticle rows per segment (Fig. 6F). These observations suggest that some Hh-transducing activity is still present in *ptc ci* mutant embryos and could be taken as evidence of a Ci-independent output of the Hh signal transduction pathway. Alternatively, a small amount of maternally provided Ci protein might account for the residual Hh-transducing activity in *ptc ci* mutant animals. As shown above, this maternal Ci protein is apparently inconsequential when Hh is secreted normally by *en*-expressing cells, but it may be relevant when the pathway is prematurely activated in *ptc* mutant animals. To test this possibility, we generated embryos that lack maternal *ci* in addition to zygotic *ci* and *ptc*. Such embryos have indeed a phenotype indistinguishable from that of embryos lacking only *ci* (Fig. 6H-J). Hence, in the complete absence of Ci, the loss of *ptc* activity has no effect on embryonic segmentation. This result strengthens our conclusion that the regulation of Ci is the sole output of the Hh signal.

## DISCUSSION

Complex branching schemes with multiple end points have been suggested to account for the multitude of cellular responses elicited by individual signal transduction pathways. We have analyzed whether the pathway transducing the Hh signal has more than a single end point and come to the unambiguous conclusion that this is not the case. The most stringent tool with which questions of pathway linearity can be addressed is genetics. Yet, the precision of any genetic approach depends greatly on the quality of alleles used. Our current analysis is solidly based on well characterized null alleles, for *ci* ($ci^{94}$), *hh* ($hh^{AC}$) and *ptc* ($ptc^{IIW}$). A critical assumption that has been validated by the recent completion of the genome sequence (Adams et al., 2000; Rubin et al, 2000) is the existence of a single Gli gene (*ci*) and a single *hh* gene in *Drosophila*, a condition that is not met by any other genetically amenable model organism. Finally, our analysis is based on the assumption that the sole effect of the Hh signal is the inhibition of Ptc activity. This assumption is legitimated by the observation that the phenotype of *ptc* mutant animals is not affected by the status of the *hh* gene, i.e. *ptc* mutant embryos die with identical patterning defects as *ptc hh* double mutants (Fig. 6F,L; Ramírez-Weber et al., 2000). Hence maximal activation of the pathway can be achieved by genetically removing Ptc.

### No Ci protein, no Hh signaling

The key result of our study is the observation that maximal activation of the Hh pathway (i.e. complete loss of Ptc) has no discernible effect in the absence of Ci. In our opinion, this can be taken as evidence against a distal branching in the Hh signal transduction pathway. Our results do not exclude the existence of alternative pathways between Smo and Ci (Thérond et al., 1999; Méthot and Basler, 2000), yet all these putative branches must converge at Ci.

We note that the indispensability of Ci for Hh signaling also explains how developmental compartments are formed and maintained. The essential difference between cells on opposite sides of the anteroposterior compartment boundary is the responsiveness to Hh. Posterior compartment cells do not respond to the Hh signal, even though they are amply exposed to Hh and appear to possess all but one of the components to transduce Smo activity. The lack of Ci, however, precludes any response to Hh and is thus sufficient to create a population of cells that behaves opposite to that of the anterior, Ci-expressing compartment.

### The default function of Ci

Although we conclude here that Hh signaling has no effect in the absence of Ci, we also conclude that the converse is not the case: Ci *does* have a function in the absence of Hh signaling. This can be illustrated most effectively by comparing a *hh ci* double mutant embryo with a *hh* single mutant one (Fig. 6B,D). Although both animals completely lack the Hh signal, the presence of a functional *ci* gene considerably increases the segment polarity phenotype of *hh* mutants. This effect of Ci is brought about by the default state of Ci, which is the repressor function Ci possesses in the absence of Hh input. We have shown previously that this function is critical for limb development but not essential for embryogenesis (Méthot and Basler, 1999). This is because an uncleavable form of Ci, $Ci^{U}$, can substitute for embryonic Ci in spite of the fact that it cannot form detectable amounts of Ci[rep]. The severe phenotype of *hh* mutant embryos indicates that – although not essential in a wild-type background – Ci[rep] activity can be detrimental in circumstances where Hh signaling is abolished. This situation is reminiscent of the Wg signal transduction pathway, where the nuclear mediator, dTCF/Pangolin, represses Wg target genes in the absence of Wg input (Cavallo et al., 1998; Waltzer and Bienz, 1998). These target genes are partially de-repressed in the absence of dTCF/Pangolin, resulting in a milder segment polarity phenotype compared with that of *wg* null mutants

(Cavallo et al., 1998). An analogous case was recently described for the Notch pathway, where the DNA-binding factor Suppressor of Hairless (Su(H)) has a repressive effect on *single-minded* (*sim*) transcription in the absence of Notch activity, yet mediates *sim* activation upon Notch signaling (Morel and Schweisguth, 2000). It may be a general principle that the transcriptional targets of a signaling pathway are repressed in the absence of the signal. Signal-mediated induction, therefore, requires both the abolition of this repression and the concomitant activation of transcription.

## Conclusions

Based on our analysis, some predictions can be made regarding the Hh pathway in other systems. First, loss-of-function mutations in murine *Gli* genes are likely to cause phenotypes different from equivalent mutations in Hedgehog genes. In particular, even a triple knockout of the *Gli1*, *Gli2* and *Gli3* genes, will presumably behave different from combined mutations in the Sonic, Indian and Desert hedgehog genes. The main reason for postulating this is the Hh-independent repressor function of Gli proteins, which appears to be primarily associated with Gli3. Lack of Shh signaling may lead to an increase of Gli3 repressor activity, while lack of Gli3 expression has the opposite effect. Hence a double *Shh Gli3* mutant may have a considerably milder phenotype than a *Shh* single mutant animal.

Second, given the conservation of the Hh transduction pathway in different species, it is unlikely that the mammalian Hh pathway contains end points other than Gli proteins. The critical but genetically challenging test will be the generation of *Gli* triple mutant mice and their comparison to animals which lack in addition the *Shh* or the *Ptc* gene.

Third, our results challenge several previous studies that claim the existence of Ci-independent outputs of the Hh signaling pathway (see Introduction). Some of these studies were conducted with a *ci* null allele, which removes both activator and repressor functions of Ci. We have previously shown for the wing imaginal disc that lack of Ci[rep] causes the ectopic expression of certain Hh target genes (Dominguez et al., 1996; Méthot and Basler, 1999). We now provide genetic evidence that this is also the case in embryos. We surmise that the seemingly Ci-independent expression of Hh-induced target genes observed by Suzuki and Saigo (2000) and Gallet et al. (2000) may reflect transcriptional derepression, owing to removal of Ci[rep]. In addition, we have discovered a maternal contribution of *ci* that may not only affect the consequences of loss of *ptc* function but also those of experimental *hh* over-expression. Our conclusions are difficult to reconcile, however, with the work of Lessing and Nusse, who have found a Ptc-sensitive enhancer of the *wg* gene that does not contain Ci binding sites (Lessing and Nusse, 1998). It is possible that Ci can bind to DNA via association with other transcription factors rather than by its own DNA-binding activity; more likely, however, this enhancer may be regulated indirectly, by a repressor produced in response to Ci activity.

Finally, we would like to note that although our results are satisfying because of their clear-cut outcome, it is actually surprising that an important signal transduction pathway such as the Hh pathway has only a single outlet. In the absence of parallel end points, the diversity of cellular responses to Hh must be explained by other means. The most likely source for diversity is the developmental state of the cell transducing Hh. This 'developmental state' depends on the history of the cell and can be viewed as the repertoire of transcriptional regulators that affect the interaction of Ci with target genes.

We thank I. Guerrero for anti-Ptc antibody, S. Luschnig and C. Nüsslein-Volhard for providing *FRT42 ubi-nlsGFP* and *FRT82 ubi-nlsGFP* stocks prior to publication, and D. Nellen for generating the $ci^{94}$ $wg^{CX4}$ mutant embryos. We also thank C. Dahmann, R. Mann and B. Müller for critically reading the manuscript, and A. Bejsovec for discussions. This work was supported by the Human Frontier Science Program, the Swiss National Science Foundation and the Kanton of Zürich.

## REFERENCES

**Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al. (**2000). The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185-2195.

**Alcedo, J. and Noll, M.** (1997). Hedgehog and its Patched-Smoothened receptor complex: a novel signalling mechanism at the cell surface. *Biol. Chem.* **378**, 583-590.

**Alves, G., Limbourg-Bouchon, B., Tricoire, H., Brissard-Zahraoui, J., Lamour-Isnard, C. and Busson, D.** (1998). Modulation of Hedgehog target gene expression by the Fused serine-threonine kinase in wing imaginal discs. *Mech. Dev.* **78**, 17-31.

**Aza-Blanc, P., Ramirez-Weber, F. A., Laget, M. P., Schwartz, C. and Kornberg, T. B.** (1997). Proteolysis that is inhibited by *hedgehog* targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89**, 1043-1053.

**Aza-Blanc, P. and Kornberg, T. B.** (1999). Ci – a complex transducer of the Hedgehog signal. *Trends Genet.* **15**, 458-462.

**Baker, N. E.** (1988). Localization of transcripts from the *wingless* gene in whole *Drosophila* embryos. *Development* **103**, 289-298.

**Bejsovec, A. and Martinez Arias, A.** (1991). Roles of *wingless* in patterning the larval epidermis of *Drosophila*. *Development* **113**, 471-485.

**Bejsovec, A. and Wieschaus, E.** (1993). Segment polarity gene interactions modulate epidermal patterning in *Drosophila* embryos. *Development* **119**, 501-517.

**Blair, S. S.** (1992). *engrailed* expression in the anterior lineage compartment of the developing wing blade of *Drosophila*. *Development* **115**, 21-33.

**Blair, S. S. and Ralston, A.** (1997). Smoothened-mediated Hedgehog signalling is required for the maintenance of the anterior-posterior lineage restriction in the developing wing of *Drosophila*. *Development* **124**, 4053-4063.

**Capdevila, J., Estrada, M. P., Sanchez-Herrero, E. and Guerrero, I.** (1994). The *Drosophila* segment polarity gene *patched* interacts with *decapentaplegic* in wing development. *EMBO J.* **13**, 71-82.

**Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M. and Bejsovec, A.** (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature* **395**, 604-608.

**Chen, Y. and Struhl, G.** (1996). Dual roles for *patched* in sequestering and transducing Hedgehog. *Cell* **87**, 553-563.

**Cole, E. S. and Palka, J.** (1982). The pattern of campaniform sensilla on the wing and haltere of *Drosophila melanogaster* and several of its homeotic mutants. *J. Embryol. Exp. Morphol.* **71**, 41-61.

**Dahmann, C. and Basler, K.** (2000). Opposing transcriptional outputs of Hedgehog signaling and *engrailed* control compartmental cell sorting at the *Drosophila* A/P boundary. *Cell* **100**, 411-422.

**Denhardt, D. T.** (1996). Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling. *Biochem. J.* **318**, 729-747.

**DiNardo, S., Sher, E., Heemskerk-Jongens, J., Kassis, J. A. and O'Farrell, P. H.** (1988). Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis. *Nature* **332**, 604-609.

**Dominguez, M., Brunner, M., Hafen, E. and Basler, K.** (1996). Sending and receiving the *hedgehog* signal: control by the *Drosophila* Gli protein Cubitus interruptus. *Science* **272**, 1621-1625.

**Forbes, A. J., Nakano, Y., Taylor, A. M. and Ingham, P. W.** (1993). Genetic analysis of *hedgehog* signalling in the Drosophila embryo. *Development* **118**, Suppl. 115-124.

**Gallet, A., Angelats, C., Kerridge, S. and Thérond, P.P.** (2000). Cubitus interruptus-independent transduction of the Hedgehog signal in Drosophila. *Development* **127**, 5509-5522.

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

**Gritzan, U., Hatini, V. and DiNardo, S.** (1999). Mutual antagonism between signals secreted by adjacent *wingless* and *engrailed* cells leads to specification of complementary regions of the *Drosophila* parasegment. *Development* **126**, 4107-4115.

**Hammerschmidt, M., Brook, A. and McMahon, A. P.** (1997). The world according to *hedgehog*. *Trends Genet.* **13**, 14-21.

**Hidalgo, A.** (1994). Three distinct roles for the *engrailed* gene in *Drosophila* wing development. *Curr. Biol.* **4**, 1087-1098.

**Hidalgo, A. and Ingham, P.** (1990). Cell patterning in the *Drosophila* segment: spatial regulation of the segment polarity gene *patched*. *Development* **110**, 291-301.

**Ingham, P. W.** (1993). Localized hedgehog activity controls spatial limits of wingless transcription in the Drosophila embryo. *Nature* **366**, 560-562.

**Ingham, P. W.** (1998). Transducing Hedgehog: the story so far. *EMBO J.* **17**, 3505-3511.

**Ingham, P. W., Baker, N. E. and Martinez-Arias, A.** (1988). Regulation of segment polarity genes in the *Drosophila* blastoderm by *fushi tarazu* and *even skipped*. *Nature* **331**, 73-75.

**Ingham, P. W., Taylor, A. M. and Nakano, Y.** (1991). Role of the *Drosophila patched* gene in positional signalling. *Nature* **353**, 184-187.

**Kingsley, D. M.** (1994). The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev.* **8**, 133-146.

**Krishnan, V., Elberg, G., Tsai, M. J. and Tsai, S. Y.** (1997a). Identification of a novel *sonic hedgehog* response element in the chicken ovalbumin upstream promoter-transcription factor II promoter. *Mol. Endocrinol.* **11**, 1458-1466.

**Krishnan, V., Pereira, F. A., Qiu, Y., Chen, C. H., Beachy, P. A., Tsai, S. Y. and Tsai, M. J.** (1997b). Mediation of Sonic hedgehog-induced expression of COUP-TFII by a protein phosphatase. *Science* **278**, 1947-1950.

**Lawrence, P. A., Casal, J. and Struhl, G.** (1999). The *hedgehog* morphogen and gradients of cell affinity in the abdomen of *Drosophila*. *Development* **126**, 2441-2449.

**Lawrence, P. A. and Struhl, G.** (1996). Morphogens, compartments, and pattern: lessons from *Drosophila*? *Cell* **85**, 951-961.

**Lee, J. J., von Kessler, D. P., Parks, S. and Beachy, P. A.** (1992). Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene *hedgehog*. *Cell* **71**, 33-50.

**Lessing, D. and Nusse, R.** (1998). Expression of *wingless* in the *Drosophila* embryo: a conserved cis-acting element lacking conserved Ci-binding sites is required for *patched*-mediated repression. *Development* **125**, 1469-1476.

**Lewis, K. E., Drossopoulou, G., Paton, I. R., Morrice, D. R., Robertson, K. E., Burt, D. W., Ingham, P. W. and Tickle, C.** (1999). Expression of *ptc* and *gli* genes in *talpid3* suggests bifurcation in Shh pathway. *Development* **126**, 2397-2407.

**Luschnig, S., Krauss, J., Bohmann, K., Desjeux, I. and Nusslein-Volhard, C.** (2000). The *Drosophila* SHC adaptor protein is required for signaling by a subset of receptor tyrosine kinases. *Mol. Cell* **5**, 231-241.

**Marigo, V., Scott, M. P., Johnson, R. L., Goodrich, L. V. and Tabin, C. J.** (1996). Conservation in *hedgehog* signaling: induction of a chicken *patched* homolog by Sonic hedgehog in the developing limb. *Development* **122**, 1225-1233.

**Mason, I. J.** (1994). The ins and outs of fibroblast growth factors. *Cell* **78**, 547-552.

**McMahon, A. P.** (2000). More surprises in the Hedgehog signaling pathway. *Cell* **100**, 185-188.

**Méthot, N. and Basler, K.** (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. *Cell* **96**, 819-831.

**Méthot, N. and Basler, K.** (2000). Suppressor of Fused opposes Hedgehog signal transduction by impeding nuclear accumulation of the activator form of Cubitus interruptus. *Development* **127**, 4001-4010.

**Morel, V. and Schweisguth, F.** (2000). Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the Drosophila embryo. *Genes Dev.* **14**, 377-388.

**Müller, B. and Basler, K.** (2000). The repressor and activator forms of Cubitus interruptus control Hedgehog target genes through common generic Gli-binding sites. *Development* **127**, 2999-3007.

**Neumann, C. and Cohen, S.** (1997). Morphogens and pattern formation. *BioEssays* **19**, 721-729.

**Nüsslein-Volhard, C. and Wieschaus, E.** (1980). Mutation affecting segment number and polarity in Drosophila. *Nature* **287**, 795-801.

**Nüsslein-Volhard, C., Wieschaus, E. and Kluding, H.** (1984). Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster I Zigotic loci on the second chromosome. *Roux's Arch. Dev. Biol.* **193**, 267-282.

**Phillips, R. G., Roberts, I. J., Ingham, P. W. and Whittle, J. R.** (1990). The *Drosophila* segment polarity gene *patched* is involved in a position-signalling mechanism in imaginal discs. *Development* **110**, 105-114.

**Ramírez-Weber, F.-A., Casso, D. J., Aza-Blanc, P., Tabata, T. and Kornberg, T. B.** (2000). Hedgehog signal transduction in the posterior compartment of the *Drosophila* wing imaginal disc. *Mol. Cell* **6**, 479-485.

**Rodriguez, I. and Basler, K.** (1997). Control of compartmental affinity boundaries by *hedgehog*. *Nature* **389**, 614-618.

**Rubin, G. M., et al. (**2000). Comparative genomics of the eukaryotes. *Science* **287**, 2204-2215.

**Ruiz i Altaba, A.** (1999). Gli proteins and Hedgehog signaling: development and cancer. *Trends Genet.* **15**, 418-425.

**Sanchez-Herrero, E., Couso, J. P., Capdevila, J. and Guerrero, I.** (1996). The *fu* gene discriminates between pathways to control *dpp* expression in *Drosophila* imaginal discs. *Mech. Dev.* **55**, 159-170.

**Sanicola, M., Sekelsky, J., Elson, S. and Gelbart, W. M.** (1995). Drawing a stripe in *Drosophila* imaginal disks: negative regulation of *decapentaplegic* and *patched* expression by *engrailed*. *Genetics* **139**, 745-756.

**Sanson, B., Alexandre, C., Fascetti, N. and Vincent, J. P.** (1999). *engrailed* and *hedgehog* make the range of Wingless asymmetric in *Drosophila* embryos. *Cell* **98**, 207-216.

**Schamel, W. W. and Dick, T. P.** (1996). Signal transduction: specificity of growth factors explained by parallel distributed processing. *Med. Hypoth.* **47**, 249-255.

**Schweitzer, R. and Shilo, B.-Z.** (1997). A thousand and one roles for the *Drosophila* EGF receptor. *Trends Genet.* **13**, 191-196.

**Selbie, L. A. and Hill, S. J.** (1998). G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways. *Trends Pharmacol. Sci.* **19**, 87-93.

**Slusarski, D. C., Motzny, C. K. and Holmgren, R.** (1995). Mutations that alter the timing and pattern of *cubitus interruptus* gene expression in *Drosophila melanogaster*. *Genetics* **139**, 229-240.

**Strigini, M. and Cohen, S. M.** (1997). A Hedgehog activity gradient contributes to AP axial patterning of the *Drosophila* wing. *Development* **124**, 4697-4705.

**Suzuki, T. and Saigo, K.** (2000). Transcriptional regulation of *atonal* required for *Drosophila* larval eye development by concerted action of *eyes absent*, *sine oculis* and *hedgehog* signaling independent of *fused kinase* and *cubitus interruptus*. *Development* **127**, 1531-1540.

**Tabata, T. and Kornberg, T. B.** (1994). Hedgehog is a signaling protein with a key role in patterning *Drosophila* imaginal discs. *Cell* **76**, 89-102.

**Tabata, T., Schwartz, C., Gustavson, E., Ali, Z. and Kornberg, T. B.** (1995). Creating a *Drosophila* wing de novo, the role of *engrailed*, and the compartment border hypothesis. *Development*, **121**, 3359-3369.

**Thérond, P.P., Limbourg-Bouchon, B., Gallet, A., Dussilol, F., Pietri, T., van den Heuvel, M. and Tricoire, H.** (1999). Differential requirements of the Fused kinase for Hedgehog signaling in the Drosophila embryo. *Development* **126**, 4039-4051.

**Waltzer, L. and Bienz, M.** (1998). Drosophila CBP represses the transcription factor TCF to antagonize Wingless signalling. *Nature* **395**, 521-525.

**Wodarz, A. and Nusse, R.** (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.

**Xu, T. and Rubin, G. M.** (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.

**Zecca, M., Basler, K. and Struhl, G.** (1995). Sequential organizing activities of *engrailed*, *hedgehog* and *decapentaplegic* in the *Drosophila* wing. *Development* **121**, 2265-2278.

**Zhang, Y. and Derynck, R.** (1999). Regulation of Smad signalling by protein associations and signalling crosstalk. *Trends Cell Biol.* **9**, 274-279.

**Zwick, E., Wallasch, C., Daub, H. and Ullrich, A.** (1999). Distinct calcium-dependent pathways of epidermal growth factor receptor transactivation and PYK2 tyrosine phosphorylation in PC12 cells. *J. Biol. Chem.* **274**, 20989-20996.