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RESEARCH ARTICLE

Ihog and Boi elicit Hh signaling via Ptc but do not aid Ptc in sequestering the Hh ligand

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ABSTRACT

Hedgehog (Hh) proteins are secreted molecules essential for tissue development in vertebrates and invertebrates. Hh reception via the 12-pass transmembrane protein Patched (Ptc) elicits intracellular signaling through Smoothened (Smo). Hh binding to Ptc is also proposed to sequester the ligand, limiting its spatial range of activity. In *Drosophila*, Interference hedgehog (Ihog) and Brother of ihog (Boi) are two conserved and redundant transmembrane proteins that are essential for Hh pathway activation. How Ihog and Boi activate signaling in response to Hh remains unknown; each can bind both Hh and Ptc and so it has been proposed that they are essential for both Hh reception and sequestration. Using genetic epistasis we established here that Ihog and Boi, and their orthologs in mice, act upstream or at the level of Ptc to allow Hh signal transduction. In the *Drosophila* developing wing model we found that it is through Hh pathway activation that Ihog and Boi maintain the boundary between the anterior and posterior compartments. We dissociated the contributions of Ptc from those of Ihog/Boi and, surprisingly, found that cells expressing Ptc can retain and sequester the Hh ligand without Ihog and Boi, but that Ihog and Boi cannot do so without Ptc. Together, these results reinforce the central role for Ptc in Hh binding *in vivo* and demonstrate that, although Ihog and Boi are dispensable for Hh sequestration, they are essential for pathway activation because they allow Hh to inhibit Ptc and thereby relieve its repression of Smo.

KEY WORDS: Pattern formation, Morphogenesis, Signaling, *Drosophila*

INTRODUCTION

Hedgehog (Hh) proteins are secreted morphogens that are essential for many events in tissue development and cellular physiology. Despite significant progress, gaps remain in the characterization of the molecular mechanisms underlying Hh signal transduction. Key points of control include the reception of the Hh signal at the cell surface and the distribution of Hh within tissues, both of which involve Patched (Ptc), a conserved 12-pass transmembrane protein. In humans, mutations of the Ptc ortholog PTCH1 cause Gorlin syndrome, holoprosencephaly, basal cell carcinoma and medulloblastoma. Therefore, understanding exactly how Ptc participates in Hh reception and distribution is essential to distinguish between normal Hh pathway function in development and aberrant Hh signaling in disease (Briscoe and Therond, 2013).

In the absence of Hh, Ptc maintains the 7-pass transmembrane protein Smoothened (Smo) in a repressed state by unknown means. Under these conditions, the Cubitus interruptus (Ci) transcription factor is proteolytically cleaved and acts as a transcriptional repressor. As part of the canonical signaling pathway that is activated in the presence of Hh, Ptc-mediated repression of Smo is relieved, leading to Smo accumulation at the cell surface and stabilization of full-length Ci (Ci155), which activates transcription of Hh target genes. The current view holds that Ptc is involved in sensing the extracellular Hh concentration (Briscoe and Therond, 2013), and that target genes respond in relation to the degree of induced pathway activation. One direct transcriptional target of Hh signaling is Ptc itself; Ptc is upregulated in response to high levels of Hh, and is required for the sequestration of Hh, which limits the distribution of Hh and its range of action within tissues via a poorly understood mechanism.

Experiments have yet to demonstrate unequivocally that Hh binds directly to Ptc in *Drosophila*; therefore, both Hh reception and sequestration have been proposed to involve Interference hedgehog (Ihog) and Brother of ihog (Boi), two closely related type I transmembrane proteins of the immunoglobulin superfamily (Zheng et al., 2010). Ihog and Boi both have an ectodomain consisting of four immunoglobulin-like (Ig) domains and two fibronectin type III (FN3) repeats, a transmembrane domain and a cytoplasmic tail (Kang et al., 2002; Lum et al., 2003; Yao et al., 2006). Their orthologs in vertebrates are Cell adhesion molecule downregulated by oncogenes (Cdon) and Brother of cdon (Boc). Intriguingly, the first FN3 domain of Ihog binds directly to Hh (McLellan et al., 2006; Yao et al., 2006), whereas the second FN3 domain interacts with Ptc (Zheng et al., 2010). This raises the possibility that Ihog and Boi are cofactors for Ptc in a multiprotein complex serving as the functional Hh receptor. This view is supported by phenotypes caused by loss-of-function mutations of the *ihog* and *boi* genes in *Drosophila*, which mimic those of *smo* mutations (Camp et al., 2010; Zheng et al., 2010). For example, we and others have found that *ihog* and *boi* are essential for Hh pathway activation in developing wing imaginal disks, where Hh produced and secreted by cells in the posterior compartment is received and sequestered by cells in the anterior compartment. Anterior cells respond by adopting fates according to their degree of Hh exposure and by maintaining the boundary separating these cells from the

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posterior compartment. Evidence indicates that Ihog and Boi are functionally redundant with one another (Camp et al., 2010; Yan et al., 2010; Zheng et al., 2010), and that they act upstream of Smo (Camp et al., 2010; Zheng et al., 2010).

Although it is clear that Ihog and Boi are crucial for Hh pathway activity *in vivo*, their mechanism of action is poorly understood and could be illuminated by genetic approaches that clearly establish their position in the pathway relative to Ptc. A previous experiment to establish the epistatic relationship of Ihog and Ptc was confounded by the lack of a strong and penetrant *ihog* mutant phenotype (Yao et al., 2006), later found to be due in part to functional redundancy with Boi (Camp et al., 2010; Zheng et al., 2010). Using genetic epistasis experiments in flies and mice, we here directly demonstrate that Ihog and Boi (or Cdon and Boc in mice) act upstream or at the level of Ptc to allow Hh pathway activation. To better understand the interplay of Ihog and Boi with Ptc, we also used genetic approaches in *Drosophila* to dissociate the contributions of Ihog and Boi from functions attributed to Ptc within developing wing disks. Like Ptc, we find that Ihog and Boi allow transduction of the Hh signal, and that through their essential role in Hh pathway activation they maintain the anterior-posterior compartment boundary. Surprisingly, we find that, unlike Ptc, Ihog and Boi are dispensable for Hh retention and sequestration by responding cells.

RESULTS

Ihog and Boi function upstream or at the level of Ptc for Hh signal transduction

To examine Ihog and Boi expression in wing imaginal disks of third instar larvae (L3), we performed immunohistochemistry for Ihog and Boi with newly developed antisera. Each was expressed broadly in both the anterior and posterior compartments (Fig. 1A,C), and overall these patterns of endogenous Ihog and Boi expression are consistent with previously published results (Yan et al., 2010; Zheng et al., 2010; Bilioni et al., 2013). We confirmed the specificity of these patterns by their absence in $ihog^{DC1}$ or boi^{C1} mutants (Fig. 1B,D), which allowed the use of these antisera to monitor Ihog and Boi levels in clones generated in mosaic animals.

Expression of Ihog and Boi in the anterior compartment is consistent with their essential role for pathway activation in Hh-responding cells (Fig. 1A-D). Cells adjacent to the compartment boundary respond to high levels of Hh by upregulating the expression of Ptc, a high-threshold target of the canonical signaling pathway. Cells positioned more anteriorly are further away from the Hh source and respond to lower-threshold levels of Hh marked by increased accumulation of Ci155. According to these high- and low-threshold reporters of Hh signaling activity, *boi;ihog* double mutant clones lack Hh pathway activation altogether (Camp et al., 2010; Zheng et al., 2010). By contrast, *ptc* mutations fully activate the pathway (Phillips et al., 1990; Ingham et al., 1991). These opposing effects allowed us to unequivocally address the epistatic relationship between Ihog, Boi and Ptc by examining pathway activation in cells that were triple mutants for all three genes. We reasoned that if triple mutant cells were to mimic *ptc* mutants and fully activate the pathway, it would indicate that Ihog and Boi act upstream or at the same level as Ptc, but not downstream. For this we studied the boi^{C1} and $ihog^{DC1}$ mutations that we had reported previously (Camp et al., 2010), and the ptc^{S2} missense mutation, which is indistinguishable from null alleles, but gives rise to a dysfunctional Ptc protein that can be detected with anti-Ptc (Phillips et al., 1990; Jiang and Struhl, 1995; Li et al., 1995; Chen and Struhl, 1996). As Ptc is an Hh pathway target, activation of the

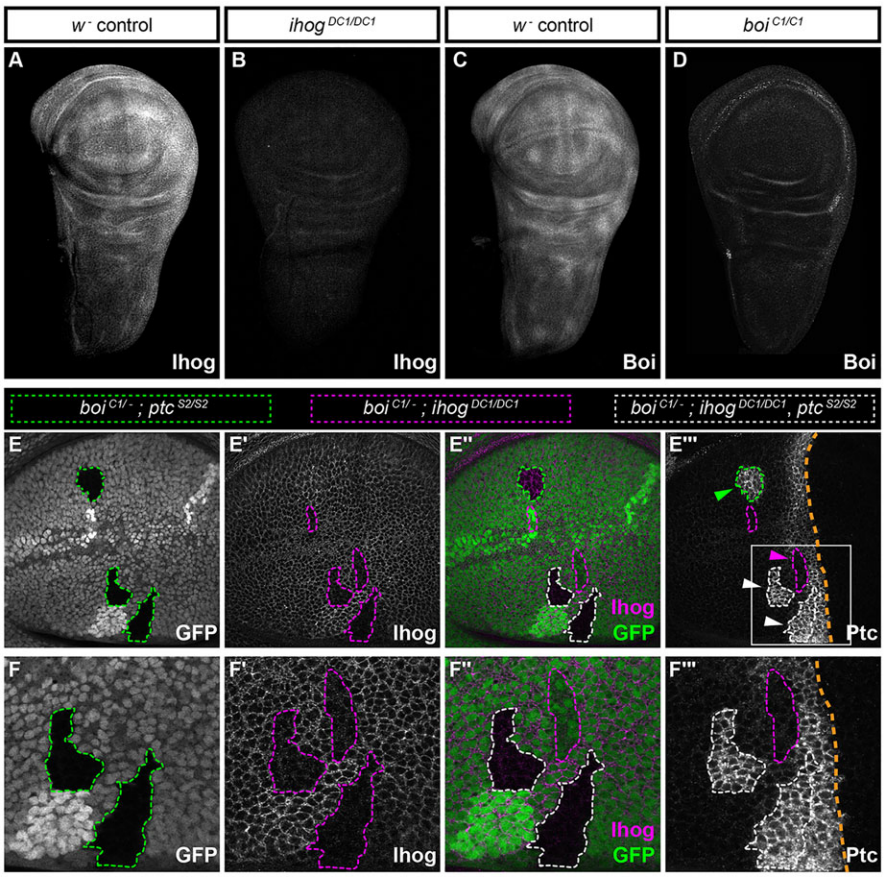

Fig. 1. Ihog and Boi function upstream or at the level of Ptc. (A-D) Immunohistochemistry for Ihog or Boi in w^{1118} (w^{-}) controls and in homozygous mutants for *ihog* or *boi*. (E-E‴) Homozygous mutant clones generated in a $boi^{C1/-}$ mutant background: *ptc* clones, *ihog* clones or *ihog;ptc* double mutant clones. (E) $ptc^{S2/S2}$ clones (GFP-negative cells outlined in green) and (E′) $ihog^{DC1/DC1}$ clones (Ihog-negative cells outlined in magenta). (E″) Merged image, in which clones mutant for both ptc^{S2} and $ihog^{DC1}$ lack both GFP and Ihog (outlined in white), and are triple mutants for *boi*, *ihog* and *ptc*. (E‴) In the two triple mutant clones shown (white arrowheads) Ptc is expressed highly, similar to ptc^{S2} mutant cells (green arrowhead) or to control anterior cells adjacent to the compartment boundary. Cells lacking both *ihog* and *boi* (magenta arrowhead) lacked Hh pathway activation and did not express Ptc. (F-F‴) Higher magnification views of the areas in E-E‴.

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pathway (as in ptc^{S2} mutants) can be detected readily as strong upregulation of Ptc expression and immunoreactivity. We studied mosaic wing disks, in which the MARCM system was used to generate ptc^{S2} and/or $ihog^{DC1}$ mutant clones in boi^{C1} hemizygotes. Because all cells in these animals were mutant for *boi*, clones generated by FLP-mediated mitotic recombination in the wing disk were either (1) double mutant for *boi;ptc*, (2) double mutant for *boi;ihog* or (3) triple mutant for *boi;ptc;ihog.* As expected, double mutant *boi;ptc* clones within the anterior compartment activated the pathway even at a distance from the compartment boundary (i.e. in the absence of Hh ligand) (Fig. 1E,E″ and green arrowhead in E‴), whereas double mutant *boi;ihog* clones abolished pathway activation even when close to the boundary (i.e. near the source of Hh ligand) (Fig. 1E′,E″ and magenta arrowhead in E‴). Like double mutant *boi;ptc* clones (green arrowhead in Fig. 1E‴), all cells within triple mutant *boi;ptc;ihog* clones fully activated the pathway (white arrowheads in Fig. 1E‴), as indicated by Ptc expression levels comparable to fully activated, wild-type cells nearest to the boundary (Fig. 1F-F‴). Therefore, *ptc* is epistatic to *ihog* and *boi in vivo*, which positions Ihog and Boi upstream or at the level of Ptc for Hh signal transduction.

Cdon and Boc act upstream or at the level of Ptch1 in vertebrate Hh signal transduction

We wondered whether the epistatic relationship of *ptc* to *ihog* and *boi* is conserved across species, in particular with regard to the mouse orthologs *Ptch1*, *Cdon* and *Boc*. To test this, we generated mice lacking *Ptch1*, *Cdon* and *Boc* and examined their gross anatomy relative to controls (Fig. 2). Compared with wild-type embryos (Fig. 2A) at E9.5, *Cdon;Boc* double mutants develop normally for the most part (Fig. 2B), but have forebrain defects indicative of holoprosencephaly as described previously (Zhang et al., 2011). By contrast, *Ptch1* mutant mice display significant defects at E9.5, including the failure to complete the turning process, exencephaly, neural tube closure defects and abnormal heart development (Fig. 2C) (Goodrich et al., 1997). Notably, *Ptch1;Cdon* and *Ptch1;Boc* double mutants, and in particular *Ptch1;Cdon;Boc* triple mutants, grossly phenocopy *Ptch1* mutants (Fig. 2D-F). This suggests that loss of *Cdon* and *Boc* is insufficient to reduce Hh signaling in the absence of *Ptch1*.

To precisely assess Hh pathway activity in these embryos, we performed a neural patterning analysis using a panel of Sonic hedgehog (Shh)-dependent markers of neural progenitors. In *Cdon;Boc* double mutant embryos, we confirmed previous results demonstrating reduced Hh signaling (Allen et al., 2011), as indicated by reduced Foxa2 expression (Fig. 2G,H) and the ventral shift of Nkx2.2 (Fig. 2M,N) and Pax6 expression (Fig. 2S,T). Conversely, $Ptch1^{-/-}$ embryos have increased Hh signaling, as reflected by expanded expression of Foxa2 and Nkx2.2, and the loss of Pax6 throughout the neural tube (Fig. 2I,I′,O,O′,U,U′). Like $Ptch1^{-/-}$ mutants, double mutants ($Ptch1^{-/-};Cdon^{-/-}$ or $Ptch1^{-/-};Boc^{-/-}$) and triple mutants ($Ptch1^{-/-};Cdon^{-/-};Boc^{-/-}$)

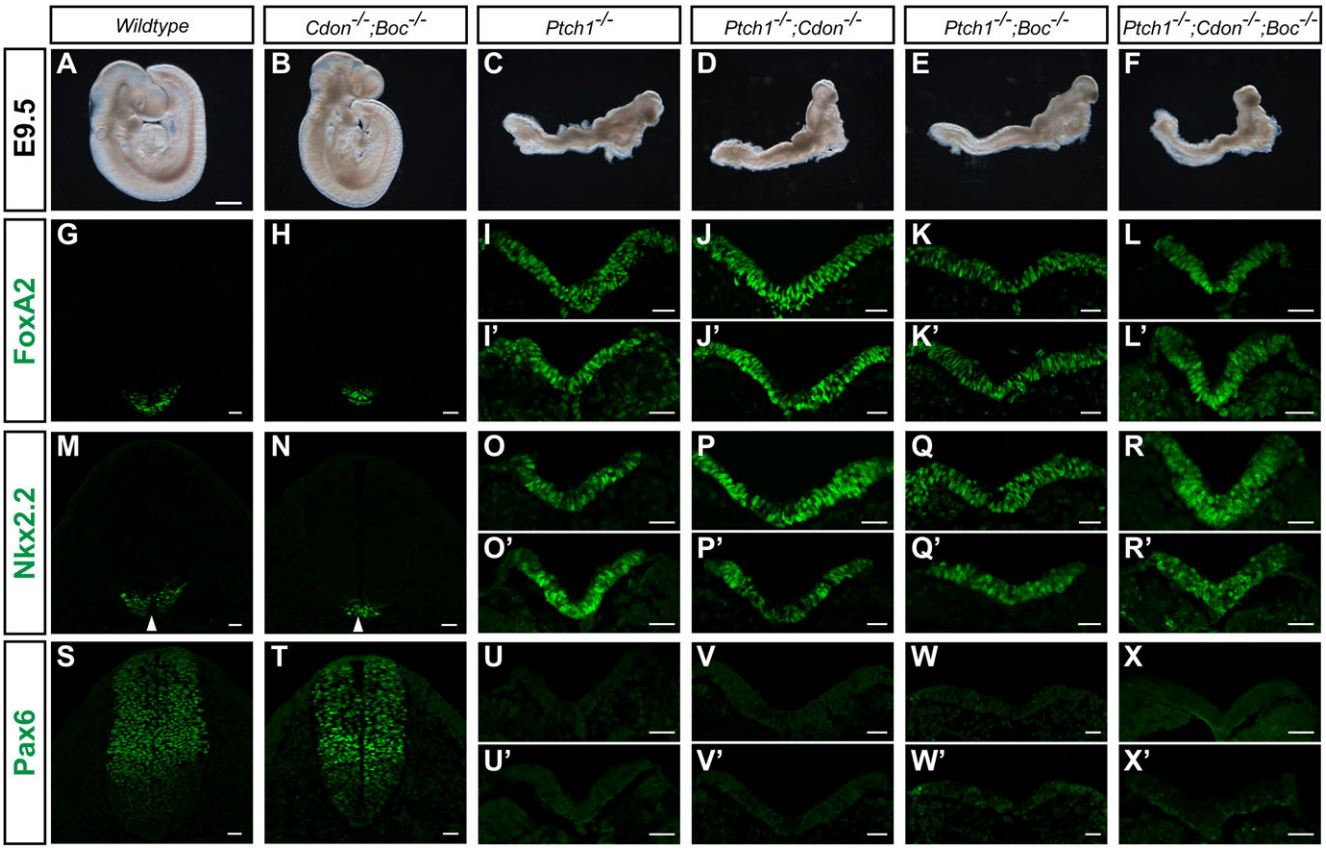

Fig. 2. *Ptch1* is epistatic to *Cdon* and *Boc* during Shh-dependent ventral neural tube patterning. (A-F) Lateral view of E9.5 mouse embryos. Wild-type (A) and $Cdon^{-/-};Boc^{-/-}$ (B) embryos have completed the turning process and display closed neural tubes, whereas $Ptch1^{-/-}$ (C), $Ptch1^{-/-};Cdon^{-/-}$ (D), $Ptch1^{-/-};Boc^{-/-}$ (E) and $Ptch1^{-/-};Cdon^{-/-};Boc^{-/-}$ (F) embryos fail to turn and exhibit open neural tubes including excencephaly. Scale bar: 500 µm. (G-X′) Antibody detection of Foxa2 (G-L′), Nkx2.2 (M-R′) and Pax6 (S-X′) in transverse sections from E9.5 mouse embryos. Genotypes are indicated at the top of each column. Arrowheads denote floor plate expression of Nkx2.2 in $Cdon^{-/-};Boc^{-/-}$ (N), but not wild-type (M) embryos. Images for two different animals are shown in I-L,O-R, U-X. Scale bars: 25 µm.

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exhibited complete activation of the Hh pathway in the neural tube (Fig. 2J-L′,P-R′,V-X′), demonstrating Ptch1 to be epistatic to Cdon and Boc. These experiments indicate that Cdon and Boc act upstream or at the same level as Ptch1, and confirm that their relationship within the Hh pathway is conserved between flies and mice.

Hh can be retained and sequestered by cells lacking Ihog and Boi

If Ihog and Boi and their orthologs in mice act upstream or at the level of Ptc, how might they function in pathway activation? To address this, we returned to experiments in *Drosophila*, for which it has been proposed that Ihog and Boi are essential for Hh signaling because without them Ptc cannot bind Hh with high affinity (Zheng et al., 2010). We focused on sequestration of the Hh signal by clones of cells near the anterior-posterior compartment boundary of developing wing disks, as sequestration must involve Hh binding at some level. Indeed, we and others have previously shown that *boi; ihog* double mutant cells cannot sequester Hh, and instead permit ectopic pathway activation in more anterior cells that would normally be too distant from the morphogen source (Camp et al., 2010; Zheng et al., 2010). The requirement for Ihog and Boi in Hh sequestration could be explained in two ways: they could bind Hh directly themselves, and/or they could act indirectly through pathway activation to influence another Hh-binding factor. To explore this, we used the MARCM technique to test whether Hh sequestration could be restored to *boi;ihog* double mutant cells simply by activating the pathway through expression of a constitutively active form of Smo (Smo^{SD123}) (Jia et al., 2004). To assess sequestration, we identified anterior clones spanning the usual domain of Hh-induced pathway activation (as revealed by upregulation of Ptc), and then examined pathway activation in cells immediately anterior to these clones. In cells just anterior to control clones that sequester Hh ($boi^{C1/+}$;$ihog^{DC1/DC1}$), pathway activation was normal, as assessed by Ptc expression levels (Fig. 3A-A″,D,E). By contrast, Ptc was upregulated in cells anterior to double mutant clones ($boi^{C1/-}$;$ihog^{DC1/DC1}$) (Fig. 3B-B″,E), as we have shown previously (Camp et al., 2010). This indicates that cells lacking Ihog and Boi cannot activate the pathway and cannot sequester Hh. Similarly, smo^{3} mutant clones also fail to sequester Hh (Chen and Struhl, 1996) (Fig. 3E), indicating that Ihog and Boi are insufficient to sequester Hh without pathway activation. Remarkably, we found that the ability of *boi;ihog* double mutant clones to sequester Hh was fully restored by expressing Smo^{SD123} to rescue pathway activation (Fig. 3C-C″,E). As the boi^{C1} allele is a nonsense mutation at Trp626

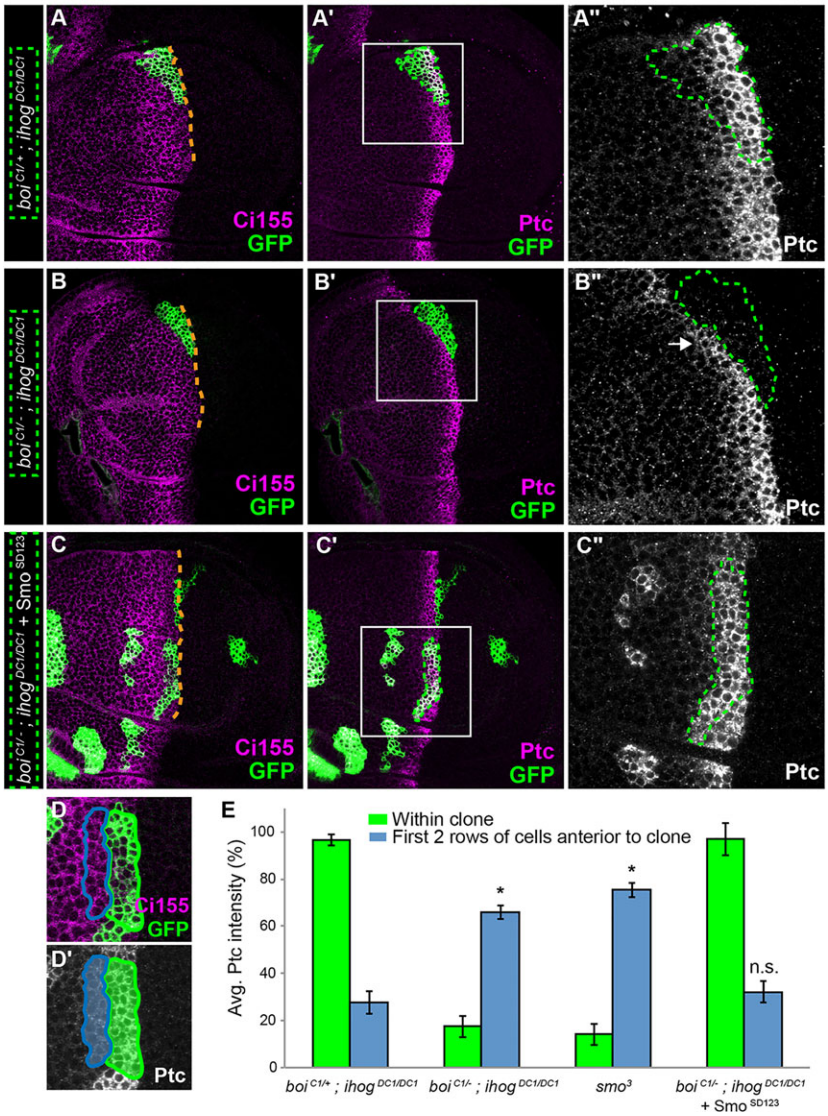

Fig. 3. Ihog and Boi are dispensable for Hh sequestration. (A-A″) In a control $boi^{C1/+}$ heterozygote, the MARCM technique was used to generate a GFP-positive $ihog^{DC1/DC1}$ clone adjacent to the anterior-posterior compartment boundary (green outline in A′,A″). (A) Ci155 (magenta) accumulates in response to Hh signaling and marks the anterior-posterior boundary. (A′,A″) The high-threshold Hh target Ptc is expressed normally within this control clone, and there is no ectopic Ptc expression in cells just anterior to the clone: this indicates correct sequestration of the Hh signal by cells within the clone. (A″) Higher magnification view of boxed area in A′. (B-B″) An $ihog^{DC1/DC1}$ clone (green outlines in B′,B″) in a $boi^{C1/-}$ mutant. (B″) Higher magnification view of boxed area in B′. Clones lacking both *ihog* and *boi* did not express Ptc (GFP-positive, green outline), but were identifiable as anterior in origin, as they express basal levels of Ci155. Failure to sequester Hh is indicated by high levels of Ptc in cells immediately anterior to the clone (arrow in B″). (C-C″) A *boi;ihog* double mutant clone adjacent to the anterior-posterior compartment boundary that is expressing *UAS-*smo^{SD123} (green outline in C′,C″). (C″) Higher magnification view of boxed area in C′. Expression of smo^{SD123} in cells lacking *boi* and *ihog* rescues pathway activation (Ptc expression within clone, C″) and is sufficient to sequester Hh. Orange dashed lines (A,B,C) mark the normal position of the anterior-posterior boundary. (D,D′) Quantification example. Average Ptc intensity within the clone (within green outline in D and green shaded region in D′) is measured within the usual high-Ptc expression zone. Average Ptc intensity anterior to the clone (within magenta outline in D and magenta shaded region in D′) consists of the first two rows of cells anterior to area measured in green. (E) Quantification of average Ptc intensity within (green bars) and anterior to (blue bars) the observed clones. Clones lacking Ihog and Boi but expressing Smo^{SD123} (n=11) show similar patterns of Ptc intensity than control clones (n=5). By contrast, clones lacking Ihog and Boi (n=8), or the pathway activator Smo (n=9), displayed low Ptc within the clone and high Ptc anterior to it (ANOVA, F3,29=9.017, P=0.0002). All measurements were normalized to the average levels of Ptc expression that were observed in areas of equivalent size outside the clones but near the compartment boundary.

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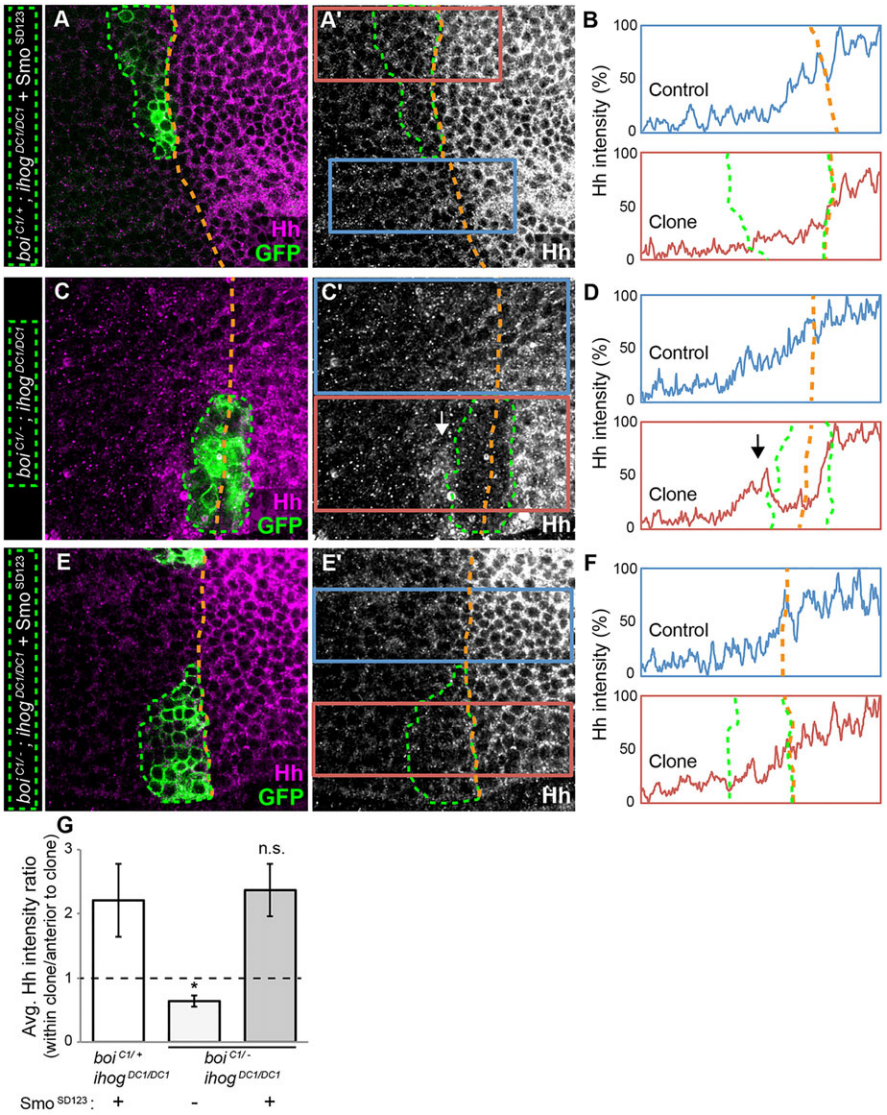

Fig. 4. Ihog and Boi are dispensable for Hh retention. (A,A′) In a $boi^{C1/+}$ control, an $ihog^{DC1/DC1}$ clone (green outline) expressing Smo^{SD123} displays a normal Hh gradient. (B) Average Hh staining intensity of control (blue) and clone (red) areas boxed in A′. (C,C′) In a $boi^{C1/-}$ mutant, an $ihog^{DC1/DC1}$ MARCM clone (green outline) is shown to cross the anterior-posterior boundary (orange dashed line). The clone lacks Hh staining, which instead accumulates immediately anterior to it (arrow in C′ and D), indicating the inability of the *boi;ihog* double mutant cells within the clone to retain Hh. (D) Average Hh staining intensity of control (blue) and clone (red) areas boxed in C′. (E,E′) In a $boi^{C1/-}$ mutant, an $ihog^{DC1/DC1}$ clone (green outline) expressing Smo^{SD123} shows that pathway activation in this manner rescues the normal Hh gradient within the clone. (F) Average Hh staining intensity of control (blue) and clone (red) areas boxed in E′. (G) Ratio of average Hh intensity within clones versus cells just anterior to the clones (ANOVA, F2,14=6.369, P=0.0108). All measurements were normalized to Hh intensity in the posterior compartment of the wing. Genotypes $boi^{C1/+}$; $ihog^{DC1/DC1}$; *UAS-smo*^{SD123} (n=5 clones), $boi^{C1/-}$; $ihog^{DC1/DC1}$ (n=7 clones) and $boi^{C1/-}$; $ihog^{DC1/DC1}$; *UAS-smo*^{SD123} (n=6 clones). n.s., nonsignificant.

(Camp et al., 2010), we considered that it might encode an intact first FN3 domain that could theoretically bind Hh in this experiment. To test this, we replaced boi^{C1} with $boi^{KO2\text{-}1}$, an allele designed by gene targeting to delete the translation initiation codon, the signal sequence and the four Ig domains from the coding sequence of Boi (Zheng et al., 2010). In results that are identical to those for boi^{C1}, we restored Hh sequestration to *boi;ihog* double mutant cells by activating the pathway (supplementary material Fig. S1). This confirms that Ihog and Boi are dispensable for sequestration if Hh signaling is activated, and implicates an Hh-binding factor other than Ihog or Boi in this process.

To investigate how sequestration of the Hh signal (as measured by Ptc levels) relates to retention of the Hh protein, we measured the labeling intensity of anti-Hh immunoreactivity within MARCM clones, and in cells just anterior to them. As cells of the anterior compartment do not produce Hh, we interpret Hh immunoreactivity within anterior compartment clones as an indication of their capacity to bind Hh produced and secreted by cells of the posterior compartment. In control animals heterozygous for *boi*, we noted that Smo^{SD123} expression in *ihog* clones (genotype: $boi^{C1/+}$;$ihog^{DC1/DC1}$) did not influence Hh immunoreactivity compared with normal levels seen just outside the clone (genotype: $boi^{C1/+}$;$ihog^{DC1/+}$) (Fig. 4A-B). However, *boi;ihog* double mutant clones lacked Hh staining (Fig. 4C-D), suggesting that Hh secreted from cells in the posterior compartment was not retained by cells within the clone. Instead, Hh accumulated immediately anterior to them (Fig. 4C-D,G), as if the movement of Hh from the posterior compartment was unimpeded by *boi;ihog* double mutant cells. Importantly, Hh staining was restored to *boi;ihog* double mutant cells that expressed Smo^{SD123}, and the graded distribution of Hh within these clones was identical to nearby control cells outside the clones (Fig. 4E-F,G). Thus, pathway activation due to Smo^{SD123} renders Ihog and Boi dispensable for cells to retain and sequester Hh.

Studies of cultured cells transfected with Ptc indicate that its localization at the plasma membrane is improved by co-expression of Ihog (Zheng et al., 2010). If Ihog/Boi were required for Ptc trafficking to the plasma membrane *in vivo*, we anticipated that the subcellular distribution of Ptc might be altered in clones of wing disk epithelial cells lacking Ihog and Boi. However, we found no evidence that the distribution of Ptc was influenced by the presence or absence of Ihog/Boi (supplementary material Fig. S2).

Ptc is essential for Hh sequestration *in vivo*, also in the absence of Ihog and Boi

If cells lacking Ihog and Boi can sequester Hh once the pathway has been activated, then another Hh-binding factor is likely to play a

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principal role in restricting the diffusion of Hh and thereby limiting the zone of pathway activation. Although it is controversial whether Ptc binds to Hh directly in *Drosophila*, Ptc is a prime candidate due to its upregulation following pathway activation. In *boi;ihog* double mutant cells, perhaps Ptc levels are simply insufficient to sequester Hh because Ptc is not upregulated, due to a lack of signaling through the Hh pathway. We made several observations in support of a primary role for Ptc in Hh sequestration. First, cells that are mutant for the null allele ptc^{16} activate the pathway fully but do not sequester Hh, allowing ectopic pathway activation in cells just anterior to ptc^{16} clones (Chen and Struhl, 1996) (Fig. 5A-A″ and arrow in B). In addition, neither Ihog nor Boi protein levels are affected within ptc^{16} clones compared with neighboring cells (Fig. 5C-F), and so a lack of Hh sequestration in ptc^{16} clones cannot be readily explained by loss of Ihog and Boi. Furthermore, we found that overexpression of Ihog was insufficient to sequester Hh in smo^{3} mutant clones, in which Ptc expression is not elevated (Fig. 5G-H). Finally, to determine whether Ptc can sequester Hh in cells lacking Ihog and Boi, we studied ptc^{S2}, a missense mutation in the intracellular sterol-sensing domain that, like ptc^{16}, cannot repress Smo and results in full pathway activation. Unlike ptc^{16} cells, cells homozygous for ptc^{S2} retain the ability to sequester Hh (Chen and Struhl, 1996). As expected of the controls, $boi;ptc^{S2}$ double mutant cells did sequester Hh, as there was no ectopic Ptc just anterior to these clones (Fig. 6A-A″), whereas *boi;ihog* double mutant cells did not sequester Hh (supplementary material Fig. S3). Triple mutant $boi;ptc^{S2};ihog$ clones spanning the anterior-posterior boundary were rare, but we found five examples, two of which are shown (Fig. 6B-B‴, supplementary material Fig. S3). In every case they fully sequestered the Hh signal, confirming that Ptc is essential for Hh sequestration *in vivo*, also in the absence of Ihog and Boi.

Ihog and Boi act via Hh pathway activation to control compartment-specific cell affinity

Ihog and Boi are essential for activation of Hh signaling, which results in upregulation of Ptc, a high-threshold target that is at least partially responsible for Hh sequestration, independently of Ihog and Boi. If Ihog and Boi are not directly required to sequester the Hh signal, we wondered whether they might also be dispensable for the process by which cells of the anterior and posterior compartments are segregated from one another. Anterior cells adjacent to the boundary require Hh signaling to remain within the anterior compartment, as cells mutant for *smo* or *ci* segregate from wild-type anterior cells, form tight borders with both anterior and posterior cells and fail to integrate with either compartment (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Dahmann and Basler, 2000). Likewise, *boi;ihog* double mutant cells also segregate away from the anterior compartment (Fig. 7A,A′) (Camp et al., 2010), phenocopying *smo* mutations (Rodriguez and Basler, 1997). This could reflect the requirement for Ihog and Boi in Hh signal transduction. However, as the boundary is thought to be implemented by mechanisms controlling compartment-specific cell affinity and adhesion (Dahmann and Basler, 1999), it could also reflect a direct role for Ihog and Boi in cell adhesion via their

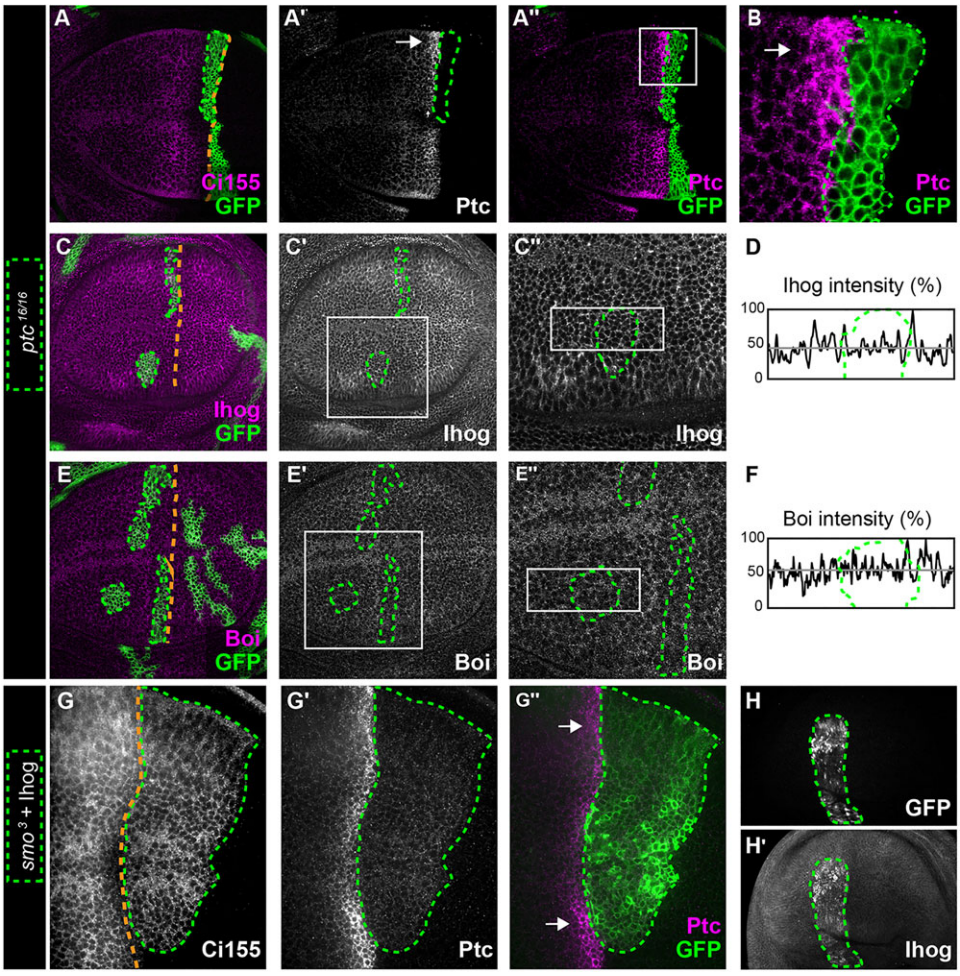

Fig. 5. Ihog and Boi are unable to sequester Hh in cells lacking Ptc. (A) A GFP-positive MARCM clone homozygous for the ptc^{16} null mutation (green outline) is situated within the anterior compartment, adjacent to the anterior-posterior boundary (orange dashed line), as assessed by Ci155 immunostaining (magenta). (A′-B) The $ptc^{16/16}$ null clone does not express Ptc and fails to sequester Hh, as indicated by ectopic activation (Ptc expression) in cells anterior to the clone (arrows in A′ and B). (B) Higher magnification view of boxed area in A″. (C-F) GFP-positive $ptc^{16/16}$ MARCM clones stained for Ihog (C-C″) or Boi (E-E″), focusing on clones of the anterior compartment (green outlines). (C″,E″) Higher magnification view of boxed area in C′ and E′, respectively. (D,F) Average staining intensity of boxed areas in C″ and E″, respectively. Gray line indicates total average intensity. Note that Ihog and Boi levels are unchanged in $ptc^{16/16}$ clones compared with neighboring cells. (G-G″) A GFP-positive MARCM clone homozygous for the smo^{3} null mutation and overexpressing Ihog (green outline) of anterior origin is situated within the posterior compartment, adjacent to the anterior-posterior boundary (orange dashed line), as assessed by Ci155 (G) and Ptc immunostaining (G′). Despite the presence of Ihog, the clone is unable to sequester Hh as indicated by the upregulation of Ptc anterior to the clone (arrows in G″). (H,H′) Higher levels of Ihog (H′) are visible in a smo^{3} clone overexpressing Ihog (green outline).

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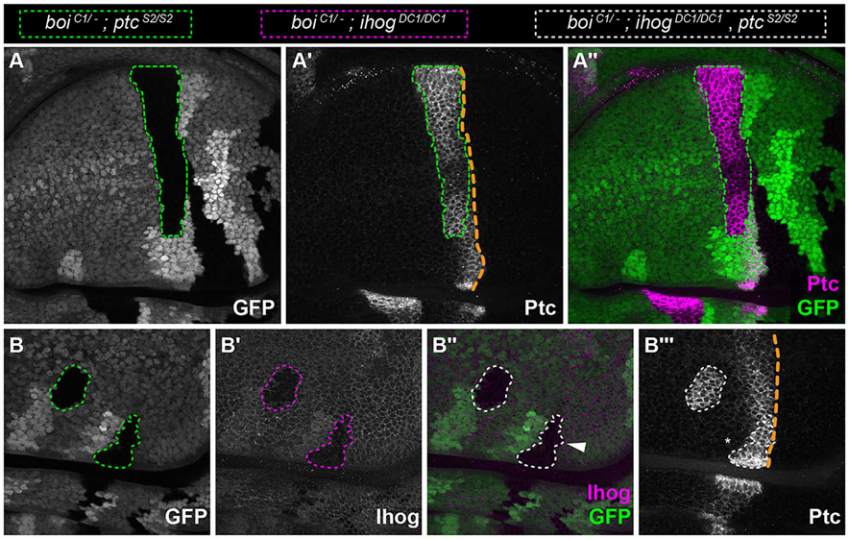

Fig. 6. Hh is sequestered by triple mutant cells for Ihog, Boi and Ptc^{S2}. (A-A″) In a *boi*$^{C1/-}$ mutant, a large GFP-negative clone homozygous for the *ptc*$^{S2/S2}$ missense mutation is situated within the anterior compartment, adjacent to the anterior-posterior boundary (orange dashed line). (A′,A″) Ptc is highly expressed within the clone because Ptc^{S2} protein can be detected with anti-Ptc antibody, and because the Hh signaling pathway is highly active due to this mutation. Lack of ectopic Ptc staining just anterior to the clone indicates full Hh sequestration within the clone. (B-B‴) Two clones mutant for both *ptc* and *ihog* are indicated by white outlines in B‴. These are triple mutants, as they are homozygous for *ptc*$^{S2/S2}$ (GFP-negative, marked by green outlines in B), *ihog*$^{DC1/DC1}$ (Ihog-negative, marked by magenta outlines in B′) and occur in a *boi*$^{C1/-}$ wing disk. (B‴) A triple mutant clone (white outline and arrowhead) near the compartment boundary can sequester Hh, as indicated by the absence of Ptc just anterior to the clone (white asterisk).

Ig and FN3-containing ectodomains. To address this, we tested whether segregation of double mutant cells (*ihog*$^{DC1/DC1}$ clones in *boi*$^{C1/-}$ hemizygotes) could be rescued by pathway activation within those cells through selective expression of Smo^{SD123} or Ptc^{1130}, a dominant-negative form of Ptc that cannot repress Smo. In either instance, pathway activation in every double mutant clone examined (n>10 for each genotype) was sufficient to prevent mis-segregation of anterior cells into the posterior compartment (Fig. 7B-C′). These results indicate that *boi* and *ihog* are dispensable for the increased cell affinity and adhesion that accompanies compartmentalization of the developing wing disk. Reinforcing this interpretation was our examination of clones in the most anterior regions of the anterior compartment, where cells are exposed to little or no Hh. Control clones and *boi*;*ihog* double mutant clones in this

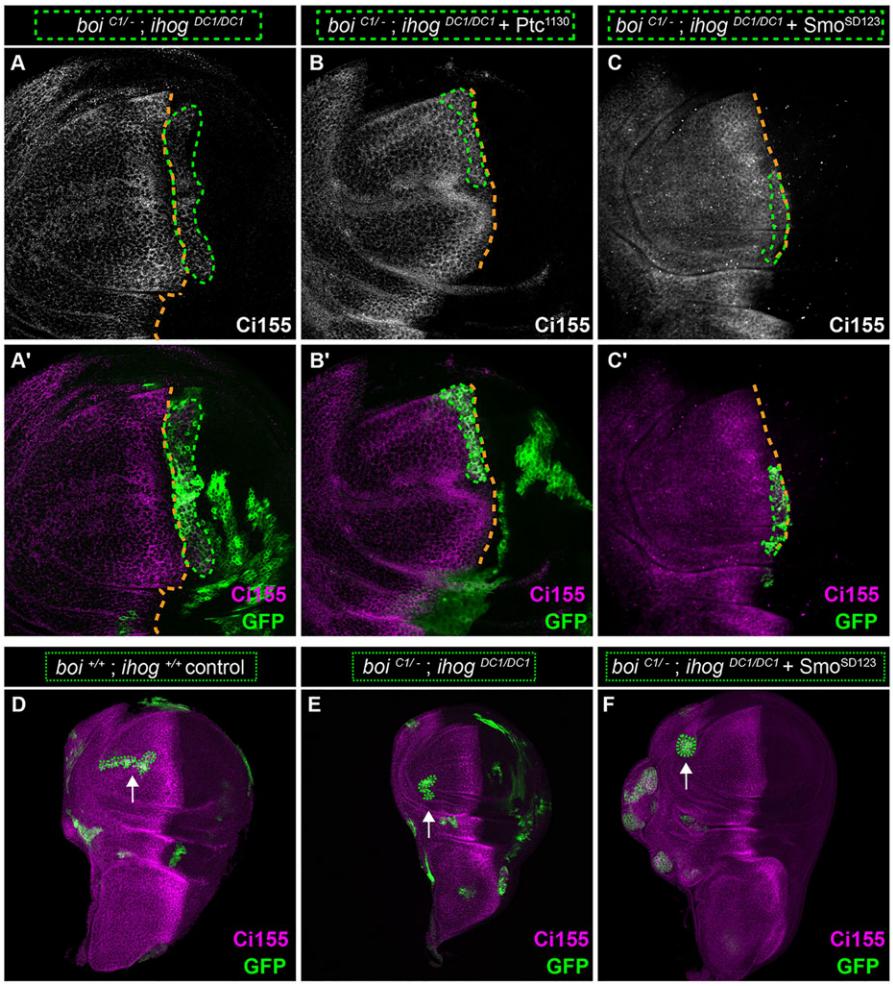

Fig. 7. Ihog and Boi act via the Hh pathway to maintain the anterior-posterior boundary. (A,A′) In a *boi*$^{C1/-}$ mutant, a large *ihog*$^{DC1/DC1}$ clone (green outline) is shown to segregate away from the anterior compartment toward posterior territory (boundary marked by orange dashed line). These *boi;ihog* double mutant cells are known to be of anterior origin because they all express basal levels of Ci155. (B-C′) By contrast, *boi;ihog* double mutants segregate normally and remain in anterior territory when Hh signaling is activated, using either Ptc^{1130} (B,B′) or Smo^{SD123} (C,C′). (D) In anterior clones that are so far removed from the boundary that they cannot receive the Hh signal, control MARCM clones, in which both the *boi* and *ihog* genes are intact and homozygous (genotype: *boi*$^{+/+}$;*ihog*$^{+/+}$), have irregular shapes (green outlines of clones marked by white arrows). (E) Likewise, similarly positioned MARCM clones that are double mutant for *boi;ihog* also have irregular shapes. (F) By contrast, activation of Hh signaling in *boi;ihog* double mutant cells with Smo^{SD123} causes such clones to appear spheroid (green outlines of clones marked by white arrows), probably reflecting that pathway activation causes cells within the clone to acquire higher affinity for one another than for cells outside the clone, even in the absence of Ihog and Boi.

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region were randomly shaped, reflecting that cells within these clones had equal affinity for each other as they did with surrounding cells outside the clone (arrows in Fig. 7D,E). By contrast, *boi;ihog* double mutant clones that were activated by Smo^{SD123} were spheroid and formed smooth boundaries with neighboring cells, revealing stronger affinity of cells within the clone for one another (arrow in Fig. 7F). We conclude that Ihog and Boi are required for compartment segregation because they are necessary to activate Hh signaling. Any potential role they might have in cell adhesion in this context is dispensable upon Hh pathway activation. Interestingly, upregulation of Ptc in response to pathway activation also appears dispensable, as *ptc* mutant clones respect the compartment boundary (Fig. 5A) (Rodriguez and Basler, 1997). This is strikingly distinct from Hh sequestration, for which our data suggest that Ptc upregulation is required.

DISCUSSION

We and others showed previously that Ihog and Boi are absolutely essential within Hh-responding cells for activation of the Hh signaling pathway, acting upstream of Smo (Camp et al., 2010; Zheng et al., 2010). In the experiments described here, we advance our understanding of Ihog and Boi function by drawing three major conclusions:

First, in genetic epistasis experiments we found that Ihog and Boi also act upstream or at the level of Ptc, supporting the idea that they function through Ptc to relieve suppression of Smo. This epistatic relationship appears conserved in evolution, as we found that Cdon and Boc also function upstream or at the level of Ptch1 for Hh signal transduction in mice. These genetic findings establish this relationship unequivocally, and so have profound implications for future studies to further clarify how these co-receptors participate in Hh reception and pathway activation.

Second, based on our experiments to dissect the relative contributions of Ihog and Boi in processes involving Ptc, we conclude that it is through their essential roles in Hh signal transduction that Ihog, Boi and Ptc contribute to anterior-posterior compartment segregation: once the pathway is activated, all three proteins are dispensable for maintenance of the compartment boundary, implicating other, yet unidentified, cell surface recognition molecules in compartment-specific cell affinity and adhesion.

Our third conclusion is that Ihog and Boi, unlike Ptc, are completely dispensable for the sequestration and retention of Hh. We show that cells lacking Ihog and Boi can sequester and retain the Hh signal if the pathway is activated and Ptc is upregulated. They do so via physiological levels of endogenous Ptc induced either by pathway activation in ptc^{S2} mutants or by expression of Smo^{SD123}. Incidentally, we also found that Hh sequestration was rescued in *boi;ihog* double mutant clones overexpressing Ptc^{1130} (Fig. 7B), a dominant-negative that fully activates the Hh pathway and upregulates endogenous, wild-type Ptc. This third conclusion is not consistent with the view that Ihog and Boi aid in addressing Ptc to the cell surface and that, once there, they are required for Ptc to bind and sequester Hh (Zheng et al., 2010). This view is based primarily on Ptc and Ihog overexpression in cultured cells, and on an experiment that failed to restore Hh sequestration to *boi;ihog* double mutant cells with mutation of cAMP-dependent protein kinase 1 (Pka-C1), which upregulates Ptc and other target genes because loss of Pka-C1 disinhibits the activity of the transcription factor Ci (Ohlmeyer and Kalderon, 1997; Chen et al., 1998; Price and Kalderon, 1999; Wang et al., 1999). It is unclear why Ptc upregulation in *Pka-C1* mutants was unable to rescue Hh sequestration in *boi;ihog* double mutants, whereas Ptc upregulation in our experiments was able to do so. In cells lacking Ihog and Boi, perhaps the level to which Ptc is upregulated in *Pka-C1* mutants is inadequate. Regardless, our data indicate that Ptc has a central role in the binding and sequestration of Hh, whereas Ihog and Boi are dispensable, despite their requirement for Hh signal transduction.

Our results are consistent with vertebrate systems, in which current models strongly favor direct contacts between Hh and Ptc, primarily because: (1) expression of the Ptc ortholog Ptch1 promotes binding of Shh to transfected cells (Marigo et al., 1996; Stone et al., 1996), (2) radiolabeled Shh can be chemically cross-linked to Ptch1 expressed on the cell surface (Fuse et al., 1999) and (3) Ptch1 can reach the cell surface in the absence of the Ihog/Boi-related proteins Cdon and Boc (Izzi et al., 2011). Whether Ptc is sufficient on its own to bind Hh remains an important question that awaits technically challenging studies using purified proteins. An alternative possibility is that Hh could have additional receptor(s), with candidates including the proteoglycans Dally and Dally-like (Dlp), and Shifted, a secreted protein of the Wnt inhibitory factor 1 (WIF1) family (Han et al., 2004; Yan et al., 2010; Ayers et al., 2012; Avanesov and Blair, 2013).

Our results clearly distinguish a role for Ptc that relies on Ihog/Boi (Hh reception/signal transduction) from one that does not (Hh sequestration), and so they contribute to an emerging view of the function of Ihog, Boi and related proteins. In non-responding cells, others have shown that Ihog and Boi are involved in restricting the movement of Hh (Hartman et al., 2010; Bilioni et al., 2013), and so may contribute to its overall distribution. Within Hh-responding cells, where Ptc is co-expressed with Ihog and Boi, we find that Ihog and Boi are essential for Hh signal transduction, but not Hh sequestration and retention. As Ihog and Boi act upstream or at the level of Ptc, they must mediate a crucial, rate-limiting step in the inhibition of Ptc in response to Hh. However, as they are not essential for Ptc to bind Hh, how they affect Ptc function remains to be elucidated. Whereas the precise molecular mechanism remains elusive, several lines of evidence provide important clues. First, an Ihog variant lacking the cytoplasmic tail can rescue *boi;ihog* double mutants (Zheng et al., 2010; our results, data not shown). Second, the second Fn3 domain of Ihog or Boi interacts physically with Ptc and is quite distinct from the first Fn3 domain that harbors the Hh-interacting surface (McLellan et al., 2006; Zheng et al., 2010). Third, the presence of Ihog or Boi potentiates co-immunoprecipitation of Hh and Ptc (Zheng et al., 2010). Together, these results suggest that the primary role of Ihog and Boi in Hh signaling involves the ability of their ectodomains to form favorable protein complexes with Ptc or Hh, or with both simultaneously. Although our data indicate that Ptc does not need Ihog and Boi to bind Hh *in vivo*, we surmise that it is through these multimolecular complexes that Ihog and Boi allow Hh to inhibit Ptc and thereby relieve its suppression of Smo and the Hh signaling cascade.

MATERIALS AND METHODS

Fly stocks

Mutant and transgenic strains are described in the following references: boi^{C1} and $ihog^{DC1}$ (Camp et al., 2010), ptc^{16} (Strutt et al., 2001), ptc^{S2} (Martin et al., 2001), smo^{3} (Chen and Struhl, 1998), $boi^{KO2\text{-}1}$ (Zheng et al., 2010), *UAS-*Smo^{SD123}*-CFP* (Jia et al., 2004), *UAS-Ptc* (Johnson et al., 1995) and *UAS-*ptc^{1130} (Johnson et al., 2000).

Mosaic analysis

Mitotic recombination was used to generate clones of mutant cells within the developing wing disk that could be scored by either the absence or presence of green fluorescent protein (GFP). Clones marked by the absence of GFP were generated by crossing $boi^{C1/C1}$, *hs-FLP*; *FRT40A*, *FRT42B*/+ females to $ihog^{DC1}$, *FRT40A*, *FRT42B*, ptc^{S2}, *Ubi-GFP*/+ males. In this way, three

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types of clones could be generated with a standard heat-shock protocol: (1) $ihog^{DC1/DC1}$, (2) $ptc^{S2/S2}$ and (3) $ihog^{DC1/DC1}$, $ptc^{S2/S2}$. For controls, these clones were generated in $boi^{C1/+}$ heterozygotes (female progeny), with which clones were compared generated in $boi^{C1/Y}$ hemizygotes (male progeny) that are denoted $boi^{C1/-}$ in this paper for simplicity.

Clones marked by the presence of GFP were generated using a mosaic analysis of a repressible cell marker (MARCM) (Lee and Luo, 2001). MARCM clones were obtained by crossing $boi^{C1/C1}$, *hs-FLP* ; *FRT40A*, *tubP-GAL80*; *actP-GAL4*, *UAS-mCD8GFP/TM6B,Tb* females to $ihog^{DC1}$, *FRT40A*; *UAS-transgene-of-interest/TM6B,Tb* males. In this way, our controls for these experiments were $ihog^{DC1/DC1}$ clones generated in $boi^{C1/+}$ heterozygotes (female progeny), with which were compared $ihog^{DC1/DC1}$ clones generated in $boi^{C1/-}$ hemizygotes (male progeny). Within these MARCM clones, the induced GFP expression could be accompanied by induced expression of an additional transgene (*UAS-*Smo^{SD123}*-CFP* or *UAS-*ptc^{1130}). In an experiment testing whether boi^{KO2-1} gave identical results to boi^{C1}, we crossed boi^{KO2-1}, *hs-FLP, UAS-mCD8GFP*; *FRT40A*, *tubP-GAL80/In(2LR)Gla, wg[Gla-1] Bc[1]*; *actP-GAL4/TM6B,Tb* females to either $ihog^{DC1}$, *FRT40A/CyO,P[Dfd-GMR-nvYFP]2* males (controls) or $ihog^{DC1}$, *FRT40A/CyO,P[Dfd-GMR-nvYFP]2*; *UAS-*Smo^{SD123}*-CFP/TM6B,Tb* males.

Antibody generation and immunohistochemistry for *Drosophila*

Wandering L3 larvae were dissected, fixed in 2% formaldehyde and stained according to standard immunohistochemistry procedures. Rabbit or guinea pig polyclonal antisera were generated against Ihog-GST and Boi-GST fusion proteins, respectively. For Ihog, the fusion protein consisted of amino acids 736-886 of the cytoplasmic tail of Ihog-PA (FlyBase), and for Boi it comprised amino acids 874-998 of the cytoplasmic tail of Boi-PB (FlyBase). Anti-Ihog was used at a dilution of 1:10,000, and anti-Boi at 1:500. Monoclonal antibodies obtained from the Developmental Studies Hybridoma Bank (DSHB) included: mouse anti-Ptc (Apa 1, 1:50) and rat anti-Ci155 (2A1, 1:2000). Rabbit anti-Hh (1:200) was a gift from Philip Ingham (Institute of Molecular and Cell Biology, Singapore). Secondary antibodies were: Alexa Fluor 488-conjugated goat anti-rabbit (A-11034, 1:500); Alexa Fluor 647-conjugated goat anti-mouse (A-21236, 1:500); and Alexa Fluor 568-conjugated goat anti-rat (A-11077, 1:500), all from Molecular Probes, and DyLight 549-conjugated anti-guinea pig (106-505-003, 1:500) from Jackson Laboratories. Confocal images were collected on an Olympus FV1000 confocal microscope. All images show a single focal plane through the basolateral region of the wing disk epithelium, with anterior to the left and dorsal to the bottom. Measurements of fluorescence intensity were performed in ImageJ (NIH). Average Ptc and Hh intensity within clones was measured in the first two (Hh) or three (Ptc) rows of cells just anterior to the compartment boundary. Average Ptc and Hh intensity anterior to the clone was measured in the first two rows of cells just anterior to the clone. Background levels of fluorescence were subtracted prior to all measurements.

Mice and analysis of spinal cord tissues by immunofluorescence

Cdo^{LacZ-2} (Cole and Krauss, 2003), Boc^{AP-2} (Zhang et al., 2011) and $Ptch1^{LacZ}$ (Goodrich et al., 1997) mice have been described previously. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All of the animals were handled according to approved institutional animal care and use committee (IACUC) protocols of the University of Michigan. The protocol was approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan (PRO00004017). *Cdon* mice were maintained on a C57BL/6 background; *Boc* mice were maintained on a mixed C57BL6;129S6/SvEvTac background; $Ptch1^{LacZ}$ mice were maintained on a mixed Swiss Webster;129S4/SvJaeJ;C57BL/6 background. Noon of the day on which a vaginal plug was detected was considered as E0.5. For all analyses, a minimum of three embryos of each genotype was examined; representative images are shown. Immunofluorescent analyses of E10.5 mouse neural tubes were performed essentially as described previously (Jeong and McMahon, 2005). The following antibodies were obtained from the DSHB and used at a 1:20 dilution: mouse IgG1 anti-Foxa2 (4C7), mouse IgG2b anti-Nkx2-2 (74.5A5) and mouse IgG1 anti-Pax6 (PAX6). Secondary antibodies were used at a dilution of 1:500 (as above). Primary antibodies were incubated overnight at 4°C. DAPI (D1306, Molecular Probes) was used at a dilution of 1:30,000. A Leica SP5X confocal microscope was used to visualize fluorescence. For each genotype a minimum of three embryos were analyzed. Whole mouse embryos were imaged with a Nikon SMZ1500 stereomicroscope.

Graphs and statistics

All bar graphs express data as mean±s.e.m. Statistical analyses were carried out in Prism (GraphPad). All data were analyzed for statistical significance using a one-way ANOVA. Statistically significant differences in ANOVA ($P<0.05$) were followed by Dunnett's post-hoc tests, and asterisks in graphs denote significance of P-values comparing indicated group with controls (* for $P<0.05$).

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Competing interests

The authors declare no competing financial interests.

Author contributions

D.C. conceived and performed experiments and wrote the manuscript; B.H.H. and S.L. performed *Drosophila* experiments; I.W.A. and A.M.H. performed the mouse neural patterning analyses; B.L.A. conceived the mouse experiments and edited the manuscript; F.C. and D.J.v.M. conceived experiments and wrote the manuscript. F.C. and D.J.v.M. contributed equally to this work.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.103564/-/DC1

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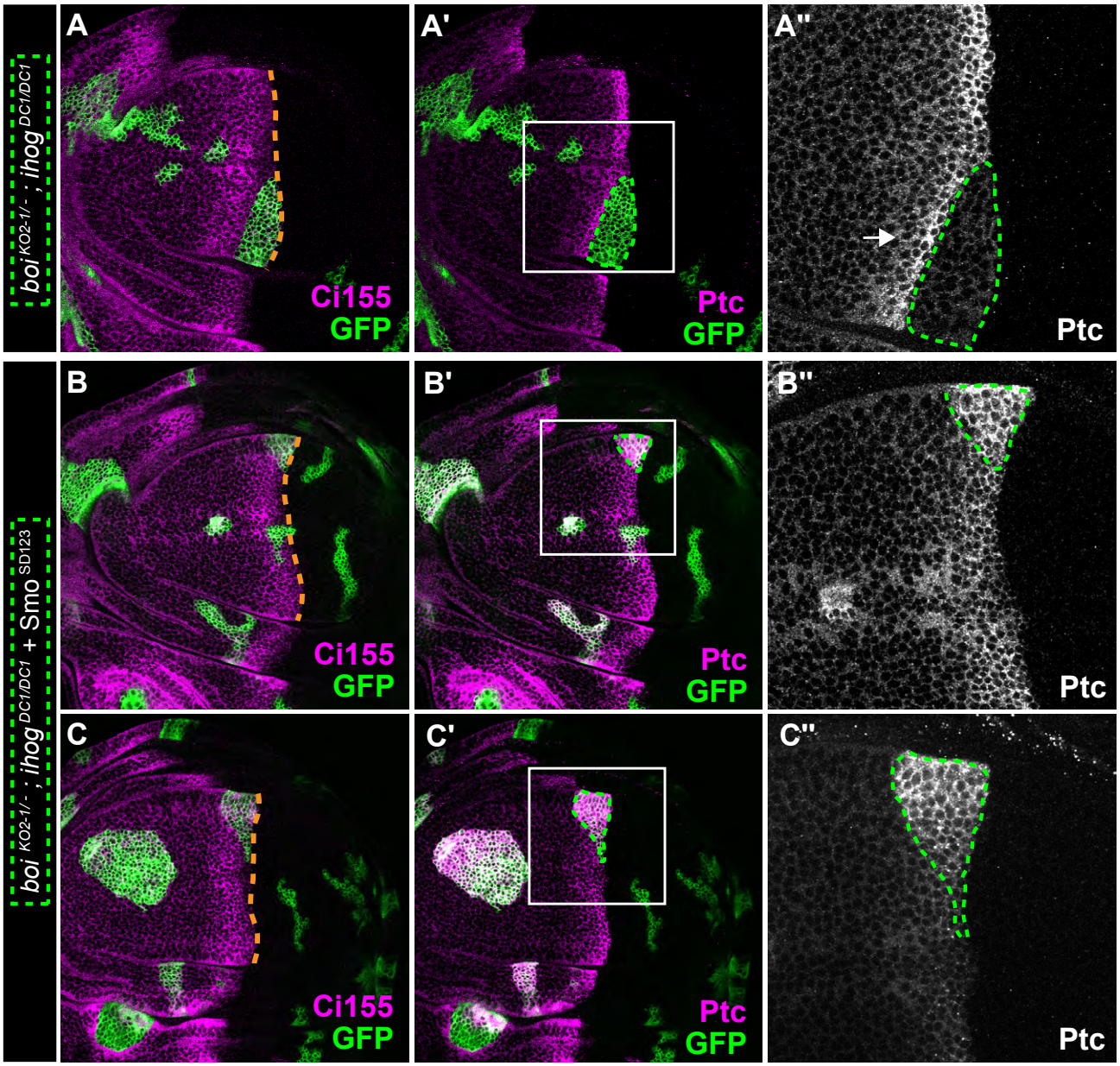

Figure S1. Confirmation with *boi*$^{KO2-1}$ that Ihog and Boi are dispensable for Hh sequestration.

(A-A") A control *ihog*$^{DC1/DC1}$ clone (green outlines in (A') and (A'')) in a *boi*$^{KO2-1/-}$ mutant. (A'') Higher-power view of boxed area in (A'). As expected, *boi*$^{KO2-1/-}$; *ihog*$^{DC1/DC1}$ double mutant cells failed to sequester Hh, as indicated by high levels of Ptc in cells immediately anterior to the clone (arrow in (A'')). Also as expected, they did not upregulate Ptc (GFP-positive, green outline), but were identifiable as anterior in origin since they express basal levels of Ci155 and Ptc. **(B-C")** Two examples of *boi*$^{KO2-1/-}$ *ihog*$^{DC1/DC1}$ double mutant clones expressing *UAS-smo*^{SD123} (green outline in (B' and C') and (B'' and C'')). (B'' and C'') Higher-power view of boxed area respectively in (B' and C'). Each example is adjacent to the anterior-posterior compartment boundary. Expression of *smo*^{SD123} in cells lacking *boi* and *ihog* rescues pathway activation (Ptc expression within clone (B'' and C'')) and is sufficient to sequester Hh as shown by the absence of high levels of Ptc anterior to the clone. Orange dashed lines in (A), (B) and (C) mark the normal position of the anterior-posterior boundary.

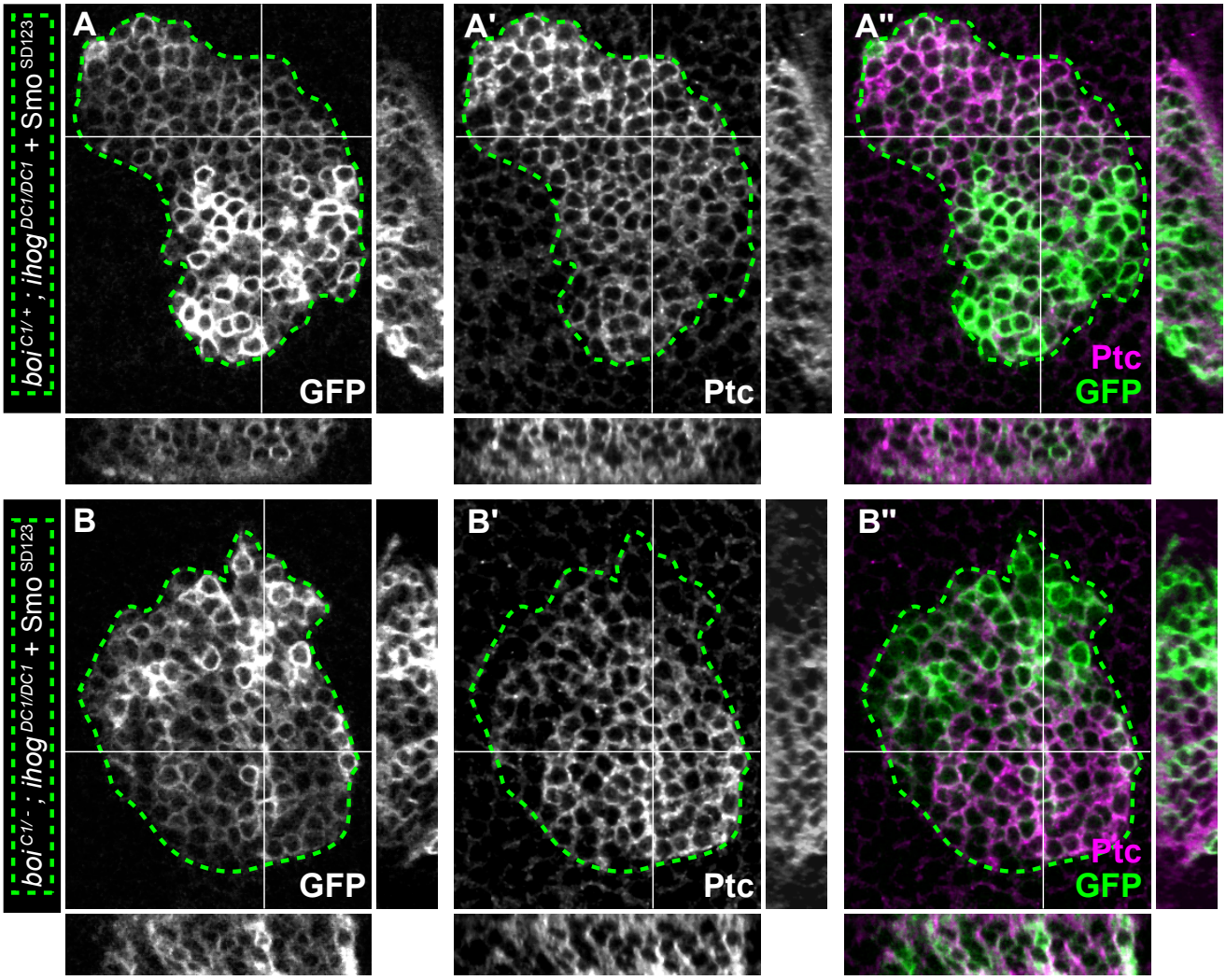

Figure S2. Ptc distribution appears unaffected in cells lacking Ihog and Boi.

(A-A") A control *boi*$^{C1/+}$, *ihog*$^{DC1/DC1}$ clone expressing the membrane-targeted reporter mCD8-GFP (green outline) and *UAS-smo*^{SD123}, which elicits pathway activation and thereby ensures adequate expression of Ptc in these controls and in double mutant clones in B-B''). The distribution of Ptc correlated well with that of mCD8-GFP. **(B-B")** In comparison, neither the degree of Ptc upregulation nor its subcellular distribution were altered when *smo*^{SD123} was expressed in *boi*$^{C1/-}$, *ihog*$^{DC1/DC1}$ double mutant clones (green outlines). The clones displayed were located in the anterior compartment of wing discs. White lines in A-B'' indicate the focal plane at which the representative confocal cross-sections were taken.

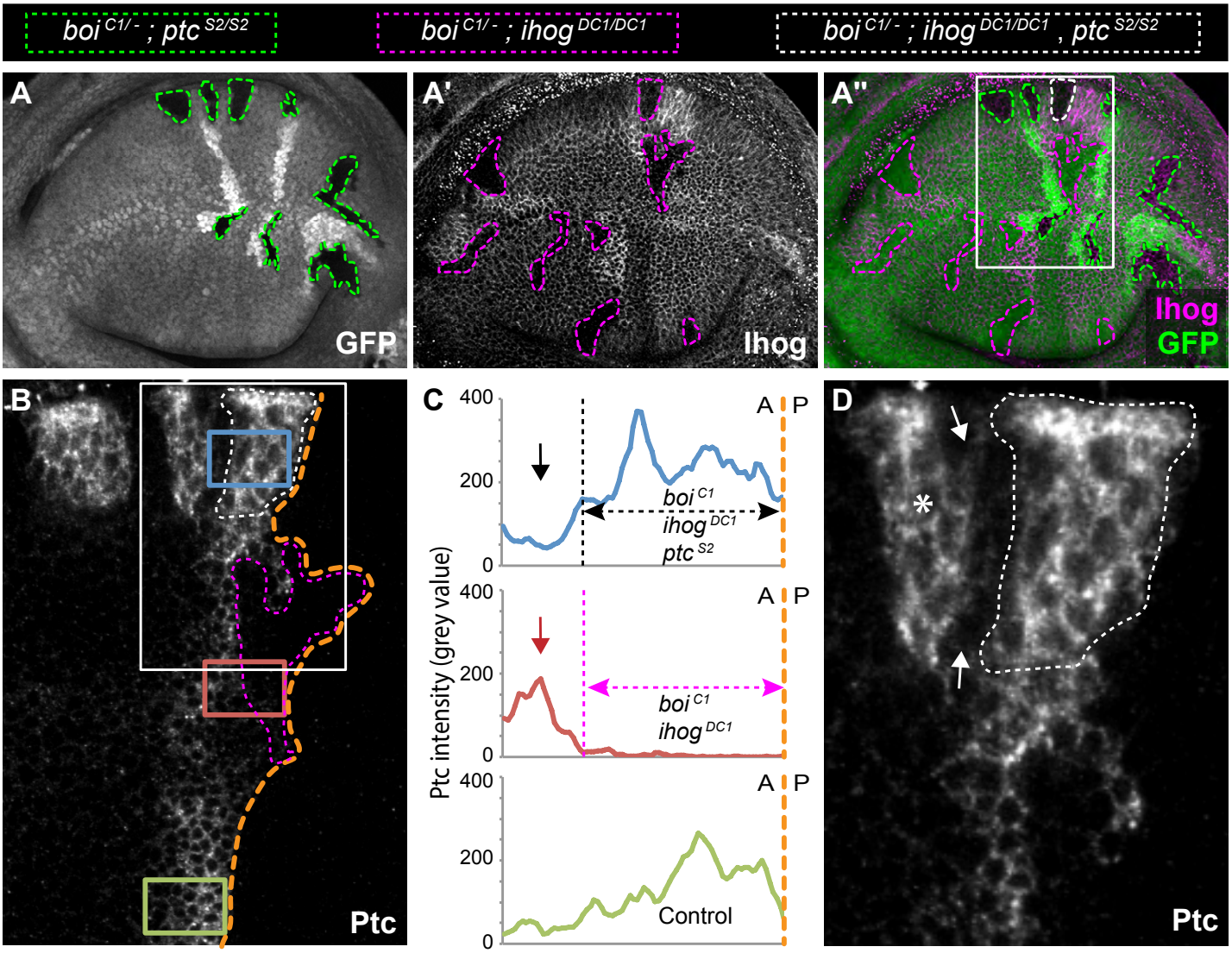

Figure S3. Hh is sequestered by triple mutant cells for Ihog, Boi and Ptc^{S2}.

(A-A") Wing disc clones generated in a *boi*$^{C1/-}$ larva, including *ptc*$^{S2/S2}$ clones (GFP-negative marked by green outlines in (A) and (A")) and *ihog*$^{DC1/DC1}$ clones (Ihog-negative marked by magenta outlines in (A') and (A")). A clone mutant for both *ptc* and *ihog* is indicated by white outlines in (A"). This is triple mutant because it occurs in a *boi*$^{C1/-}$ wing disc. **(B)** Higher-power view of boxed area in (A") showing a *boi*$^{C1/-}$; *ihog*$^{DC1/DC1}$; *ptc*$^{S2/S2}$ triple mutant clone (white outline) and a *boi*$^{C1/-}$; *ihog*$^{DC1/DC1}$ double mutant clone (magenta outline) generated adjacent to anterior-posterior boundary. Colored boxes: region of interest including five rows of cells starting from the anterior-posterior boundary (orange dashed line). Green box: control area containing no clones. Blue and red boxes: first three rows of cells located within a clone followed by 2 rows of cells just anterior to it. **(C)** Average Ptc intensity within corresponding colored boxed areas in (B). Top: Ptc intensity is elevated in within triple mutant clones (marked black double-headed arrow) but not just anterior (black arrow), indicating Hh sequestration. Middle: Hh is not sequestered by cells lacking Ihog and Boi (magenta double-headed arrow), and instead there is elevated Ptc expression (marking pathway activation) just anterior to the clone (red arrow). **(D)** Higher-power view of white boxed area in (B). A triple mutant clone (white outline) near the compartment boundary can sequester Hh, as indicated by low levels of Ptc just anterior to the clone (region between white arrows). More anteriorly, elevated Ptc staining (white asterisk) was the result of ectopic Ptc expression induced within another, separate *ihog*$^{DC1/DC1}$, *ptc*$^{S2/S2}$ clone.