A positive role for Patched-Smoothened signaling in promoting cell proliferation during normal head development in Drosophila

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Accepted 14 January 2002

SUMMARY

The transmembrane receptor Patched regulates several developmental processes in both invertebrates and vertebrates. In vertebrates, Patched also acts as a tumor suppressor. The Patched pathway normally operates by negatively regulating Smoothened, a G-protein-coupled receptor; binding of Hedgehog ligand to Patched relieves this negative interaction and allows signaling by Smoothened. We show that Ptc regulates Drosophila head development by promoting cell proliferation in the eye-antennal disc. During head morphogenesis, Patched positively interacts with Smoothened, which leads to the activation of Activin type I receptor Baboon and stimulation of cell proliferation in the eye-antennal disc. Thus, loss of Ptc or Smoothened activity affects cell proliferation in the eye-antennal disc and results in adult head capsule defects. Similarly, reducing the dose of smoothened in a patched background enhances the head defects. Consistent with these results, gain-of-function Hedgehog interferes with the activation of Baboon by Patched and Smoothened, leading to a similar head capsule defect. Expression of an activated form of Baboon in the patched domain in a patched mutant background completely rescues the head defects. These results provide insight into head morphogenesis, a process we know very little about, and reveal an unexpected non-canonical positive signaling pathway in which Patched and Smoothened function to promote cell proliferation as opposed to repressing it.

Key words: patched, Head, Drosophila

INTRODUCTION

Much of the adult structures in Drosophila are formed by imaginal discs, which are composed of cells with special proliferative and differentiation properties. The cells of these imaginal discs undergo a highly organized and controlled program of cell proliferation and developmental patterning during post-embryonic larval and pupal stages. This process, combined with the histolysis of larval structures, leads to the formation of adult body parts (reviewed by Bodenstein, 1950; Bryant, 1978; Cohen, 1993; Fristrom and Fristrom, 1993). As the adult structures that comprise a large number of cells are formed from a small number of imaginal cells, regulated proliferation of cells is one of the most important aspects of imaginal development. Following the 16th embryonic cell division, imaginal discs undergo G1 arrest (Foe et al., 1993). The future mitotic growth of imaginal cells (re-entry into cell-cycle) is tightly controlled and varies between different discs.

Of all the adult structures, morphogenesis of the Drosophila adult head capsule is a poorly understood developmental process. The eye-antennal discs primarily contribute to the formation of adult head, while the labial discs give rise to most of the proboscis. In the early larvae, the two contralateral eye-antennal discs that develop into the two halves of the adult head, are connected by a thin layer of squamous cells (Madhavan and Schneiderman, 1977). The proliferation of the eye-antennal disc begins only after 14 hours into the first instar larval stage (Madhavan and Schneiderman, 1977). With the progression of metamorphosis, the disc cells spread out to fuse with the contralateral disc along the midline. This results in a contiguous epidermal layer and a lumen forming the head sac. At about 12 hours after puparium formation (APF), the head sac evaginates, exposing the primordial eye, the antenna and the head capsule cells to the surface, each of which continue to develop and differentiate into respective structures. An extensive clonal analysis by Haynie and Bryant (Haynie and Bryant, 1986) has revealed a detailed fate map of the eye-antennal disc and the primordia of different head structures. The antennal half has imaginal cells for the three segments of the antenna, which are arranged in three concentric rings, for the maxillary palp and part of the head capsule. The cells surrounding the eye primordium together with the peripodial cells form the rest of the head capsule including the ocellar and the frontal regions. This patterning of the eye-antennal disc to give rise to adult structures occurs much later during larval-pupal transition stage.

Despite these studies, very little is known about the regulation of formation of the adult head structures. Only a few genes that regulate the events that lead to formation of the head capsule from the eye-antennal disc have been identified (Weaver and White, 1995; Royet and Finkelstein, 1996; Amin et al., 1999). For example, we are not yet certain even how...
many segments contribute to it. The dissection of the developmental events that lead to the formation of the head capsule has been complicated mainly because of the fact that the head capsule is a difficult and complex structure. Moreover, the genes that might be required for the development of the head are likely to be also required for embryonic development. Mutations in these genes will lead to embryonic lethality, and one never gets to analyze their role in post-embryonic stages of development in a genetically straightforward manner, thus, contributing to the difficulty in analyzing the adult head development.

Previous results indicate that several segment polarity genes such as hedgehog, wingless and orthodenticle are involved in the patterning of the ocellar and frontal regions of the head capsule (Royet and Finkelstein, 1996; Amin et al., 1999). Over the past few years, we have been examining the role of one of the segment polarity genes, patched (ptc), during the development of the nervous system (Bhat, 1996; Bhat and Schedl, 1997; Bhat, 1999). The ptc gene, which encodes a transmembrane protein, regulates a number of developmental events in both invertebrates and vertebrates. In vertebrates, loss of Ptc activity leads to nevoid basal cell carcinoma, medulloblastoma, spina bifida and several other developmental defects (Hahn et al., 1996; Johnson et al., 1996; Goodrich and Scott, 1998; Bhat, 1999). The Ptc protein has been shown to be a receptor for Hedgehog (Hh). Interaction of Hh with Ptc relieves the Ptc-mediated suppression of Smoothened (Smo), a G-protein coupled seven-pass transmembrane molecule, allowing Smo to activate downstream target genes. In the absence of Hh activity, Ptc functions as a repressor of Smo and thus, the repressor of downstream target genes.

In a modifier screen for genes that interact with ptc (see Materials and Methods), we isolated a mutation in the ptc gene, which, in combination with various loss-of-function alleles of ptc, showed severe head capsule defects. In this pathway, Ptc positively interacts with Smo and activates Baboon (Babo), the Activin type I receptor, to promote cell proliferation. Thus, when Ptc or Smo activities are eliminated, cell proliferation in the eye-antennal disc is affected and the adults show severe head capsule defects. Moreover, reducing the dose of smo in ptc background enhances the head defects. However, gain-of-function Hedgehog interferes with the activation of Babo by Ptc and Smo, causing head defects. That the role of Ptc-Smo signaling in head morphogenesis is to promote cell proliferation via activation of Babo is indicated by the fact that expression of an activated form of Babo in the ptc domain in a ptc mutant background completely rescues the head defects. These results provide insight into head morphogenesis and reveal an unexpected positive role for Ptc-Smo signaling in promoting cell proliferation.

### MATERIALS AND METHODS

**ptc**<sup>hdl</sup> allele

ptc<sup>hld</sup> allele was isolated in an F<sub>1</sub> modifier screen using the standard procedures and screening against ptc<sup>IN108</sup>/Cyo (ptc<sup>IN108</sup> is a null allele of ptc; Cyo is a second chromosome balancer). The progeny were screened for trans-heterozygous flies (ptc<sup>+</sup>; * could be on the second or on the other chromosomes) that showed any type of visible defects. One of the individuals that we obtained in this screen had unilateral head capsule defects. This ptc<sup>+</sup> individual survived and produced progeny, allowing us to establish the mutant line, headless (hdl). This hdl mutation was on the second chromosome and by recombination it mapped to the ptc locus. Further analysis showed that hdl is a loss of function hypomorphic allele of ptc.

**Other fly stocks and genetics**

Other stocks used were Df (2R) NP3/Cyo (eliminates both babo and ptc), Df (2R) NP1 (eliminates babo but not ptc), babo<sup>52</sup> (null allele), babo<sup>775</sup>, puni<sup>10460</sup>, rpr, wrinkle, grim [Df (3)H99], hs-hh and various ptc alleles (see Tables 1 and 2). The temperature-sensitive hh allele we used was hh<sup>ts2</sup> (Ma et al., 1996). Appropriate markers were used to identify the mutant versus the balancer-bearing individuals.

**Generating ptc and smo null mitotic clones**

ptc null clones were generated using the FRT/FLP system (Xu and Rubin, 1993). The ptc<sup>IN108</sup> allele was recombined on an FRT-containing second chromosome [w<sup>118</sup>: P (ry<sup>+</sup>, neo FRT) 42D P(w<sup>+</sup> +, piM) 45F]. These recombinants were crossed to hs-FLP stock [y, w, P hs-FLP 122: P (ry<sup>+</sup>, neo FRT) 42D P(w<sup>+</sup> +, piM) 45F] and the progeny from this cross were given heat shock for 1 hour at 37°C during second and early third instar larval stage, and then allowed to grow at 25°C until eclosion. The dead pharate adults were dissected out of the pupal case and checked for the head phenotype. Heat shock of wild type during larval or pupal stages does not cause these head capsule defects. To generate smo null clones, two smo FRT strains, y,w; smo<sup>11G16</sup>, FRT 40A/Cyo and 51Z smo<sup>11H6</sup> FRT 40A/Cyo, were used. These flies were crossed to y,w,P hs-FLP; P[w<sup>+</sup>mCt=piM]36F P [ry<sup>+</sup> (7.2=neoFRT) 40A] females. The progeny were given heat shock for 1 hour at 37°C during second to early third instar larval stage. After the heat shock, larvae were allowed to develop at 25°C.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Transheterozygotes showing the phenotype (%)</th>
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<tr>
<td>ptc&lt;sup&gt;hld&lt;/sup&gt;/ptc&lt;sup&gt;52&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>ptc&lt;sup&gt;hld&lt;/sup&gt;/ptc&lt;sup&gt;9b28&lt;/sup&gt;</td>
<td>0</td>
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<td>ptc&lt;sup&gt;hld&lt;/sup&gt;/ptc&lt;sup&gt;6P43&lt;/sup&gt;</td>
<td>50</td>
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<tr>
<td>ptc&lt;sup&gt;hld&lt;/sup&gt;/ptc&lt;sup&gt;84&lt;/sup&gt;</td>
<td>100</td>
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<tr>
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<td>83</td>
</tr>
<tr>
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<td>8</td>
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<tr>
<td>ptc&lt;sup&gt;hld&lt;/sup&gt;/ptc&lt;sup&gt;4R888&lt;/sup&gt;</td>
<td>100</td>
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<td>75</td>
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<td>100</td>
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<td>100</td>
</tr>
<tr>
<td>ptc&lt;sup&gt;hld&lt;/sup&gt;/ptc&lt;sup&gt;IN12&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>ptc&lt;sup&gt;hld&lt;/sup&gt;/ptc&lt;sup&gt;IN104&lt;/sup&gt;</td>
<td>3</td>
</tr>
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Between 100 and 200 transheterozygotes were examined in each experiment. While all the ptc alleles shown here are embryonic lethals, they show different levels of complementation with ptc<sup>hld</sup> allele in terms of head capsule defects. The mutation in ptc<sup>52</sup> is a D584N amino acid change and maps to the sterol-sensing domain (Strutt et al., 2001). In ptc<sup>IN108</sup>, it is a L83Q change and maps to the first TM domain (Strutt et al., 2001). In ptc<sup>IN108</sup>, it is a S809N change and maps to the extracellular loop (Strutt et al., 2001). However, according to Hooper and Scott (Hooper and Scott, 1989), this allele carries a 0.2 kb insertion at position +165. The nature of the mutations in the other alleles is not known.
BrdU incorporation was as described elsewhere (Truman and Bate, 1988) and the staining was as described elsewhere (Prokop and Technau, 1994) with slight modifications. Mid third instar larvae were fed with 1 mg/ml BrdU in their cornmeal media and also in yeast paste. They were grown in BrdU-containing food for 12 hours and then in normal medium for 6 hours before sacrificing. The dissected eye-antennal discs were fixed in Carnoy’s fixative (3:1 ethanol:acetic acid), after rehydration, treated with 2 N HCl for 30 minutes. The discs were incubated with Biotinylated anti-BrdU (Becton-Dickinson) at 1:200 dilution for 36 hours at 4°C and developed by HRP reaction.

Analysis of gain of function effects of hh
Second instar to early third instar hs-hh transgenic larvae were given series of heat shocks at 37°C for 20 minutes with an interval of 12 hours in between, and were continuously grown at 29°C during the intervals. The unclosed pharate adults were dissected out of the pupal case to check for the head phenotype.

Rescue of the head capsule phenotype with activated babo
A constitutively activated form of Babo (Baboact) was ectopically expressed with two different gal4 drivers in a ptc mutant background. UAS-baboact carries a constitutively active form of Babo in which glutamine at position 302 is replaced by aspartic acid (Wieser et al., 1995). In the first set of experiments, UAS-baboact (on the III chromosome) was crossed to the ptc deficiency, Df (2R)NP3/Cyo. The individuals bearing Df (2R)NP3 and UAS-baboact were then crossed to ptcgal4. In the second set of crosses, ptcH84f1; UAS-baboact/+ flies were crossed to ptcH84/+; P[w+mW.hs=GawB] 69B-GAL4/+ (Gal4 in this line is expressed in embryonic epidermis and in all the imaginal discs, Bloomington Stock number 1772: w; P[w+mW.hs=GawB] 69B/TM3,Sb1). The embryos were grown at 16.5°C until early second instar larval stage in order to avoid early lethality caused by ectopic expression of activated Babo (Gal4 activity is very low at 16.5°C), and then shifted to 25°C for the rest of their developmental period.

Immunostaining, whole-mount RNA in situ
For immunostaining the discs were dissected in cold PBS, fixed on ice for 45 minutes in freshly made 4% paraformaldehyde in PBS with lysine and sodium periodate. They were incubated with primary antibody in 0.05% PBSTx containing 10% natural goat serum. Anti-Ptc antibody was used at 1:5 dilution and anti-phosphohistone3 antibody (Upstate Biotechnology) at 1:1000 dilution. FITC-conjugated anti-mouse IgG, and Cy5-conjugated anti-rabbit IgG were used as secondary antibody for anti-Ptc and anti-phosphohistone 3, respectively. The cytoplasmatic actin was marked using FITC-Phalloïdin (Molecular Probes). Whole-mount RNA in situ was carried out using the standard procedures.

RESULTS

Isolation of a new allele of ptc
In a genetic screen to identify adult specific mutations that interact in trans with a phenotypic null allele, ptcIn108, we isolated a new hypomorphic loss-of-function allele of ptc, ptcheadless (ptchdl), which showed head capsule defects in trans to null alleles of ptc (K. M. B., unpublished). In this allele, the hydrophobic amino acid valine was changed to the hydrophilic methionine at position 1129 in the intracellularly located C terminus of the Ptc protein (N. Mortimer and K. M. B., unpublished). The overt head capsule phenotype in adults transheterozygous for ptchdl and the null alleles of ptc are shown in Fig. 1. While every ptchdl/ptcIn108 (or ptchdl/ptcH84, ptcH84 – another phenotypic null allele) individual had mis-

### Table 2. Genetic interaction between ptc, smo and babo

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Transheterozygotes showing the phenotype (%)</th>
</tr>
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<tbody>
<tr>
<td>ptcH84/ptchdl</td>
<td>100</td>
</tr>
<tr>
<td>ptcH84/ptc108</td>
<td>4</td>
</tr>
<tr>
<td>ptcH84/ptcheadless</td>
<td>3</td>
</tr>
<tr>
<td>ptcH84/ptcheadless</td>
<td>0</td>
</tr>
<tr>
<td>ptcH84, smo1/ptc93H44, +</td>
<td>35</td>
</tr>
<tr>
<td>ptcH84, smo1/ptcheadless, +</td>
<td>100</td>
</tr>
<tr>
<td>ptcH84, smo1/+, +</td>
<td>2</td>
</tr>
<tr>
<td>babo7777/Df(2R)NP1</td>
<td>100</td>
</tr>
<tr>
<td>ptcH84, +/+; babo-22</td>
<td>5</td>
</tr>
<tr>
<td>ptcH84/Df(2R)NP3</td>
<td>67</td>
</tr>
<tr>
<td>ptcH84/Df(2R)NP3; babo7777, +</td>
<td>100</td>
</tr>
<tr>
<td>ptcH84/Df(3L)H99/ptcheadless</td>
<td>100</td>
</tr>
<tr>
<td>ptcH84, smo1/+, +</td>
<td>0</td>
</tr>
<tr>
<td>ptcH84, smo1/+, babo7777, +</td>
<td>10</td>
</tr>
<tr>
<td>ptcH84, Df(2R)NP3/babo7777, +</td>
<td>100</td>
</tr>
<tr>
<td>ptcH84/punt</td>
<td>0</td>
</tr>
<tr>
<td>ptcH84, Df (3L) H99/ptchdl, +</td>
<td>100</td>
</tr>
<tr>
<td>ptcH84/Df(2R)NP3; UAS baboact</td>
<td>0</td>
</tr>
<tr>
<td>ptcH84/69B-gal4/ptcheadless, UAS-baboact</td>
<td>0</td>
</tr>
<tr>
<td>ptcH84/69B-gal4/ptchdl</td>
<td>100</td>
</tr>
</tbody>
</table>

Between 100 and 200 transheterozygotes were examined in each experiment. Df (2R)NP3 eliminates babo but not ptc, whereas Df (2R)NP3 eliminates both ptcH84 and ptcheadless gives identical results, thus considered equivalent and null as far as the head capsule phenotype is concerned (see also Table 1). Df (3L) H99 eliminates reaper, wrinkled and grim.

### Sequencing of the ptc gene in ptchdl allele

ptchdl homozygous embryos were identified by the presence of CNS defects (visualized by Eve staining) and lack of balancer-specific staining. Genomic DNA from 12 homozygous embryos were individually prepared and genomic DNA corresponding to ptc exons was amplified from these individual DNA preparations. A total of 14 nested pairs of oligos were used. Embryos that are homozygous for the balancer as well as wild-type Canton S flies were used as control. The amplified DNA fragments were then sequenced in both directions.

### In vitro culture of eye-antennal discs

Late 3rd instar (getting immobile or nearly immobile with only its head showing slight movement) larval discs from mutant and wild type were dissected in insect cell culture medium CCM3 (HyClone). It is very important to dissect the culture discs out in the cold culture medium itself and transfer to the fresh medium with the mouth hook, brain and leg discs intact so that the structures are least disturbed. The discs are incubated with the CCM3 medium in a sterile 24-well tissue culture plate (the wells were thoroughly cleaned with alcohol). The culture plate was placed in a clean, alcohol cleaned humid box, and incubated at 25°C overnight. As control, we used ptcH84/CyoO or ptcH84/CyoO and wild type (Canton-S). The discs were then fixed in 4% paraformaldehyde for 30 minutes and stained with FITC-conjugated Phalloïdin (1:50 dilution). The mouth hook and the brain were cleaned off and the discs were mounted in Vector Shield mounting medium. Note that discs from wandering 3rd instar larvae did not show any significant signs of differentiation in vitro. It seems therefore that the stage of larval development is important for in vitro culturing of discs. Also, the leg discs seem to respond the most in vitro since we could make out the leg-primordium following overnight culture. Discs for the in vivo analysis were dissected out from late third instar larvae. In both cases the discs were subsequently fixed in freshly made 4% paraformaldehyde and then stained with FITC-conjugated Phalloïdin (1:50 dilution) and examined by confocal microscopy.

BrdU incorporation experiment and staining

BrdU incorporation was as described elsewhere (Truman and Bate,
formed head capsules (Fig. 1B-D), between individuals, the head capsule defects ranged from mild (Fig. 1B, 51%, \( n = 220 \)), moderate (Fig. 1C, 10%) to severe (Fig. 1D, 39%). This variability in the severity of head capsule defects could be either due to the hypomorphic nature of the \( ptc^{hdl} \) allele or a genetic redundancy for the Ptc-pathway during head morphogenesis.

Furthermore, as shown in Fig. 1E, the same head capsule defect is observed in individuals that are transheterozygous for \( ptc \) null alleles and \( ptc_{gal4} \) [\( ptc_{gal4} \) is a hypomorphic allele where the \( gal4 \) gene is inserted in the regulatory sequence of \( ptc \) (Wilder and Perrimon, 1995)]. Moreover, \( ptc^{hdl} \) or \( ptc^{gal4} \) showed head capsule defects in trans with each other and also with several different \( ptc \) alleles (see Tables 1 and 2). We also generated \( ptc \)-null mitotic clones using the FLP-FRT technique (see Materials and Methods) and as shown in Fig. 1F, we observed the same head capsule defect in these individuals as in the \( ptc^{hdl}/ptc^{-} \) null combination. These results indicate that \( ptc^{hdl} \) is a hypomorphic loss-of-function allele and that Ptc-signaling is required for normal head development.

The eye-antennal discs are affected in \( ptc \) mutant individuals

As shown schematically in Fig. 2A,B, the head capsule in \( Drosophila \) is generated by the eye-antennal disc (Haynie and Bryant, 1986; Weaver and White, 1995; Royet and Finkelstein, 1996; Karim and Rubin, 1998; Amin et al., 1999). While the eye and the antennal primordia develop into respective adult structures, the marginal regions of these primordia along with part of the peripodial layer give rise to the head capsule structures. As the \( ptc^{hdl}/ptc^{-} \) null individuals suffer from head capsule defects, first we examined the expression of Ptc in the 3rd instar eye-antennal disc with anti-Ptc antibody. As shown in Fig. 2, Ptc is expressed in cells from the regions that contribute to the head capsule (Fig. 2C,D compare with 2A,B).

To determine when in development the eye-antennal disc becomes visibly affected in \( ptc \) mutants, we examined the eye-antennal discs of \( ptc^{hdl}/ptc^{-} \) individuals from the 2nd and 3rd instar larval stages. While we did not detect any size difference between wild-type and mutant discs in the 2nd instar larval stages, by the mid to late 3rd instar larval stages, the difference between wild-type and mutant discs was morphologically
discernible. The discs from the mutants were small and lacking organized folds and swirls of proliferating cells (Fig. 3B, compare with the wild type in 3A). These in vivo results suggest that cells in the mutant discs either fail to proliferate or suffer from extensive cell death. We next examined the discs by culturing very late 3rd instar larval eye-antennal discs from ptcΔ/Δpct−/− individuals in vitro for 15-16 hours. As shown in Fig. 3C,D, discs from both the control (or wild type) and the mutant showed clear signs of differentiation following overnight incubation (n=12 for the number of pairs of discs cultured), about 45% of the pairs of discs showed clear fusion of the two contra-lateral counterparts along the mid line. This indicates that the differentiation of the eye-antennal discs is not significantly affected in the mutant. Moreover, as shown in Fig. 3C, following in vitro culture, the control (and the wild type) discs showed increase in size and presence of folds in an organized manner, suggestive of cell proliferation (but not conclusive, which requires monitoring the size of clones that are marked). In contrast, the mutant discs lacked any folds (Fig. 3D).

The eye-antennal defects are due to loss of cell proliferation and not due to abnormal cell death

To distinguish between cell proliferation defects and abnormal cell death, we conducted BrdU incorporation experiments. Consistent with the possibility that the proliferation of cells is affected in ptcΔ/Δpct−/− individuals, the discs were smaller and showed lower levels of incorporation of BrdU (Fig. 4B,C, discs on the right side). Moreover, the areas of lack of cell proliferation as indicated by the lack of BrdU-positive cells generally corresponded to the regions where Ptc is expressed (Fig. 4D; see also Fig. 2C). We further examined the mutant discs at various developmental time points using an anti-phosphohistone 3 antibody. We found that 1 hour after puparium formation, very few cells express phosphohistone 3 in mutant discs (Fig. 4F,G) as opposed to a large number of cells in wild type (Fig. 4E). We next examined if there was any abnormal cell death in these mutant discs. Examination of discs from 2nd instar larvae to early pupae with the TUNNEL assay did not reveal any abnormal cell death in the mutant discs.

3C, following in vitro culture, the control (and the wild type) discs showed increase in size and presence of folds in an organized manner, suggestive of cell proliferation (but not conclusive, which requires monitoring the size of clones that are marked). In contrast, the mutant discs lacked any folds (Fig. 3D).

Fig. 4. Cell-proliferation is affected in the eye-antennal disc of ptc mutant individuals. (A-D) BrdU incorporation in wild-type and ptc mutant eye-antennal discs from late 3rd instar stages, stained with anti-BrdU. (A) Wild type. (B) A pair of eye-antennal discs from the same ptcΔ/ΔpctH84 individual; the disc on the right shows reduced BrdU incorporation (the bilateral difference in the incorporation is consistent with the unilateral head capsule defect). (C) Another pair of ptcΔ/ΔpctH84 eye-antennal discs; the disc on the right has very few BrdU-positive cells. (D) Domains of cells in a 3rd instar larval eye-antennal disc where Ptc expression is very strong. Note that there is a low level of Ptc expression in most of the cells in the eye-antennal disc. The loss of BrdU incorporation corresponds generally to the Ptc-expression domains. (E-G) Anti-Phosphohistone-3 (PH3) staining (red; green is Phalloidin staining) of the eye-antennal discs 1 hour after puparium formation. (E) Wild type, note that many cells are dividing. (F,G) ptcΔ/ΔpctH84 discs, a few cells are undergoing mitosis.

Fig. 5. Ptc-signaling during head morphogenesis. Pharate adults are shown. (A) Hs-hh individual. (B) The head capsule defect in smo+ pharate adult with smo− mitotic clones. (C) babo32/Df (2R) NP1 individual. (D) ptcH84Δ/Δ, babo32 individual. (E,F) ptcΔ/Δ/Df (2R) NP3: UAS-baboact individual, ventral and dorsal views. Note that the head capsule defect is completely rescued by the expression of activated Babo in the Ptc-domain from the ptcgal4 (see text). (G) ptcΔ/Δ; ptcH84Δ/Δ: UAS-baboact69B-GAL4, the head capsule defect is also rescued by the expression of activated Babo using a disc-specific driver (see text). (H) The genetic epistatic relationship between hh, ptc, smo and babo during head development. This genetic pathway does not necessarily indicate a linear relationship between Ptc-Smo signaling and Babo signaling, as there are other possibilities that have not been ruled out (see text).
compared with wild type (data not shown). Finally, halving the doses of reaper, wrinkler and grim [Df (3L) H99] was used to halve the doses of these three genes, as all three are uncovered by this small deficiency, which promote cell death (White et al., 1996; Chen et al., 1996), in a ptcH84/ptcn-fall background did not suppress the head capsule defects (Table 2). Based on these results, we conclude that the head capsule defect in ptc mutants is due to a lack of precursor cell proliferation in the eye-antennal disc.

**Gain-of-function for hh induces the same head capsule phenotype as loss of function for ptc**

Interaction of Hh with Ptc relieves the suppression of Smo by Ptc; this in turn allows Smo to trigger activation of downstream target genes. To determine if this scenario also applies to head development, we examined the expression of Hh in the eye-antennal disc. We observed that the expression of Hh was much more restricted in the eye-antennal disc compared with the expression of Ptc. That is, Hh is present in cells adjacent to Ptc-expressing cells in only certain regions of the eye-antennal disc. This suggests that if the interaction of Hh with Ptc (and the de-suppression of Smo) is required during cell proliferation in the eye-antennal disc, Hh has to function as a long-range signaling molecule. To determine if Hh activity is required during head capsule development, we examined if hh mutants show any head capsule defects using a temperature-sensitive allele of hh (Ma et al., 1993). Shifting of hh mutants from the permissive to the restrictive temperatures at various developmental time points failed to generate any individuals with head capsule defects. We also considered the alternative possibility where Hh is not required for cell proliferation during head morphogenesis, and it is the suppression of Smo by Ptc in the absence of Hh that allows cells to proliferate. To determine if this possibility is true, we examined the gain-of-function effects of hh. Specifically, we determined if gain of function for hh would cause the same head capsule defects as loss of function for ptc. Indeed, when an hh transgene was expressed at high levels using a heat shock 70 gene promoter in larval stages of development (see Materials and Methods), head capsule defects similar to ptc mutants were observed (Fig. 5A).

**Reducing the dose of smo enhances the head capsule defects**

The above hhGOF result would argue that during head morphogenesis, Ptc promotes cell proliferation by suppressing Smo. If this interpretation were correct, reducing the dose of smo in the ptc mutant background would result in the suppression of the head capsule phenotype. However, halving the copy numbers of smo in ptc background (ptcH84 smo1/ptcH84 smo1/ptcH84 + ; the ptcH84 insertion allele was used here because only 4% of the ptcH84/ptcH84 individuals show the phenotype, see Table 2) enhanced the head phenotype to 34.8% (Table 2; note that 2% of the individuals transheterozygous for ptcH84 and smo show the head capsule phenotype). This enhancement can be observed with other alleles of smo or ptc (thus, in different genetic backgrounds, data not shown), indicating that this is not a background effect. Finally, we generated loss-of-function smo clones in smo1+ individuals using the FLP-FRT technique and, as shown in Fig. 5B, such individuals developed similar head capsule defects as ptc mutants. These results indicate a non-canonical relationship between Ptc and Smo during head development where Ptc in concert with Smo promotes cell proliferation in the eye-antennal disc. How one would account for the gain-of-function hh phenotype? The inappropriate expression of hh in hhGOF individuals must be disrupting the positive interaction between Ptc and Smo, thus, rendering Ptc unable to promote cell proliferation.

**baboon, which encodes an Activin type I receptor, genetically interacts with ptc during head capsule development**

We next addressed how this Ptc-Smo pathway regulates cell proliferation. Previous results indicate that in *Drosophila*, the Activin pathway mediated by Babo, which is a type I Activin receptor, regulates cell proliferation in larval tissues such as the brain and wing discs (Brummel et al., 1999). We sought to determine if the Ptc-Smo pathway promotes cell proliferation via the Activin pathway. This was first addressed genetically by examining whether individuals that are mutant for babo show the head capsule phenotype. Indeed, as shown in Fig. 5C and Table 2, individuals that are transheterozygous for babo and a deficiency that eliminates babo [babop/Df (2R) NP1] – this deficiency leaves ptc intact) showed the head capsule phenotype, indicating that babo is required for head morphogenesis (neither of the two babo alleles survive to pupal stages as homozygotes or transheterozygotes). This result is also consistent with the fact that babo is expressed in all the cells of the eye-antennal disc (Brummel et al., 1999). In order to explore the possibility that the Ptc-Smo pathway interacts with the Activin pathway to promote cell proliferation, we next determined if the head capsule phenotype can be observed in individuals transheterozygous for ptc and babo (ptc, +/+; babo). As shown in Fig. 5D and Table 2, we indeed observed the head capsule phenotype in individuals that were transheterozygous for ptc and babo. Moreover, while the number of ptcH84/ptcn-fall individuals showing the head capsule phenotype is 4%, the penetrance was enhanced to 67% in ptcH84/ptcn-fall, +/babores individuals [Df (2R) NP3 was used to eliminate ptc and babo; see Table 2] and fully penetrant in ptcH84/+; babo/babores [babore773 or babore72/Df (2R) NP3] individuals (Table 2).

Finally, consistent with the finding that reducing the dose of smo did not suppress but, instead, enhanced the head capsule phenotype in ptc mutants, heterozygosing smo in a babo homozygous and ptc heterozygous condition [smo1, Df (2R) NP3+/+, babore773 ] did not suppress the head capsule defects either (Table 2). These results provide further evidence that Ptc, together with Smo, positively interacts with Babo to promote cell proliferation during head morphogenesis.

During Activin signaling, the Type II receptor binds to its ligand, which in turn promotes physical interaction between Type II and Type I receptors and the phosphorylation of Type I receptor (Wrana et al., 1994). We examined if ptc interacts in trans with punt, the Type II receptor (Childs et al., 1993). However, unlike the interaction between ptc and babo, no such transheterozygous interaction was observed between ptc and punt (Table 2).

**An activated form of Babo rescues the head capsule defects in ptc mutants**

Previous results have indicated that substitution of glutamine
at position 302 of the Babo protein with aspartic acid results in a constitutively active form of Babo, as assayed by its ability to interact with Smad2 protein (Wieser, et al., 1995; Brummel et al., 1999). This suggests that for Babo to become active, specific conformational changes in the protein might be required. It is possible that Ptc-Smo signaling interfaces with Babo by promoting the activation of Babo or it might function upstream of the activation of Babo. First, we also examined if the transcription of babo is affected in ptc mutants. Whole-mount RNA in situ of the eye-antennal disc with a babo probe did not reveal any reduction in the levels of babo RNA compared with wild type (data not shown). This indicates that the Ptc-Smo pathway does not regulate babo at the transcription level. It is generally the case that if 50% reduction of two separate genes shows a phenotype not revealed in the individual heterozygous mutant, the transheterozygous interaction is very specific and indicative of a very close interaction (Artavanis-Tsakonas et al., 1995; Winberg et al., 1999). Given that ptc and babo show transheterozygous interaction (see above and Table 2), it is possible that Ptc and Smo interaction with Babo is at the activation of Babo level, although we cannot rule out the possibility that Ptc-Smo pathway regulates expression of one of the Activin-like ligands.

Finally, we sought to determine if the Ptc-Smo pathway during head morphogenesis operates upstream of activation of Babo by determining if an activated form of babo can rescue the head capsule defects in ptc mutants. We introduced a babo transgene that encodes the activated form of Babo into ptc background (UAS-baboact). The constitutively active form of the Babo has glutamine at position 302 replaced by aspartic acid (Wieser et al., 1995). Induction of babo in ptc mutant background in the exact Ptc expression domain using the UAS-baboact transgene and ptc-GAL4 driver (ptcgal4), we used this allele not only to generate the mutant combination but also to express baboact in the Ptc-domain), completely rescued the head phenotype in ptc mutant individuals (Fig. 5E,F; Table 2). Moreover, expression of UAS-baboact in ptcnull/ptcnull background using a disc-specific GAL4 driver (69B-GAL4) also rescued the head capsule defects (Table 2; Fig. 5G).

**DISCUSSION**

In this paper, we have uncovered a novel pathway by which Ptc promotes proliferation of cells in the eye-antennal disc to generate the *Drosophila* head capsule. Ptc, together with the enigmatic transmembrane protein Smo, promotes activation of Babo, the Activin type I receptor, to stimulate cell proliferation. Previous studies have shown that Ptc is a repressor of Smo, and the interaction of Hh and Ptc relieves this repression on Smo, allowing Smo to activate downstream genes. Ptc signaling is also known to be a suppressor of cell proliferation and loss of function for Ptc in vertebrates, for example, leads to nevoid basal carcinomas (Hahn et al., 1996; Johnson et al., 1996). The results described here show that Ptc signaling, in concert with Smo, can also promote cell proliferation and that this is via activation of downstream genes. Thus, our results reveal an intriguing and non-canonical mode of action by this pathway during head morphogenesis (see Fig. 5H).

**Ptc-signaling promotes cell proliferation in the eye-antennal disc**

Previous results indicate that Ptc is a repressor of cell proliferation. Our results, however, show that during head development in *Drosophila*, Ptc functions to promote cell proliferation. The loss of the head capsule in *ptc* mutants is not due to cell death, as we did not observe any inappropriate and massive cell death in the eye-antennal disc by the TUNEL assays. However, we observed a lack of BrdU incorporation as well as fewer phospho-histone-positive cells in the eye-antennal disc. Lack of differentiation of cells of the eye-antennal discs can also give rise to similar head capsule defects. For example, pharate adult mutant for the headcase gene show severe head capsule defects with resemblance to *ptc* mutants (Weaver and White, 1995). However, in headcase mutants, the morphology, the size and the shape of the eye-antennal discs are normal and the head capsule defects appear to be due to a failure in the differentiation of cells of the eye-antennal disc (Weaver and White, 1995). In *ptc* mutants, our results indicate that the morphology, organization, and size of the eye-antennal disc are severely affected by late 3rd instar larvae and the primary cause for the head capsule defects is loss of cell proliferation. This conclusion is further supported by the fact that an activated form of Babo completely rescues the head capsule defects in *ptc* mutants. *babo* is a known player in promoting cell proliferation and it has been previously shown to be required only for cell proliferation but not for cell differentiation in the imaginal discs (Brummel et al., 1999). Moreover, our in vitro culture of eye-antennal discs indicate that the differentiation per se is not affected in *ptc* mutants (see Fig. 3D). Therefore, we conclude that Ptc promotes cell proliferation in the eye-antennal disc during head development.

**Ptc, together with Smo, promotes cell proliferation in the eye-antennal disc**

Previous studies indicate that Ptc is likely to complex with Smo and repress Smo from activating downstream target genes. Binding of Hh to Ptc frees Smo from Ptc repression, which then goes on to activate downstream target genes. Thus, Ptc has been always viewed as a suppressor of gene activity via suppressing Smo. For example, during the development of the embryonic nerve cord, loss of *ptc* activity leads to losing RP2 neurons. This is due to the ectopic activation of Gsb in the neuroectoderm from which the RP2 precursor neuroblast (NB4-2, a row 4 NB) delaminates; ectopic Gsb prevents Wingless signaling from specifying NB4-2 identity and therefore the loss of RP2 neurons (Bhat, 1996; Bhat and Schedl, 1997; Bhat et al., 2000). Consistent with the possibility that Smo is downstream of Ptc, ectopic expression of Gsb in row 4 in *ptc* mutants and the consequent loss of RP2 neurons is rescued in *ptc*, smo double mutants (K. M. B., unpublished). If this signaling also occurs during the head development, loss of Ptc will lead to inappropriate activation of Smo, leading to the head capsule defects; loss of Smo activity in a *ptc* mutant background, therefore, should suppress the head capsule defects. However, reducing the dose of Smo in a *ptc* mutant background (smo+/+, ptcnull/ptcnull), instead of suppressing the head defects (or at the least reducing the severity), enhanced the head capsule defects. Moreover, our results show that loss of Smo activity leads to the same head capsule defects as in *ptc* mutants.
Previous results have indicated that Ptc might negatively regulate levels of Smo via vesicular trafficking of Smo from the cell surface (Denef et al., 2000; Ingham et al., 2000; Martin et al., 2001; Strutt et al., 2001). Thus, in ptc mutants it has been inferred that the level of Smo on the membrane is high, leading to the inappropriate activation of downstream target genes. That a similar mechanism might operate during head capsule development is unlikely for the following reasons. First, we found that reducing the dose of smo in ptc mutant background enhances the phenotype. Second, in one of the ptc alleles, ptcS2, the mutation is an amino acid change from a charged to an uncharged residue in the sterol-sensing domain (Martin et al., 2001). We found that ptcS2 fully complements ptcnull and the transheterozygotes have no head capsule defects (Table 1). Moreover, in ptcnull/ptcnull mutant eye-antennal disc, the level of Smo is not upregulated (N. Mortimer and K. M. B., unpublished). Based on these results, we conclude that a positive signaling by Ptc and Smo regulates cell proliferation during head development.

Gain-of-function Hh interferes with the positive signaling by Ptc-Smo in head morphogenesis

In the conventional Ptc-signaling, interaction of Hh with Ptc relieves the repression on Smo, thus allowing Smo to function. When Hh is ectopically expressed, it interacts with Ptc to relieve the repression on Smo. This in turn is thought to cause phenotypes in hh gain-of-function situations. Thus, in the CNS, for example, loss of Ptc activity from the RP2 neuronal precursor cell leads to missing RP2 neurons (see above); ectopic expression of Hh in adjacent rows of cells leads to loss of RP2 neuron via inappropriate activation of Gsb in the neuroectoderm from which NB4-2 is delaminated (K. M. B., unpublished). The results described in this paper, that during head development gain-of-function Hh mimics a loss of function ptc phenotype, are not inconsistent with the finding that Ptc, together with Smo, promotes cell proliferation. That is, ectopic expression of Hh will bind to Ptc and this will interfere with the positive signaling by Ptc and Smo. One possibility is that Ptc and Smo are physically associated with one another, and binding of Hh to Ptc will break this physical association, rendering Ptc or Smo unable to positively regulate cell proliferation in the eye-antennal disc.

Interaction between Babo signaling and Ptc-Smo signaling

Our results indicate that Ptc-Smo signaling leads to the activation of Babo. During Activin signaling, Activin binds to Activin type II receptor, which promotes physical interaction between type II and type I receptors and the phosphorylation of type I receptor. Both type I and type II receptors are transmembrane serine/threonine kinases. Phosphorylation of the type I receptor results in the activation of its kinase activity and the phosphorylation of downstream transcription activators such as the Smad proteins, resulting in their nuclear localization (Wrana et al., 1994; Heldin et al., 1997). In Drosophila, analysis of null mutants for the type I receptor babo, as well as analysis of babo germline clones, indicates that babo is not required during embryogenesis but is essential during pupal development and adult viability (Brummel et al., 1999). The major defect in babo mutants is a reduction of cell proliferation in the imaginal discs and brain tissue. It has also been shown that in tissue culture experiments, a constitutively active form of Babo can signal to vertebrate TGF-β/Activin, but not to BMP-responsive promoters (Brummel et al., 1999). The activated Babo then interacts with Drosophila Smad2 to effect the nuclear localization of this transcription factor.

Our results, that expression of an activated form of Babo in the ptc-expression domain in the eye-antennal disc of ptc mutants completely rescues the head capsule defects, indicates that Ptc-Smo signaling ultimately leads to activation of Babo and promotes cell proliferation in the eye-antennal disc. As babo and ptc show transheterozygous interaction, it is tempting to speculate that the interaction between Ptc and Babo might be direct. A transheterozygous interaction is generally observed in several cases where the two proteins associate with one another, in cases such as the receptor-ligand pairs Notch and Delta (Artavanis-Tsakonas et al., 1995; Winberg et al., 1998; Kidd et al., 1999; Bashaw et al., 2000). However, it is also possible that Ptc-Smo signaling and Babo signaling represent parallel pathways that converge at the point of cell cycle control. In this scenario, partial reduction in each could have a synergistic negative affect on cell proliferation, while overexpression of one (i.e. activated Babo) could compensate for loss of the other. Yet another possibility would be that the Pt-Smo pathway activates one of the Activin-like ligands. While our results indicate that there is no transheterozygous genetic interaction between ptc and punt (the inferred type II receptor for Activin), we cannot rule out the possibility that the Ptc-Smo pathway does not interact with Punt. This is due to the fact that a lack of transheterozygous interaction does not mean that the two players do not interact, as it actually depends on what is limiting. Nonetheless, our finding that Ptc, together with Smo stimulates cell proliferation and the interfacing of Ptc-signaling with Babo-signaling in this process provides new insight into the process of head development.

We thank Drs Mike O’Connor for babo alleles, babo cDNA and information on these lines; and Markus Noll, Phil Ingham, Bob Holmgren and Manfred Frasch for various antibodies. We greatly appreciate the comments and suggestions from Dr Tom Kornberg on this work. We also thank Bloomington Drosophila stock center and the Umeå stock center for fly stocks. We appreciate help from Nathan Mortimer in sequencing the ptc gene in ptcnull allele, and other members of the Bhat laboratory for various help during this study. K. M. B. thanks Prema Bhat for her help during the screen for ptcnull allele. We also thank Dr Guy Benian for comments on the manuscript. This project is supported in part by grants from NIH and the Ara Parseghian Medical Research Foundation to K. M. B.

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