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The *Drosophila* WIF1 homolog Shifted maintains glypican-independent Hedgehog signaling and interacts with the Hedgehog co-receptors Ihog and Boi

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SUMMARY

Hedgehog (Hh) family proteins are secreted signaling ligands whose short- and long-range activities transform cellular fates in multiple contexts in organisms ranging from metazoans to humans. In the developing *Drosophila* wing, extracellular Hh binds to cell-bound glypican heparan sulfate proteoglycans (HSPGs) and the secreted protein Shifted (Shf), a member of Wnt inhibitory factor 1 (WIF1) family. The glypicans and Shf are required for long-range Hh movement and signaling; it has been proposed that Shf promotes long-range Hh signaling by reinforcing binding between Hh and the glypicans, and that much or all of glypican function in Hh signaling requires Shf. However, we will show here that Shf maintains short-range Hh signaling in the wing via a mechanism that does not require the presence of or binding to the *Drosophila* glypicans Dally and Dally-like protein. Conversely, we demonstrate interactions between Hh and the glypicans that are maintained, and even strengthened, in the absence of Shf. We present evidence that Shf binds to the CDO/BOC family Hh co-receptors Interference hedgehog (Ihog) and Brother of Ihog, suggesting that Shf regulates short-range Hh signaling through interactions with the receptor complex. In support of a functional interaction between Ihog and members of the Shf/WIF1 family, we show that Ihog can increase the Wnt-inhibitory activity of vertebrate WIF1; this result raises the possibility of interactions between WIF1 and vertebrate CDO/BOC family members.

KEY WORDS: Hedgehog, Shifted, Interference hedgehog, Glypican, WIF1, HSPG

INTRODUCTION

Wnt inhibitory factor 1 (WIF1) family members play important roles in the extracellular regulation of developmental signaling via their effects on the movement, accumulation and access of morphogens to their receptors. Vertebrate WIF1 is a secreted protein that binds Wnts in the extracellular space, sequesters them from their receptors, and thereby reduces Wnt signaling (Hsieh et al., 1999). Knockdown of zebrafish *wif1* expression leads to elongation defects in the embryo and increased canonical Wnt signaling in the developing swimbladder (Yin et al., 2012). Although mouse *Wif1* knockouts have no overt developmental phenotype, epigenetic silencing of *Wif1* is thought to be crucial for Wnt-dependent pathologies in many human tumors (Clément et al., 2008; Gao et al., 2009; Kansara et al., 2009).

Shifted (Shf) is the sole *Drosophila* member of the WIF1 family but, surprisingly, has no known effects on *Drosophila* Wnt/Wingless signaling; instead, Shf is required for normal Hedgehog (Hh) signaling in the wing imaginal disc, the precursor of the *Drosophila* wing (Avanesov et al., 2012; Glise et al., 2005; Gorfinkiel et al., 2005). Hh is expressed in the posterior compartment of the disc, but signaling is limited to the anterior by the anterior-specific expression of the Hh receptor Patched (Ptc) and the signal-transducing Gli-family transcription factor Cubitus interruptus (Ci) (Lum and Beachy, 2004). In *shf* discs, Hh does not accumulate normally in the posterior compartment, and the range of Hh movement and signaling into the anterior compartment is strongly reduced (Glise et al., 2005, Gorfinkiel et al., 2005). Hh signaling defects are also evident in adult *shf* wings; long-range Hh activity determines the spacing between the third and fourth longitudinal veins (Mullor et al., 1997; Strigini and Cohen, 1997), and in *shf* escapers these veins are 'shifted' closer together.

Shf activity is probably based on binding Hh. Shf and Hh co-immunoprecipitate together when expressed in *Drosophila* salivary glands (Gorfinkiel et al., 2005). And, just as Hh accumulation depends on Shf, Shf accumulation in wing discs depends on Hh: the highly diffusible Shf protein accumulates more strongly on the surfaces of posterior cells where the levels of extracellular Hh are high, and Shf accumulation is lost from hh^{ts} discs (Glise et al., 2005) or from posterior *hh*-null mutant clones (data not shown).

Shf function has been further linked to glypicans, members of the heparan sulfate proteoglycan (HSPG) family. The protein cores of the two *Drosophila* glypicans, Dally and Dally-like protein (Dlp), are GPI-linked to the cell surface, and decorated with long unbranched heparan sulfate (HS) sidechains. The HS sidechains are highly negatively charged, which allows them to bind a range of ligands and ligand-binding factors (Bernfield et al., 1999; Yan and Lin, 2009). Full glypican function depends on the synthesis of these HS sidechains, but HS-independent activities of, and Hh binding to, the protein core have also been reported (Kirkpatrick et al., 2006; Williams et al., 2010; Yan et al., 2009; Yan et al., 2010). Dally and Dlp have several roles in Hh signaling in the wing: they maintain the levels of Hh in the posterior compartment, its release from posterior cells, and its movement along the apical and basolateral surfaces of the anterior epithelium (Bellaiche et al., 1998; Bornemann et al., 2004; Desbordes and Sanson, 2003; Han et al., 2004a; Han et al., 2004b; Takei et al., 2004; The et al., 1999; Ayers et al., 2010; Callejo et al., 2011; Gallet et al., 2008). Dlp may also have a role in Hh signal reception that is independent of its effects on Hh transport (Lum et al., 2003; Callejo et al., 2006; McLellan et al., 2006; Yan et al., 2010).

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Loss of Dally and Dlp, HS synthesis, or Shf all have similar effects on Hh accumulation, movement and signaling, suggesting that they have a shared function. Moreover, Shf accumulation is sensitive to glypican and HS levels (Glise et al., 2005; Avanesov et al., 2012), and its vertebrate homolog WIF1 directly binds HS sidechains with high affinity (Malinauskas et al., 2011). Therefore, it was proposed that the function of Shf depends on its ability to bridge or reinforce interactions between Hh and the glypicans (Glise et al., 2005; Gorfinkiel et al., 2005). Conversely, the function of glypicans as Hh-binding molecules was thought to be partly or wholly dependent on Shf, as HSPG-defective cells in *shf* discs do not show further reductions in Hh accumulation (Gorfinkiel et al., 2005). Interestingly, the Wnt-inhibitory activity of vertebrate WIF1 can be similarly regulated by interactions with the glypicans, in this case by forming a complex that sequesters Wnts from their receptors (Avanesov et al., 2012).

However, we will show here that Shf has a glypican-independent function, and glypicans have Shf-independent functions. The short-range Hh signaling that is normally maintained in *shf* discs or clones lacking glypican function is eliminated by the simultaneous loss of both, and the glypicans can affect Hh levels and movement in the absence of Shf. Moreover, the glypican-independent Shf activity is maintained after the loss of the HS-interacting 'EGF-like' domains of Shf without obviously affecting Hh levels, whereas the 'EGF-like' domain can stabilize Hh and increase its movement without affecting Hh signaling. Finally, we provide evidence strongly suggesting that Shf and its vertebrate homolog WIF1 binds to the BOC/CDO family Hh co-receptors Interference hedgehog (Ihog) and Brother of Ihog (Boi) (Lum et al., 2003; Yao et al., 2006; Camp et al., 2010; Yan et al., 2010; Zheng et al., 2010), providing a possible mechanism for the glypican-independent function.

MATERIALS AND METHODS

Mutant strains

Mutants used in this study, with the exception of shf^2 (Glise et al., 2005), are strong loss-of-function alleles: $botv^{103}$ (Han et al., 2004a); dlp^{A187} and $dally^{80}$ (Han et al., 2004b), $dally^{MH32}$ and dlp^{MH20} (Franch-Marro et al., 2005; Han et al., 2004b); dor^8 (an insertion) (Shestopal et al., 1997); ptc^{S2} (Strutt et al., 2001); ptc^{IIW} (Nakano et al., 1989); shf^{e33} (Glise et al., 2005); and smo^{D16} (Chen and Struhl, 1998).

Generation of mitotic recombinant clones

dor^8 clones were generated by γ-irradiation. All other clones were generated by FRT-mediated recombination (Xu and Rubin, 1993). The following lines were provided by the Bloomington *Drosophila* Stock Center: *ubi-GFP* FRT^{18A}*; hs-Flp*, *hs-Flp; ubi-GFP* FRT^{40A}, *hs-Flp;* FRT^{42D} *ubi-GFP*, *hs-Flp*; FRT^{G13} *M(2)53 πM/CyO*, *hs-Flp*; *ubi-GFP* FRT^{2A} and *hs-Flp*; *M(3)55 πM* FRT^{2A}*/TM6.* Dr Xinhua Lin (University of Cincinnati, OH, USA) kindly provided *hs-flp*; FRT^{G13}*ubi-GFP/CyO.*

Transgenic strains and constructs

Transgenic strains and constructs used were: *UAS-hh-GFP* and *UAS-*ptc^{14}*-GFP* (Torroja et al., 2004); *UAS-ptc* (Bloomington, IN, USA); *UAS-dlp* (Kirkpatrick et al., 2004); *UAS-dally* (Jackson et al., 1997); *UAS-ihog-RNAi* (VDRC, 29897); *UAS-boi RNAi* (VDRC, 869); *UAS-dlp-RNAi* (VDRC, 10299); *UAS-ihog* (Yao et al., 2006); *UAS-*boi^{EP1385} (Bloomington); *UAS-shf*, *UAS-shfΔEGF* and *UAS-shfΔWIF* (Glise et al., 2005); and *UAS-wif1* (Avanesov et al., 2012). Expression was induced using *A9-Gal4*, *ap-Gal4*, *en-Gal4*, *hh-Gal4* or *nub-Gal4* strains as described previously (Brand and Perrimon, 1993).

UAS-shf- contains a V5 epitope at the *Xcm*I restriction site proximal to the region encoding the WIF domain; it rescues *shf* nulls. *UAS-shfΔEGF-V5* was generated from *UAS-shf-v5* template by PCR. *UAS-shfΔWIF-V5* construct is C-terminally tagged. Transgenes were cloned into pUAST and the DNA constructs were injected into *Drosophila* embryos by Injection Services (Sudbury, MA, USA).

Protein expression

For in vitro protein expression in S2 cells, *UAS-shf* constructs were transfected with *paW-Gal4* (Han, 1996). At 5 days post-transfection 15 μl of medium was prepared in SDS-PAGE sample buffer, separated on SDS-PAGE and western blotted using anti-V5 or anti-Shf.

Antibody staining

Wing discs were removed from 3rd instar larvae in chilled phosphate-buffered saline (PBS, pH 7.4) and immediately fixed in 4% formaldehyde in PBS for 25 minutes, washed several times in PBST (PBS with 0.3% Triton X-100) and incubated with primary antisera overnight in blocking reagent (PBST+1% BSA) at following dilutions: 1:10 rat anti-Ci (Motzny and Holmgren, 1995); 1:200 rabbit anti-Col/Kn (Vervoort et al., 1999); 1:200 mouse anti-GFP (Chemicon); 1:200 rabbit anti-GFP (MBL International); mouse anti-Myc, 1:500 (Santa Cruz); rabbit anti-Myc, 1:500 (Santa Cruz); 1:400 rabbit anti-Hh (Tabata and Kornberg, 1994); 1:20 mouse anti-Ptc (Developmental Studies Hybridoma Bank); 1:200 mouse anti-V5, (Invitrogen); 1:250 rabbit anti-V5 (Bethyl); 1:1000 pre-absorbed rat anti-Shf (Glise et al., 2005); and 1:100 rat ant-Ihog (Yao et al., 2006). Secondary antisera from Jackson ImmunoResearch were diluted 1:200 in PBST and applied for 2 hours. Discs were mounted with their apical surfaces up in 70% (v/v) glycerol/PBS. Confocal images were collected on BioRad or Olympus confocal microscopes. Unless otherwise noted, all images show a single focal plane through the basolateral or apical surface. Measurements of fluorescent signal intensity were performed in ImageJ (NIH).

RESULTS

Short-range Hh signaling is maintained in clones lacking Dally and Dlp or HS

Drosophila embryos lacking maternal and zygotic *dlp* still retain low levels of Hh activity (Han et al., 2004b; Yan et al., 2009). Similarly, mosaic analysis shows that the Hh signaling from the posterior to the anterior compartments of wing imaginal discs is not completely eliminated from anterior clones lacking both Dally and Dlp, or lacking HS sidechains through the loss of EXT polymerases (Han et al., 2004a; Han et al., 2004b; Takei et al., 2004; The et al., 1999). Although these anterior clones lose expression of the most sensitive Hh target *engrailed* (*en*), they maintain expression of the high-threshold Hh target Ptc in a very thin domain measuring approximately one to two cells wide. The low threshold Hh target Ci^{Act} is also maintained in these anterior clones.

One possibility is that this effect is caused by contact with posterior, HSPG-expressing cells; for example, the HS chains of posterior Dlp and/or Dally might function non-autonomously to support Hh signaling in the adjacent anterior *dally dlp* mutant cells (Dejima et al., 2011; Jakobsson et al., 2006; Yan et al., 2009). To test this, we removed both Dally and Dlp, or the EXT polymerase Brother of tout-velu (Botv), from clones that straddled the compartment boundary and that included both anterior and adjacent posterior cells. These 'double-sided' HSPG-defective clones still maintained thin stripes of strong Ptc and Ci^{Act} expression anterior to the compartment boundary, even if they were quite large (Fig. 1A-C; supplementary material Fig. S1A), in agreement with published figures (Han et al., 2004b). Some non-autonomous improvement of Hh signaling was observed just inside clone boundaries, consistent with earlier observations (Callejo et al., 2006; Gallet et al., 2008). These results indicate that the *Drosophila* glypicans and HS sidechains are not required for the majority of short-range Hh activity. This data are also consistent with the retention of short-range Hh activity in single mutant *dally* or *dlp* wing discs (Ayers et al., 2010; Callejo et al., 2011; Gallet et al., 2008; Yan et al., 2010).

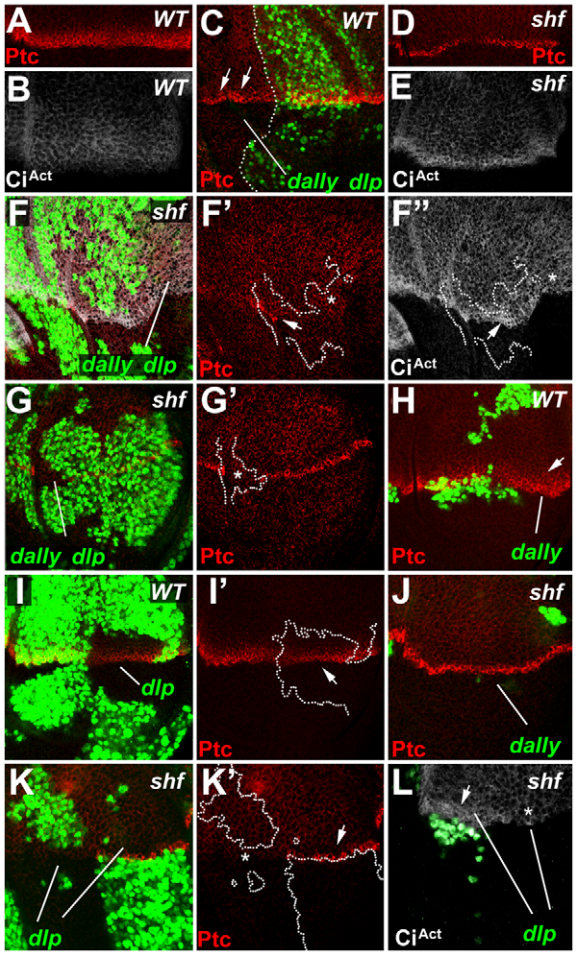

Fig. 1. Shf and glypicans independently maintain short-range Hh response. (A-L) Wing pouch region of wing imaginal discs showing anti-Ptc in red and anti-Ci^{Act} in white. Homozygous clones are marked by the absence of green marker (anti-Myc, outlined). Anterior is upwards and dorsal is leftwards. (**A**,**B**) Boundary regions of wild-type (WT) discs showing normal widths of Ptc (A) and Ci^{Act} (B) domains. Ci^{Act} is reduced next to the compartment boundary in the region of highest Hh signaling, probably through anterior *en/inv* expression (S.S.B., unpublished). (**C**) *dally dlp* clones in wild-type disc on both sides of the compartment boundary retain a thin stripe of Ptc (arrows). (**D**,**E**) Boundary regions of *shf* discs showing thinner domain of Ptc (C) and Ci^{Act} (D). Increased Ci^{Act} levels immediately adjacent to the compartment boundary are probably due to loss of anterior *en/inv* expression (Glise et al., 2005). (**F-G′**) *dally dlp* clones in *shf* discs. High Ptc and Ci^{Act} is lost in clones that straddle the boundary (asterisks). Residual staining typical of *shf* discs is retained adjacent to wild-type posterior cells (arrow in F′), and Ci^{Act} is retained adjacent to anterior wild-type cells (arrow in F″). (**H**) *dally* clones in wild-type disc. Ptc stripe inside the clone is similar to wild type (arrow, compare with A). (**I**,**I′**) *dlp* clones in wild-type disc. Ptc stripe is thinner in clones that straddle the boundary (arrow). (**J**) *dally* clones in *shf* disc. Thin Ptc stripe is not eliminated in clones. (**K-L**) *dlp* clones in *shf* discs. (K,K′) Ptc stripe is lost in clones that straddle the boundary (asterisk), but retained in purely anterior clone (arrow). (L) Ci^{Act} is reduced in clones that straddle the boundary (asterisk), but retained anterior to wild-type cells (arrow).

Shf and HSPGs have separate roles in short-range Hh signaling

We next tested whether Shf retained function in the absence of HSPGs, by examining whether Shf helped maintain the short-range Hh response in HSPG-deficient clones. Clonal analysis of *shf* function is not possible because Shf diffuses over long distances (Glise et al., 2005; Gorfinkiel et al., 2005). We therefore induced *dally dlp* mutant clones in the discs of *shf*^{x33} null (from here on referred to as *shf*) mutant larvae. Strikingly, we found that double-sided *dally dlp* mutant clones generated in *shf* discs lost the one- to two-cell wide stripe of high Ptc expression normally seen in *shf* mutant discs (Fig. 1F; supplementary material Fig. S1B). The stabilization of Ci^{Act} was also affected. Normally, *shf* discs have a thin, two- to three-cell wide stripe of anti-Ci^{Act} staining that overlaps with the expression of Ptc (Glise et al., 2005; Gorfinkiel et al., 2005). This region of heightened anti-Ci^{Act} staining was largely eliminated from double-sided *dally dlp* mutant clones in *shf* discs (Fig. 1F; supplementary material Fig. S1B). Thus, Shf is required for much of the short-range Hh signaling normally maintained in the absence of Dally and Dlp. Conversely, the glypicans were required for much of the short-range Hh signaling normally maintained in the absence of Shf.

The additive effects of losing Shf and the glypicans was not apparent unless we also eliminating the short-range non-autonomous effects of the glypicans. Purely anterior or purely posterior *dally dlp* mutant clones did not obviously alter the anti-Ptc and anti-Ci^{Act} staining in *shf* mutant discs (Fig. 1F; supplementary material Fig. S1B). The additive effects were also weaker when Shf loss was combined with the loss of HS synthesis, instead of the loss of the glypicans. Although double-sided *botv* mutant clones also lost heightened Ptc expression in *shf* mutant discs, they had *shf*-like anti-Ci^{Act} staining (supplementary material Fig. S2). This indicates that the glypican protein cores retain some ability to promote low levels of Hh signaling in the absence of the HS chains and Shf.

Dlp maintains Shf-independent short-range Hh signaling

Dally and Dlp differ in their contributions to Hh signaling: loss of Dally has much weaker effects on Hh signaling than loss of Dlp (Fig. 1H,I), and removal of both genes produces stronger defects than the removal of either one alone (Fig. 1C; supplementary material Fig. S1A) (Ayers et al., 2010; Eugster et al., 2007; Gallet et al., 2008; Han et al., 2004b). We therefore asked which of the two glypicans was required for the Shf-independent Hh response. We found that the width of the anti-Ptc staining was very similar in *shf* discs and *shf* discs that contained double-sided *dally* mutant clones, even when such clones encompassed most of the wing disc (Fig. 1H,J). By contrast, boundary anti-Ptc staining was eliminated and anti-Ci^{Act} staining was reduced in double-sided *dlp* mutant clones in *shf* mutant discs (Fig. 1K,L). These data indicate that Dlp is the predominant glypican that mediates the Shf-independent Hh response.

Interactions between glypicans and Hh can be independent of Shf

The proposal that Shf is required for interactions between glypicans and Hh was based on the finding that defects in both HS chain synthesis and *shf* function produced similar, and not additive, effects of on Hh accumulation (Gorfinkiel et al., 2005). We obtained identical results in *shf* mutant discs that contained clones lacking both Dally and Dlp (data not shown) However, it is possible that the already strong reduction of extracellular, basolateral Hh in *shf* discs may make it difficult to detect additional Hh reductions, concealing separable effects of Shf and HSPGs on Hh levels. We therefore turned to assays in which changes in HSPG expression produce the opposite effect: an increase in anti-Hh staining.

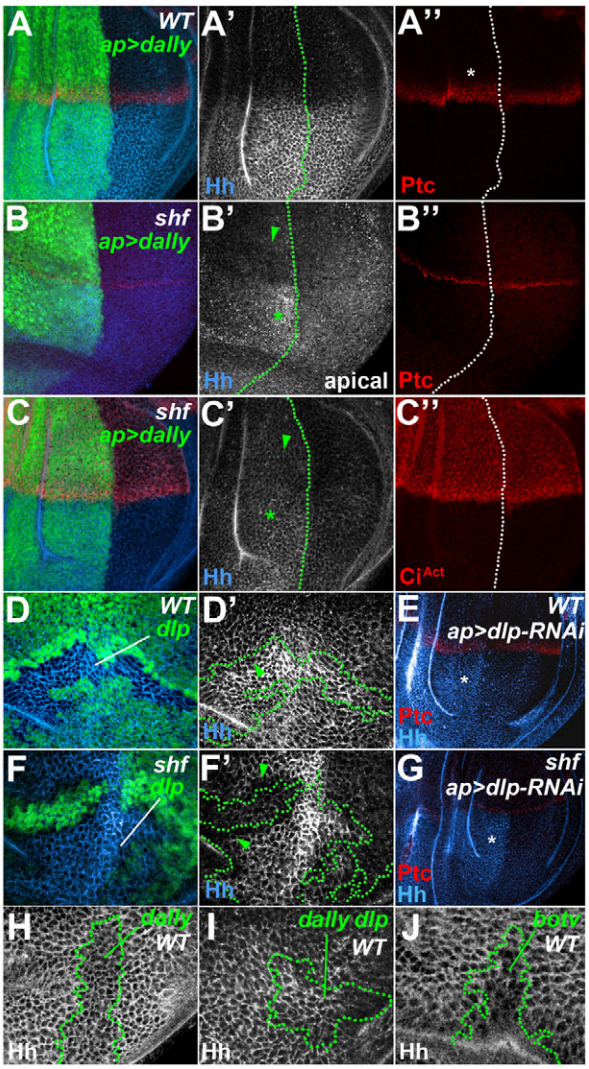

Fig. 2. Hh and glypicans interact in the absence of Shf. Anti-Hh (blue, white) and anti-Ptc or anti-Ci^{Act}t (red) staining in wing imaginal discs. B and B′ show an apical focal plane; all others show a basolateral focal plane. Anterior is upwards and dorsal is leftwards. (**A-A″**) Dorsal, *ap-Gal4*-driven overexpression of *UAS-dally* in wild-type disc (*UAS-GFP*, green) does not change Hh (A′), but increases the width of the Ptc stripe (red, asterisk) (A″). (**B-C″**) Dorsal *ap-Gal4*-driven overexpression of *UAS-dally* (*UAS-GFP*, green, outlined) in *shf* mutant discs increases anti-Hh staining: strongly in the apical and basolateral focal planes in the posterior (asterisks), and weakly in the basolateral focal planes in the anterior (arrowheads). The widths of the Ptc (B″) or Ci^{Act} (C″) stripes are not increased. (**D**,**D′**) *dlp* clone, identified by absence of Myc epitope (green, outlined), in posterior of wild-type disc. Hh increases inside the clone, but this increase is rescued at clone boundaries by surrounding wild-type cells (arrowhead). (**E**) Dorsal *ap-Gal4*-driven expression of *UAS-dlp-RNAi* increases dorsal/posterior Hh (asterisk). (**F**,**F′**) *dlp* clone, identified by absence of green Myc epitope (green, outlined), in the posterior of a *shf* disc. Hh increases inside the clone, but this increase is rescued at clone boundaries by surrounding *dlp*-expressing cells (arrowheads), despite the absence of *shf*. The contrast in Hh levels between the outside and the center of the clone is greater than in wild-type disc (compare with D′). (**G**) Dorsal *ap-Gal4*-driven expression of *UAS-dlp-RNAi* in *shf* disc increases dorsal/posterior Hh (asterisk). (**H-J**) *dally*, *dally dlp* and *botv* mutant clones (outlined) in posterior wild-type discs. *dally* mutant clones reduce Hh (H), but in the *dally dlp* clone (I), Hh is largely unaffected. *botv* mutant clones (J) reduce Hh.

Overexpression of *dally* was shown previously to elevate apical Hh levels (Ayers et al., 2010). Apical Hh levels are not reduced in *shf* mutants (Glise et al., 2005), and we often detected increased apical anti-Hh staining after expressing *UAS-dally* under the control of the dorsal *ap-Gal4* driver in *shf* mutant discs (Fig. 2B). Moreover, we also often observed increases in basolateral anti-Hh staining in both the posterior and anterior compartment (8/12 discs) (Fig. 2C). Importantly, the increased anterior Hh was never accompanied by an obvious increase in Hh signaling as judged using Ptc and Ci^{Act} (Fig. 2B,C). By contrast, the same level of *UAS-dally* overexpression in wild-type discs increased the width of anterior anti-Ptc staining, indicating that *UAS-dally* is capable of increasing Hh signaling in the presence of Shf (Fig. 3A), but we only rarely detected increased basolateral anti-Hh staining (1/8 discs). This is the opposite of what might be expected if Shf stabilized or bridged binding between Dally and Hh.

We next tested whether Shf was required for interactions between Hh and Dlp, as our data demonstrate that Dlp, and not Dally, was required for the majority of Shf-independent Hh signaling in the wing (see Fig. 1J,K). We first tried Dlp overexpression, but this did not increase anti-Hh staining in either wild-type or *shf* discs, using our strongest driver *ap-Gal4*, and actually appeared to reduce anti-Hh staining in some wild-type discs (data not shown). We therefore examined the non-autonomous effects of Dlp loss on Hh accumulation: Hh levels increase inside *dlp* mutant clones, but these increases are not seen in mutant cells near clone boundaries adjacent to wild-type Dlp-expressing cells (Callejo et al., 2011) (also in Fig. 2D). Dlp appears to have a non-autonomous role removing Hh from the surfaces of adjacent cells, accounting for the accumulation of Hh in *dlp* mutant clones and for the boundary effect (Callejo et al., 2011).

This role for Dlp in Hh movement does not require Shf. *dlp* mutant clones caused similar effects in *shf* mutant discs and, as in the wild-type background, Hh accumulation inside the clone margins was rescued by surrounding *dlp*-expressing cells. In fact, the contrast between the Hh in the center of the *dlp* clone and in the adjacent tissue was more striking in *shf* discs than in wild type (Fig. 2F). We also observed increased Hh levels after dorsal, *ap-Gal4*-driven expression of *UAS-dlp-RNAi*, and this effect was again more striking in a *shf* background (Fig. 2E,G). If anything, the more striking increases in Hh levels caused by *dlp* knock down or *dally* overexpression in *shf* mutants suggest that binding between Hh and the glypicans is more effective in the absence of Shf.

The increase in basolateral Hh levels in posterior Dlp clones is surprising, as Hh accumulation is reduced in clones lacking the HS synthesis thought to be required for much of Dlp function (Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004). However, clones lacking HS synthesis also disrupt Dally function, and although removing *dally* has only weak effects on Hh signaling, posterior Hh is markedly reduced in clones lacking *dally* alone (Fig. 2H). That said, removing HS synthesis does not simply mimic the combined loss of *dally* and *dlp*, as we did not observe a consistent reduction in Hh levels in *dally dlp* clones; the opposite effects of *dally* and *dlp* loss on Hh levels balance out (Fig. 2I). By contrast, Hh was consistently reduced in *botv* clones (Fig. 2J). One likely explanation is that, in the absence of the HS sidechains, the Dlp core protein retains some of its ability to remove Hh from the cell surface.

The ‘WIF’ domain of Shf promotes Hh signaling independently of the HSPGs

Shf, like its vertebrate ortholog WIF1, is composed an N-terminal ‘WIF’ domain, followed by five ‘EGF-like’ repeats. The ‘EGF-like’

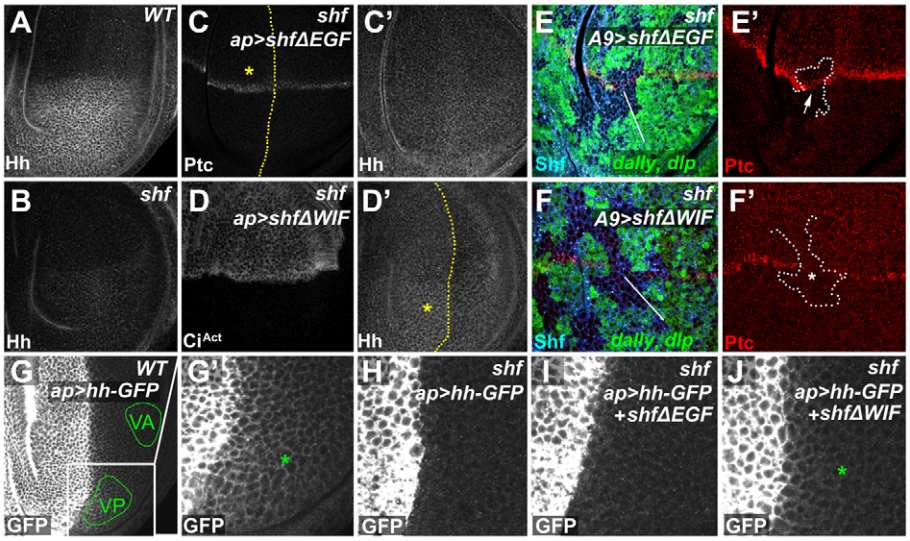

Fig. 3. The roles of the WIF and 'EGF-like' domains of Shf in signaling and Hh accumulation. Anterior is upwards and dorsal is leftwards. (**A**,**B**) Hh in wild-type (A) and *shf* (B) wing discs. (**C**,**C'**) Dorsal *ap-Gal4* expression of *UAS-shfΔEGF* widens the Ptc stripe in dorsal cells (C, asterisk) without detectably increasing Hh (C'). (**D**,**D'**) Dorsal *ap-Gal4* expression of *UAS-shfΔWIF* does not widen the Ci^{Act} stripe (D) but does increase Hh in dorsal cells (D', asterisk). Broken lines in C-D' mark dorsal/ventral boundaries. (**E-F'**) *shf* discs with widespread *A9*-Gal4-driven expression of *UAS-shfΔEGF* (E,E') or *UAS-shfΔWIF* (F,F') (identified with anti-Shf, blue) and *dally dlp* clones (absence of Myc epitope, green, outlined). *UAS-shfΔEGF* rescues Ptc (red) in *dally dlp* clone (arrow in E'); the Ptc expression domain is also wider outside the clones than in *shf* discs, owing to the effects of *shfΔEGF* on Hh signaling. *UAS-shfΔWIF* did not induce Ptc staining in *dally dlp* clones (asterisk in F'). (**G-J**) Hh-GFP after dorsal *ap-Gal4*-driven expression of *UAS-Hh-GFP*. G shows an example of the wild-type wing disc pouch, other panels show magnified details of the posterior compartments, including the wild type. Full panels, average intensities and statistics are in supplementary material Fig. S4. In wild-type discs (G,G'), Hh-GFP accumulates in ventral cells (G', asterisk), but this is largely absent in *shf* disc (H). Dorsal expression of *UAS-shfΔEGF* did not significantly improve ventral Hh-GFP in *shf* disc (I, P=0.18), but *UAS-shfΔWIF* did (J, asterisk; P=0.004), although probably not to wild-type levels (P=0.045).

domains of human WIF1 are necessary and sufficient for binding to HS (Malinauskas et al., 2011), consistent with our recent analysis of genetic interactions between HSPGs and truncated WIF1 proteins (Avanesov et al., 2012). We also showed in that study that the ability of Shf to interact with HSPGs required a normal 'EGF-like' domain. Although changes in Dlp levels did not consistently alter anti-Shf staining, anti-Shf staining decreased in *botv*, *dally dlp* (Glise et al., 2005) or *dally* mutant clones, and increased after overexpression of Dally. However, accumulation of the product of shf^2, which carries a missense mutation in its third 'EGF-like' repeat, did not respond to changes in *dally* levels (Avanesov et al., 2012).

Thus, the shf^2 product lacks detectable interactions with HSPGs. But although shf^2 has a strong effect on Hh signaling, it is much more viable than the null *shf* excision mutant shf^{x33}, which suggests that Shf^2 retains some activity. Similarly, whereas completely removing the 'EGF-like' repeats of Shf (ShfΔEGF) strongly impairs its ability to rescue *shf* mutants (Glise et al., 2005), ShfΔEGF retains some activity (Avanesov et al., 2012). *ap-Gal4* driven expression of *UAS-shfΔEGF* in *shf* nulls improved viability, increased the width of anterior anti-Ptc staining in the dorsal region of discs (Fig. 3C), and led to a modest but significant increase in the distance between the third and fourth longitudinal veins in adult wings (supplementary material Fig. S3). The activity of ShfΔEGF was independent of the glypicans: the anti-Ptc staining that is normally lost from double-sided *dally dlp* mutant clones in *shf* mutant discs was improved by the *A9-Gal4*-driven expression of *UAS-shfΔEGF* (Fig. 3E).

The improvement in signaling in *shf* discs with *ap-Gal4*-driven expression of *UAS-shfΔEGF* was not accompanied by a detectable increase in Hh levels in dorsal cells (Fig. 3C). This is in agreement with the reduced Hh levels caused by the 'EGF-like' domain mutation in Shf^2 (Glise et al., 2005). Nor did ShfΔEGF detectably improve Hh movement. In wild-type discs, dorsally expressed Hh-GFP moves well into the ventral region of the disc, and this ventral accumulation is almost abolished in *shf* discs (Glise et al., 2005; Gorfinkiel et al., 2005) (Fig. 3G,H; supplementary material Fig. S4A,B). Hh-GFP movement in *shf* discs was not detectably improved by dorsal expression of *UAS-shfΔEGF* (Fig. 3I; supplementary material Fig. S4C).

By contrast, expression of the complementary *UAS-shfΔWIF* in *shf*-null discs did not increase the width of Ptc and Ci^{Act} staining (Fig. 3D; data not shown), nor improve spacing between the third and fourth longitudinal veins in *shf* adult wings (supplementary material Fig. S3). Nor did it rescue the loss of Ptc expression caused by double-sided *dally dlp* clones in *shf* discs (Fig. 3F). This was despite the fact that both of our truncated *shf* constructs produced very comparable protein levels in the discs (Glise et al., 2005), and were secreted and remained stable when expressed by S2 cells in vitro (data not shown). Surprisingly, despite the absence of improved signaling, *ap-gal4* driven expression of *UAS-shfΔWIF* in *shf* discs did increase anti-Hh staining dorsally (Fig. 3D), and significantly improved the ventral accumulation of dorsally expressed Hh-GFP (Fig. 3J; supplementary material Fig. S4D). Both these results suggest that the 'EGF-like' domains of Shf not only bind HSPGs, but also retain some ability to bind to Hh in the absence of the 'WIF' domain (see Discussion).

ShfΔEGF did not, however, rescue signaling to wild-type levels, nor did ShfΔWIF completely rescue Hh levels or movement. Moreover, both constructs had short-range effects, unlike full-

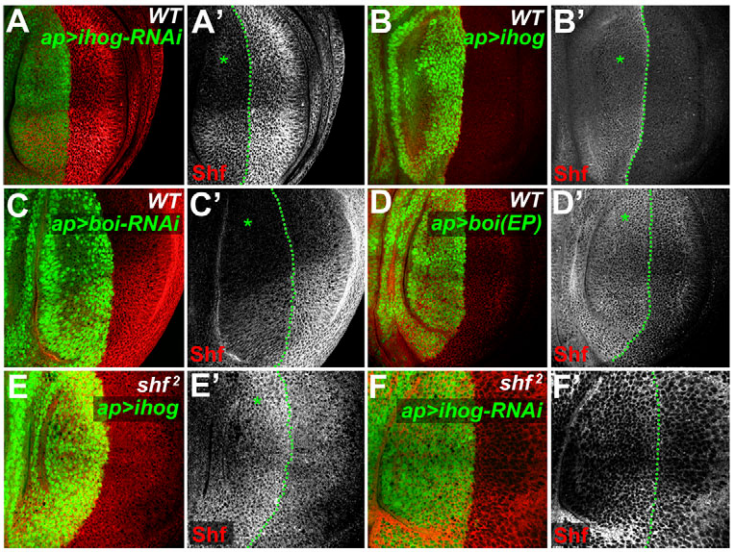

Fig. 4. Boi and Ihog regulate Shf levels. The effects of dorsal *ap-Gal4*-driven (*UAS-GFP*, green, outlined) *ihog*/*boi* knock down or overexpression on anti-Shf (red). Shf changes in far anterior regions, where anti-Hh cannot be detected, are marked with asterisks. Anterior is upwards and dorsal is leftwards. (**A**,**A′**) *UAS-ihog-RNAi* reduces Shf. (**B**,**B′**) *UAS-ihog* increases Shf. (**C**,**C′**) *UAS-boi-RNAi* reduces Shf. (**D**,**D′**) Driving *boi* expression from boi^{EP1385} increases Shf. (**E**,**E′**) *UAS-ihog* in shf^{2} disc weakly increases Shf^{2} protein (asterisk), which is recognized by anti-Shf. (**F**,**F′**) *UAS-ihog-RNAi* in a shf^{2} mutant disc does not reduce the Shf^{2} protein.

length Shf, which can rescue both signaling and Hh levels over a very long range (Glise et al., 2005). Interestingly, combined expression of both truncated transgenes in *shf* mutants did not significantly improve L3-L4 spacing compared with *shf* mutants that expressed *UAS-shfΔEGF* alone (supplementary material Fig. S3F). Thus, the two domains must be attached in order to support full Shf function.

Shf and its vertebrate ortholog WIF1 interact with the Hh co-receptor Ihog

In several of our experiments above, increased levels or movement of Hh failed to improve the Hh signaling defects in *shf* discs (Fig. 2A-C,F; Fig. 3D). This suggests that the glypican-independent activity of Shf might operate at the level of Hh signal reception, rather than at the level of Hh accumulation (see Discussion). This function would be similar to, though independent of, the co-receptor role demonstrated for Dlp (Lum et al., 2003; Yan et al., 2010).

To provide additional support for the role of Shf in Hh reception, we tested its interactions with the Hh receptor Ptc and the co-receptors Ihog and Boi. We could not find any convincing evidence of interactions between Shf and Ptc. First, the levels of cell surface anti-Shf staining were not altered in *ptc* mutant clones, even those on the anterior side of the A/P compartment boundary where Ptc is normally high (data not shown). Second, although dorsal misexpression of Ptc did reduce Shf levels in posterior (supplementary material Fig. S5B), this was more likely to be an indirect effect mediated by competition for binding to Hh: the accumulation of posterior Shf is partly dependent on Hh (Glise et al., 2005) and we did not observe the effect in the far anterior where Hh levels are extremely low. This effect is apparently not mediated by endocytosis of Ptc-Shf-Hh complexes, because it was also observed after overexpression of Ptc^{14} (supplementary material Fig. S5C), a mutant form of Ptc that is capable of Hh binding but defective in its internalization (Torroja et al., 2004). Finally, Shf did not colocalize with Ptc puncta in the endosomal compartment known to contain internalized Hh/Ptc complexes (Callejo et al., 2006; Gallet et al., 2008; Torroja et al., 2004) (supplementary material Fig. S5D), even after increasing the levels of endosomal Hh/Ptc using *deep orange* mutant clones (supplementary material Fig. S5E), which block endosomal processing (Shestopal et al., 1997; Torroja et al., 2004).

By contrast, we found that the levels of Shf protein were strongly reduced in the dorsal compartment following reductions of Ihog or Boi by *ap-Gal4*-driven expression of *UAS*-controlled *ihog* or *boi* hairpin constructs (e.g. *UAS-ihog-RNAi*) (Fig. 4A,C). As Shf accumulation partly requires Hh (Glise et al., 2005), and Hh levels were recently shown to be affected by Ihog and Boi (Yan et al., 2010), it is possible that the interaction between Shf and Ihog/Boi is mediated indirectly via effects on Hh levels, or through reductions of Hh signaling. However, this is unlikely. First, Ihog and Boi affect Shf levels, even in the far anterior region of the disc where the levels of the endogenous Hh are extremely low, and in the posterior, where canonical Ci-mediated Hh signaling does not occur. Second, owing to the redundancy of Ihog and Boi, knocking either one down individually does not appear to reduce Hh signaling (Camp et al., 2010; Yan et al., 2010; Zheng et al., 2010) (data not shown), but does affect Shf levels. Third, Shf staining was not changed in *smoothened* mutant clones, which lose all measurable responses to Hh (supplementary material Fig. S5A). Last, overexpression of Ihog or Boi increased Shf accumulation in both posterior and anterior cells (Fig. 4B,D).

Interestingly, the mutation of the EGF-like domains of Shf in Shf^{2} reduced, but did not completely eliminate, this apparent binding between Shf and Ihog. Shf^{2} levels were not obviously affected by knock down of *ihog* expression (Fig. 4A,F), but overexpression of *ihog* led to weak stabilization of Shf^{2} (Fig. 4B,E). This raises the possibility that the weak activity of the WIF domain of Shf might be mediated by interactions with Ihog/Boi (see Discussion).

The properties of Shf and Ihog/Boi, and the possibility that they have independent roles in Hh signaling, make it difficult to test genetically for functional interactions between these molecules. Removing Ihog and Boi completely blocks Hh signaling in the wing disc (Camp et al., 2010; Yan et al., 2010; Zheng et al., 2010), preventing us from assessing whether Shf activity is absolutely dependent on the presence of Ihog/Boi. Nonetheless, *ap-Gal4*-driven expression of *UAS-ihog* can increase Hh levels in discs (Yan et al., 2010), and those levels are significantly lower in *shf*-null discs (Fig. 5). This suggests that Shf increases the affinity for or access to Hh by Ihog. Overexpression of Shf does not affect signaling in wild-type wings (Glise et al., 2005; Gorfinkiel et al., 2005), but can slightly improve the reduction in L3-L4 spacing

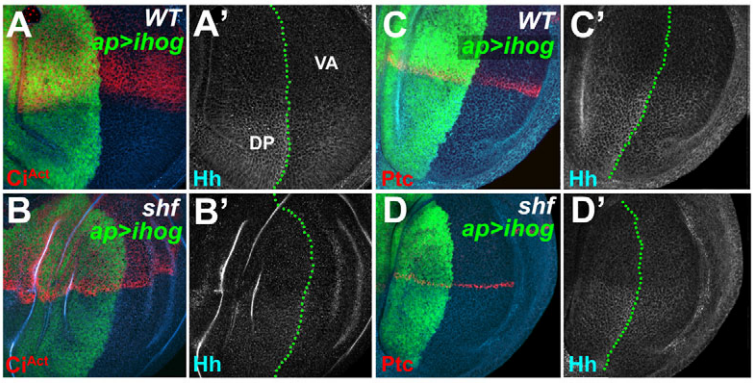

Fig. 5. Shf increases the effects of Ihog on Hh levels. Anterior is upwards and dorsal is leftwards. (**A-D'**) Dorsal *ap-Gal4*-driven expression of *UAS-ihog* (*UAS-GFP*, green, outlined) in wild-type disc increases anti-Hh staining (blue, white) in the dorsal side of the posterior compartment (DP quadrant) (A,A',C,C'), but in *shf* discs this dorsal increase is weaker (B,B',D,D'). The experiments used rabbit anti-Hh (A-B') or goat anti-Hh (C-D'). Wild-type and *shf* discs are identified using antibodies to Ci^{Act} and Ptc. The average anti-Hh staining in the DP quadrant, normalized to the background staining in the anterior-ventral (AV) quadrant, was quantified in *n* discs of each type. With rabbit anti-Hh, the averages and standard deviations were: wild type versus *shf*, 1.92±0.42 (*n*=9) versus 1.34±0.08 (*n*=4); *P*=0.02, Mann-Whitney U test. With goat anti-Hh they were: wild type versus *shf*, 1.41±0.17 (*n*=15) versus 1.23±0.14 (*n*=9); *P*=0.0035, Mann-Whitney U test.

caused by anterior, *ptc-Gal4*-driven expression of both *UAS-boi-RNAi* and *UAS-ihog-RNAi*; this could indicate that Shf improves the function of the residual Boi and Ihog (supplementary material Fig. S6). However, because Shf, Ihog and Boi all affect Hh levels and signaling, it is difficult to rule out that these effects are independent of any direct functional interaction between Shf and Ihog/Boi.

Ihog does not, however, affect signaling by the *Drosophila* Wnt Wingless (Wg), allowing us to test the role of Ihog in the activity of the vertebrate Shf homolog WIF1. WIF1 shows an obvious gain-of-function phenotype because it binds Wnts, including *Drosophila* Wg, and inhibits their activity (Hsieh et al., 1999); expressing WIF1 in the wing disc results in the loss of Wg-dependent wing margin structures (Avanesov et al., 2012; Gorfinkiel et al., 2005). We found that the inhibition of Wg signaling caused by expressing zebrafish *wif1* with *nubbin-Gal4* was significantly increased by co-expressing *UAS-ihog* (Fig. 6). Thus, Ihog can influence the function of a Shf-like molecule, and the result suggests that Ihog and WIF1 bind each other, sequestering Wg in the Hh-co-receptor complex and thereby reducing the levels available for the Wg receptors.

DISCUSSION

Shf has a glypican/HSPG-independent role in Hh signaling

In the wing disc of *Drosophila*, the secreted protein Shf helps maintain the levels of extracellular Hh around posterior Hh-secreting cells, the accumulation of Hh in the anterior, distant from the source of Hh synthesis, and the range and levels of Hh signaling (Glise et al., 2005; Gorfinkiel et al., 2005). The phenotypes observed after the loss of *shf* are very similar to those observed after removal of HS synthesis, or simultaneous loss of both of the *Drosophila* glypican HSPGs, Dally and Dlp (Bellaiche et al., 1998; Bornemann et al., 2004; Desbordes and Sanson, 2003; Han et al., 2004a; Han et al., 2004b; Takei et al., 2004; The et al., 1999). As Shf binds Hh and, like its vertebrate ortholog WIF1, interacts with the HS sidechains of glypicans (Avanesov et al., 2012; Glise et al., 2005; Malinauskas et al., 2011), this led to proposal that the role of Shf role was to reinforce binding between Hh and glypican HSPGs.

However, by examining the effects of the simultaneous loss of *shf* and glypican/HSPG function, we have shown that Shf retains

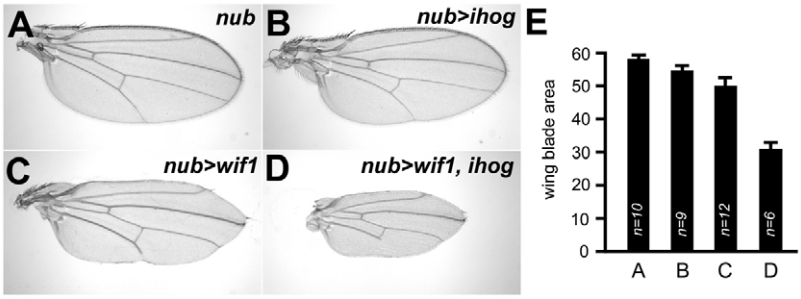

Fig. 6. Synergistic effects of Ihog and Wif1 on wing size. (**A**,**B**) Normal wing margin in *nub-Gal4* (A) and *nub-Gal4 UAS-ihog* (B). (**C**) Loss of wing margin and reduced wing size in *nub-Gal4 UAS-wif1*, which is typical of the inhibition of Wg signaling by Wif1. (**D**) Combined *nub-Gal4*-driven *UAS-wif1* and *UAS-ihog* further reduces the wing margin and wing size when compared with wings expressing either transgene alone. (**E**) Comparison of average wing blade areas and standard deviations (error bars) after the manipulations in A-D. Co-overexpression of Ihog and Wif1 (D) synergistically reduces the wing blade area when compared with the overexpression of Ihog (B) or Wif1 (C) alone. Quantifications are in arbitrary units; all differences were statistically significant using the Mann-Whitney U test (*P*<0.001).

an Hh signaling function in tissues lacking glypicans or HS synthesis (Fig. 1; supplementary material Fig. S2). Indeed, even the truncated ShfΔEGF construct that lacks the 'EGF-like' domains that are responsible for HS binding and HS-dependent genetic interactions of WIF1 family members (Avanesov et al., 2012; Malinauskas et al., 2011) can partially improve the reduced Hh signaling of *shf* mutants, whether or not those tissues have glypicans (Fig. 3C,E).

HSPGs and Shf were previously thought to regulate signaling by increasing the posterior levels of Hh and Hh movement into the anterior compartment, but our results indicate a Shf function independent of these effects. Removing the glypican-independent function of Shf is not accompanied by any additional reduction in the posterior levels of extracellular Hh, and ShfΔEGF improves signaling in *shf* discs without detectably improving the levels and movement of Hh. The complementary ShfΔWIF construct, by contrast, can improve Hh levels and movement, without improving signaling (Fig. 3D,J).

Shf has a short-range activity and interacts with the Hh co-receptors Ihog and Boi

Several findings suggest that glypican-independent function of Shf is short range. Removing the glypican-independent Shf function reduces signaling in cells immediately adjacent to the posterior region of Hh secretion (Fig. 1). Posterior cells lacking *dlp* retain high levels of basolateral Hh on their surfaces in wild-type (Callejo et al., 2011) and *shf* mutant discs (Fig. 2D-G), but this cell-bound Hh loses much of its ability to signal to immediately adjacent anterior cells in the absence of Shf. Finally, anterior overexpression of *dally* can increase Hh signaling in wild-type discs, but in *shf* discs the increase in anterior Hh caused by *dally* overexpression is not accompanied by an increase in the range of Hh signaling (Fig. 3A-C).

The short-range Hh movement mediated by Dlp (Callejo et al., 2011) is intact in *shf* mutant discs (Fig. 2F), suggesting that *shf* discs are defective in Hh reception. Although we did not see any evidence for binding between Shf and the Hh receptor Ptc (supplementary material Fig. S5), the co-receptors Ihog and Boi were both necessary and sufficient for the normal accumulation Shf on cell surfaces, independent of any effects on Hh accumulation or signaling (Fig. 4), which strongly suggests that they bind one another.

The strong Hh signaling defects caused by Ihog/Boi depletion, and lack of obvious gain-of-function phenotypes with Shf, made it difficult to address functional relevance of Ihog/Shf interactions in Hh signaling. To circumvent this problem, we used WIF1, a vertebrate Shf homolog that exhibits a strong gain-of-function phenotype in Wnt/Wg signaling. Overexpression of human or zebrafish WIF1 in the *Drosophila* wing sequesters the *Drosophila* Wnt Wg from its receptors and thereby inhibits Wg signaling (Avanesov et al., 2012; Gorfinkiel et al., 2005). Although Ihog overexpression does not affect Wg signaling on its own and has only a very slight effect on wing size, Ihog overexpression increased the Wg-inhibiting activity of WIF1 (Fig. 6). This suggests that Ihog can recruit WIF1/Shf family members and their binding partners into a complex, one that is counter-productive in the case of Wg-bound WIF1, but likely to be productive in the case of Hh-bound Shf. This result also suggests there might be similar interactions between vertebrate WIF1 and the vertebrate homologs of Ihog, BOC and CDO, providing a possible role for these proteins in the WIF1-mediated regulation of Wnt signaling.

How might the binding between Shf and Ihog/Boi regulate Hh activity? The increase in Hh accumulation caused by overexpression of Ihog was weakened by removal of Shf, and Shf overexpression, despite having no phenotype on its own, improved Hh signaling after knockdown of *ihog*/*boi* expression in Hh-sensitive cells (Fig. 5; supplementary material Fig. S6). Although Shf is clearly not absolutely required for Hh-Ihog binding, these results suggest that Shf might increase the affinity for or access to Hh by Ihog.

While it remains possible that these effects are caused by independent influences of Ihog and Shf on Hh levels, they contrast greatly with the effects that changes in glypican expression have on Hh levels, which appear stronger or more reliable in the absence of Shf (see below). If Shf strengthened the binding between Ihog and Hh, this could also explain some or all of the reduction of posterior Hh levels in *shf* mutants, since Ihog and Boi are also required to stabilize posterior Hh (Yan et al., 2010).

Finally, we note the activities of Ihog/Boi may go beyond that of simple co-receptors, complicating the possible mechanisms of Shf activity. Unlike the Hh receptor Ptc, Ihog and Boi are expressed in the posterior compartment (Yao et al., 2006), where Hh is produced but signaling blocked by the absence of the Ci transcription factor. Ihog and Boi help extracellular Hh bind to posterior cells (Yan et al., 2010), raising the possibility that Ihog and Boi increase the levels of Hh available for signaling to anterior cells. However, we do not favor this view, since signaling is not obviously reduced by large adjacent posterior clones lacking *ihog* and *boi* (Zheng et al., 2010), or by posterior, *hh-gal4*-driven expression of *UAS-ihog-RNAi* and *UAS-boi-RNAi* (supplementary material Fig. S7). In fact, there is data suggesting that Ihog/Boi reduces Hh movement into the anterior (Zheng et al., 2010), which raises the converse hypothesis that Shf-Ihog binding increases signaling by allowing Shf to free Hh from Ihog/Boi. Our evidence also does not favor this hypothesis, as the Ihog-dependent accumulation of Hh is not obviously increased in the absence of Shf (Fig. 5).

Does Shf strengthen or reduce HSPG functions in Hh signaling?

Our results do not, of course, rule out additional cooperative roles for interactions between Shf and glypican HSPGs, especially in Hh accumulation and long-range movement. However, we note that glypicans retain many of their effects on Hh levels and signaling in tissues lacking Shf: Dally overexpression can often increase the levels of apical and basolateral Hh; Dlp can still reduce Hh accumulation in adjacent cells lacking Dlp (Fig. 2), something hypothesized to depend on Dlp-dependent movement of Hh between cells (Callejo et al., 2011). In fact, the stabilization of extracellular Hh after either Dally overexpression or Dlp removal were more consistent and proportionately stronger in *shf* discs. This suggests that Shf actually reduces Hh-glypican interactions, rather than reinforcing them. This contrasts markedly with the effects of Shf on Ihog-mediated Hh stabilization.

Given the ability of Shf to bind with Hh, Ihog/Boi and HSPGs, but the inability of Shf overexpression to sequester Hh from its receptors, we suggest that Shf acts as a highly diffusible 'exchange protein' that weakly binds Hh and reinforces its delivery to other binding partners, or solubulizes Hh to allow exchange between those partners. Thus, Shf would help transfer Hh from the HSPGs to Ihog/Boi, but also between binding sites on HSPGs during a type of ligand movement that has been recently visualized with FGF2 (Duchesne et al., 2012). This is similar in principle to the role

proposed for Crossveinless 2/BMPER (Cv-2), which binds BMPs, HSPGs, the BMP-binding protein Short gastrulation (Sog) and BMP receptors, and is thought to exchange BMPs between these molecules (Serpe et al., 2008).

Specificities of the 'WIF' and 'EGF-like' domains of Shf

Like all WIF1 family members, Shf contains a 'WIF' domain followed by five 'EGF-like' domains. It has been proposed that the 'WIF' domain can interact with Hh. Most of the Wnt-binding activity of vertebrate WIF1 resides within its 'WIF' domain, which contains a hydrophobic pocket that has been suggested to bind the N-terminal palmitate added to Wnts and, by analogy, the palmitate added to Hh (Liepinsh et al., 2006; Malinauskas, 2008; but see Malinauskas et al., 2011). Binding between Hh and the WIF domain is consistent with the weak Hh signaling activity we demonstrated for a ShfΔEGF construct containing only the 'WIF' domain, and the absence of any Hh signaling activity of the complementary ShfΔWIF construct (Fig. 3; supplementary material Fig. S3) (Avanesov et al., 2012).

However, there are probably other regions in Shf that bind Hh, particularly in the 'EGF-like' domains. The Shf^2 mutant protein, which contains a mutation in the third 'EGF-like' domain, no longer accumulates in regions with high extracellular Hh (Glise et al., 2005). Substituting the 'EGF-like' domain of Shf into zebrafish WIF1 greatly improves the ability of WIF1 to regulate Hh signaling in the wing disc (Avanesov et al., 2012), indicating not only a function for the Shf EGF-like domain in Hh signaling, but also that the Hh function of the Shf WIF domain is shared by vertebrate WIF1. We show here that expression of the 'EGF-like' domains of Shf increases Hh accumulation in *shf* discs. Weak Hh binding to the 'EGF-like' domains of Shf would parallel the weak binding between Wnts and the 'EGF-like' domains of WIF1 (Malinauskas et al., 2011).

The increased accumulation and movement of Hh on the cell surface caused by the isolated 'EGF-like' domains of Shf, and the inability of the isolated 'WIF' domains to do the same, is probably due to the HSPG-binding mediated by the 'EGF-like' domains. Shf^2, which has a mutation in its 'EGF-like' domain that is likely to disrupt its conformation, no longer accumulates in response to changes in glypican expression (Avanesov et al., 2012; Glise et al., 2005). Similarly, WIF1 depleted of its 'EGF-like' domains was not able to bind HS sidechains, or to stabilize its binding partner Wg on HSPG-expressing cells (Avanesov et al., 2012; Malinauskas et al., 2011).

The 'EGF-like' domains may also help stabilize Hh by linking it to Ihog/Boi. But although the mutated 'EGF-like' domain of Shf^2 protein renders it less sensitive than wild-type Shf to Ihog/Boi levels, Shf^2 is weakly stabilized by UAS-Ihog in wing discs, suggesting that the 'WIF' domain also binds Ihog (Fig. 4). This provides a potential explanation for the weak, glypican-independent ability of ShfΔEGF to improve Hh signaling in *shf* mutant discs.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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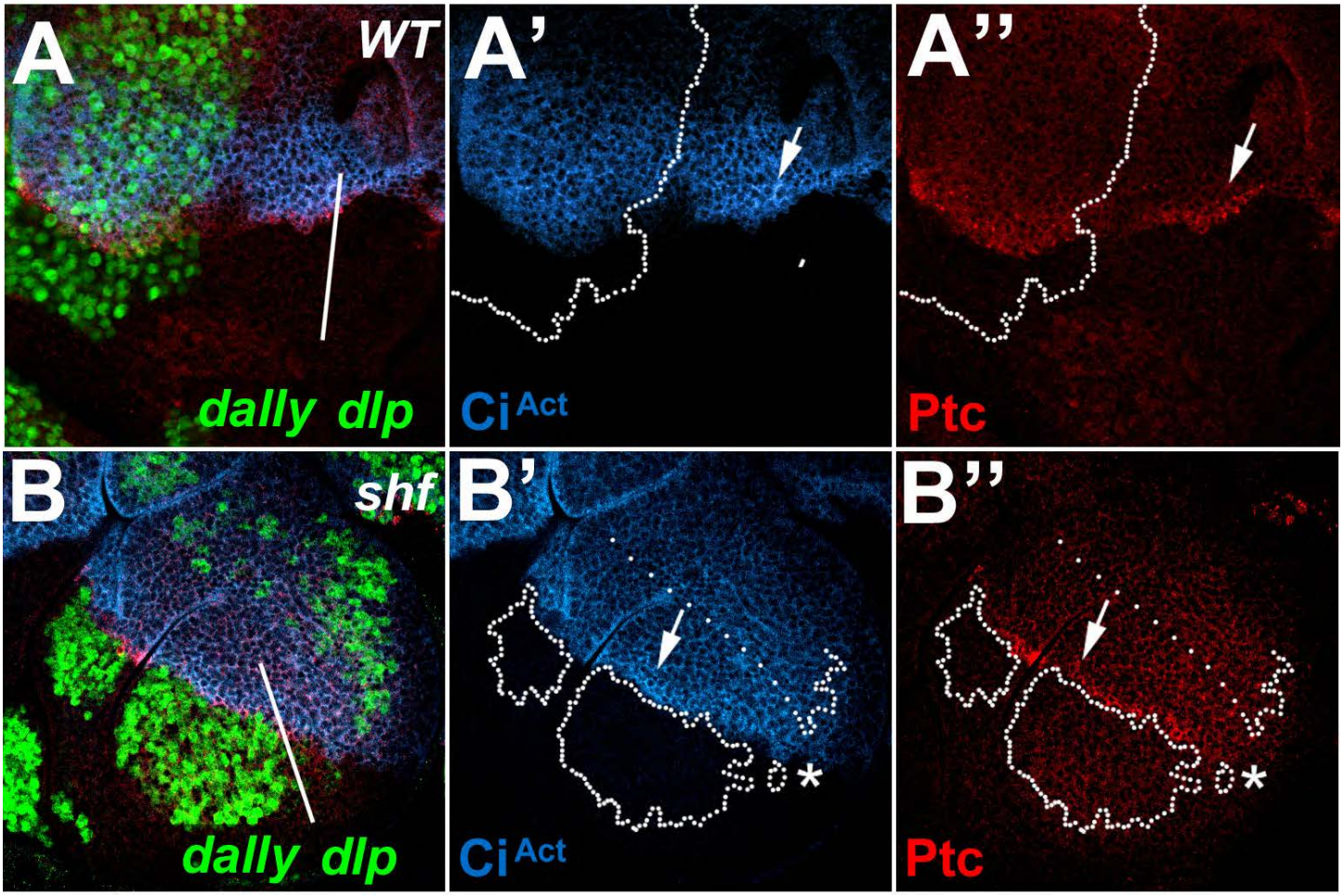


Fig. S1. Effects of losing *dally dlp* and *shf* on Hh signaling. *dally dlp* clones, marked by the absence of Myc epitope (green). Effects on Hh signaling are evaluated using Ci^{Act} (blue) and Ptc (red). (**A-A″**) In wild-type disc, double-sided clones lacking both glypicans strongly reduce long-range, but not short-range, signaling. (**B-B″**) In *shf* mutant discs, high levels of Ci^{Act} and Ptc are lost from double-sided *dally dlp* mutant clones (asterisks), but the thin Ci^{Act} and Ptc stripes typical of *shf* discs are retained in anterior clones that are touching wild-type, *dally dlp* containing posterior cells (arrows in B′,B″).

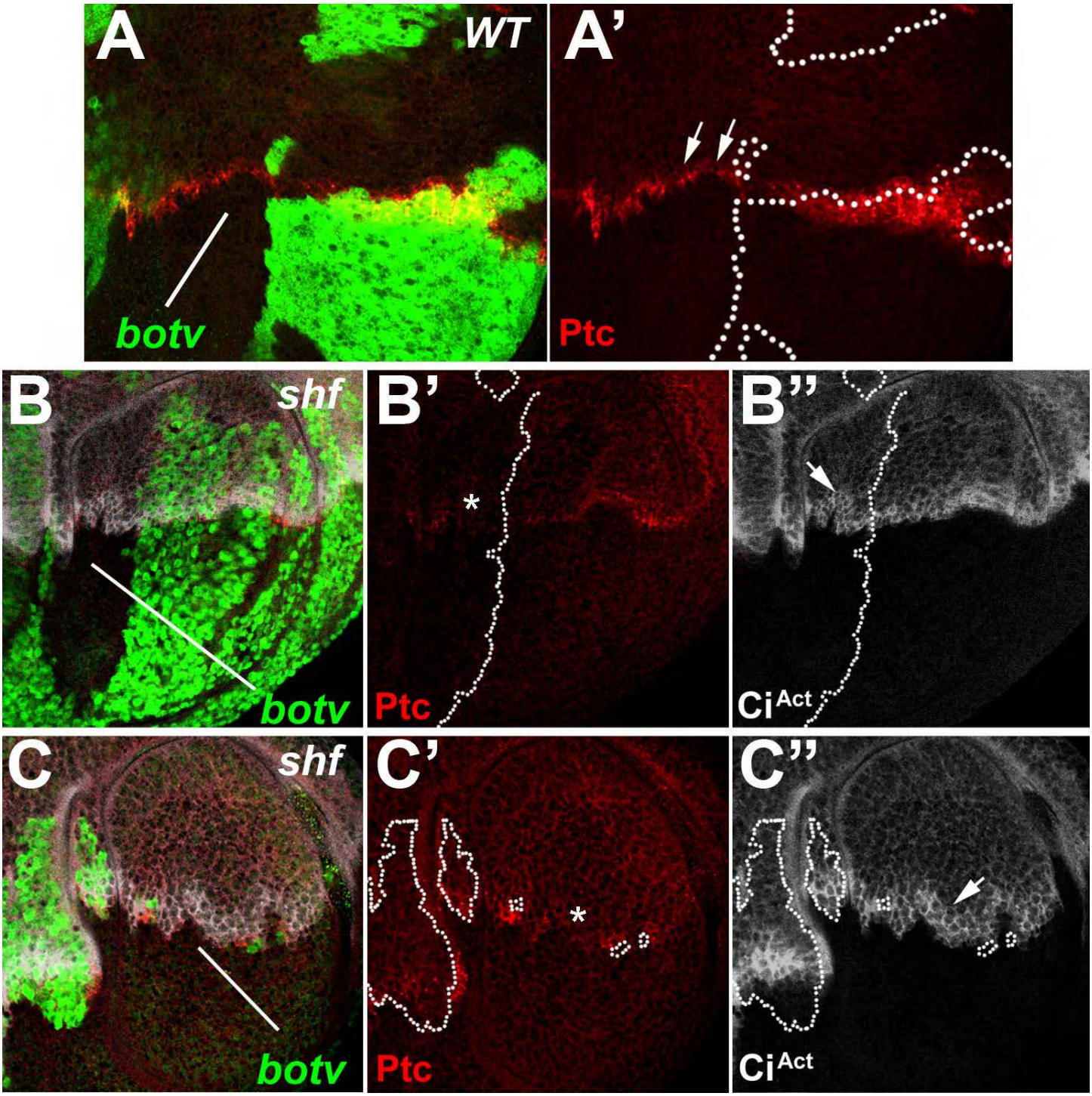


Fig. S2. Hh signaling defects in wild-type and *shf* cells lacking HS synthesis. (**A,A′**) *botv* clones, identified by the absence of GFP (green), in wild-type disc. In the region with double-sided clones, Ptc is reduced to a thin stripe. (**B-C″**) *shf* discs with *botv* clones, identified by the absence of GFP (green). Ptc (red) is lost from regions with double-sided clones, but in the same area the width of the Ci^{Act} stripe (white) is not reduced (arrows).

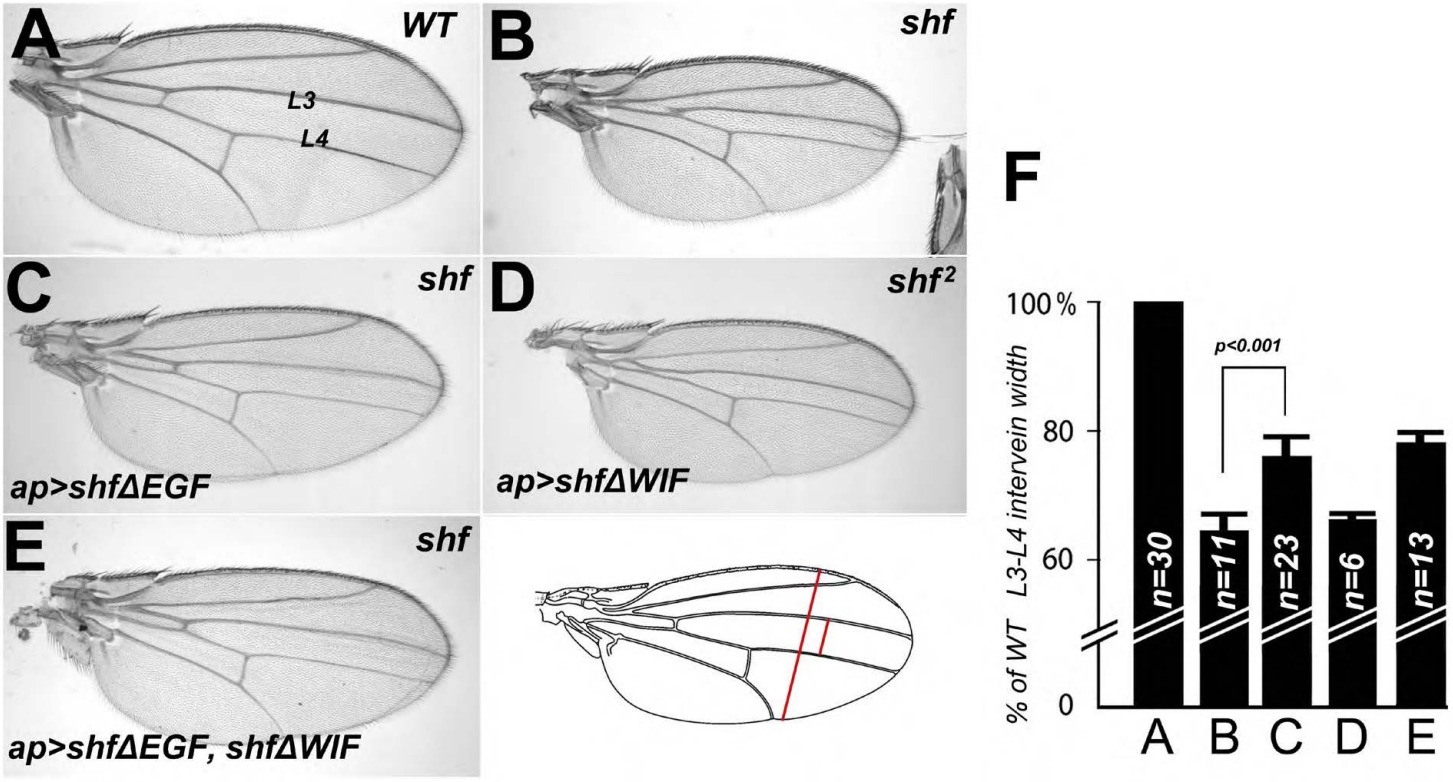
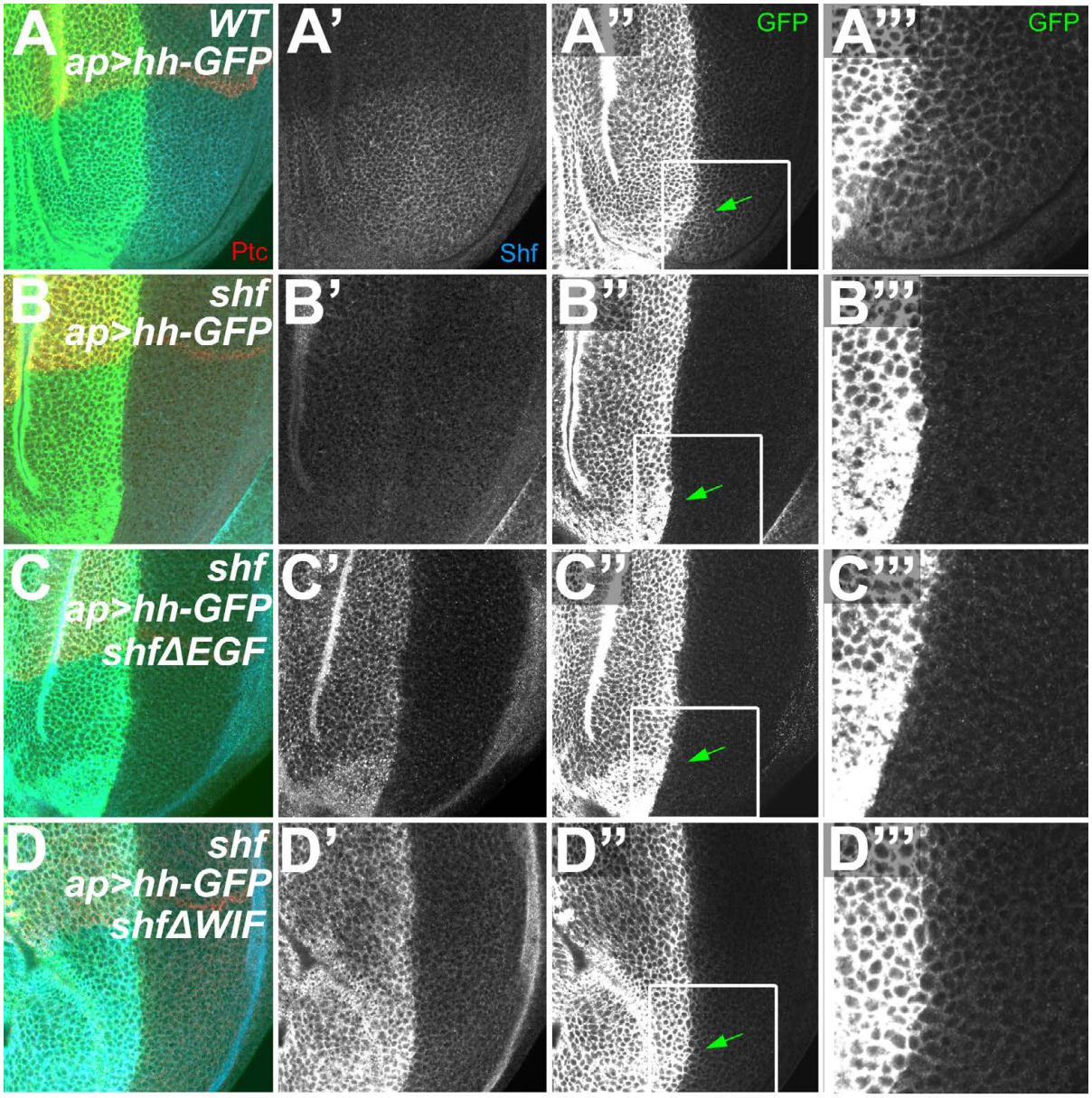


Fig. S3. Effects of expressing the 'WIF' and 'EGF-like' domains of Shf on of the Hh-dependent L3-L4 spacing in *shf* wings. (**A**) Wild-type wing showing normal spacing between L3 and L4 veins. (**B**) In *shf* mutant wings, L3 and L4 are much closer together. (**C-E**) Changes in L3-L4 spacing after *ap-Gal4*-driven expression of truncated Shf constructs in *shf* mutants. Because null shf^{x33}/Y flies expressing *UAS-shfΔWIF* did not survive to adulthood, we expressed it in shf^{2}. (**F**) Quantification of changes in L3-L4 spacing in the genotypes of A-E. L3-L4 distances were normalized to the anterior-posterior width of the entire wing (red lines in wing diagram), then expressed as percentages of the normalized wild-type L3-L4 distance. L3-L4 spacing in *shf* wings was improved only in the presence of ShfΔEGF, whereas expression of *UAS-shfΔWIF* did not improve spacing in comparison with either shf^{2} or shf^{x33}. Differences of statistical significance were determined using the Mann-Whitney U-test. Similar results were obtained with *en-Gal4* (Avanesov et al., 2012).



E **Normalized Hh-GFP accumulation in ventral-posterior quadrant (VP/VA)**

wt, ap>hh-GFP	1.6 +/- 0.3 (N=15)		
shf, ap>hh-GFP	1.2 +/- 0.1 (N=11)	P=0.18	
shf, ap>hh-GFP, shfΔEGF	1.2 +/- 0.2 (N=12)		P= 0.004
shf, ap>hh-GFP, shfΔWIF	1.4 +/- 0.2 (N=10)		P= 0.045

Fig. S4. Effects of truncated Shf constructs on Hh-GFP movement. (**A-D**) Wing pouch and detail images of wing discs from Fig. 3G-J, with dorsal *ap-Gal4*-driven expression of *UAS-hh-GFP*. Panels show Hh-GFP (green or white), Shf (blue or white) and Ptc (red). Hh-GFP in found well into the ventral compartment in wild type (A-A‴), but is largely absent ventrally in *shf* (B-B‴). *UAS-shf*Δ*EGF* does not significantly increase ventral Hh-GFP in *shf* disc (C-C‴), but *UAS-shf*Δ*WIF* does (D-D‴). (**E**) Average intensity values of Hh-GFP in ventral-posterior (VP) quadrant, normalized to the values in the ventral-anterior quadrant (VA) distant from the dorsal zone of Hh-GFP expression. Hh-GFP does not move as far in the anterior, probably due to binding to anteriorly expressed Ptc. Shown are standard deviations, numbers of images scored and significance of selected comparisons using the two-tailed Student's *t*-test.

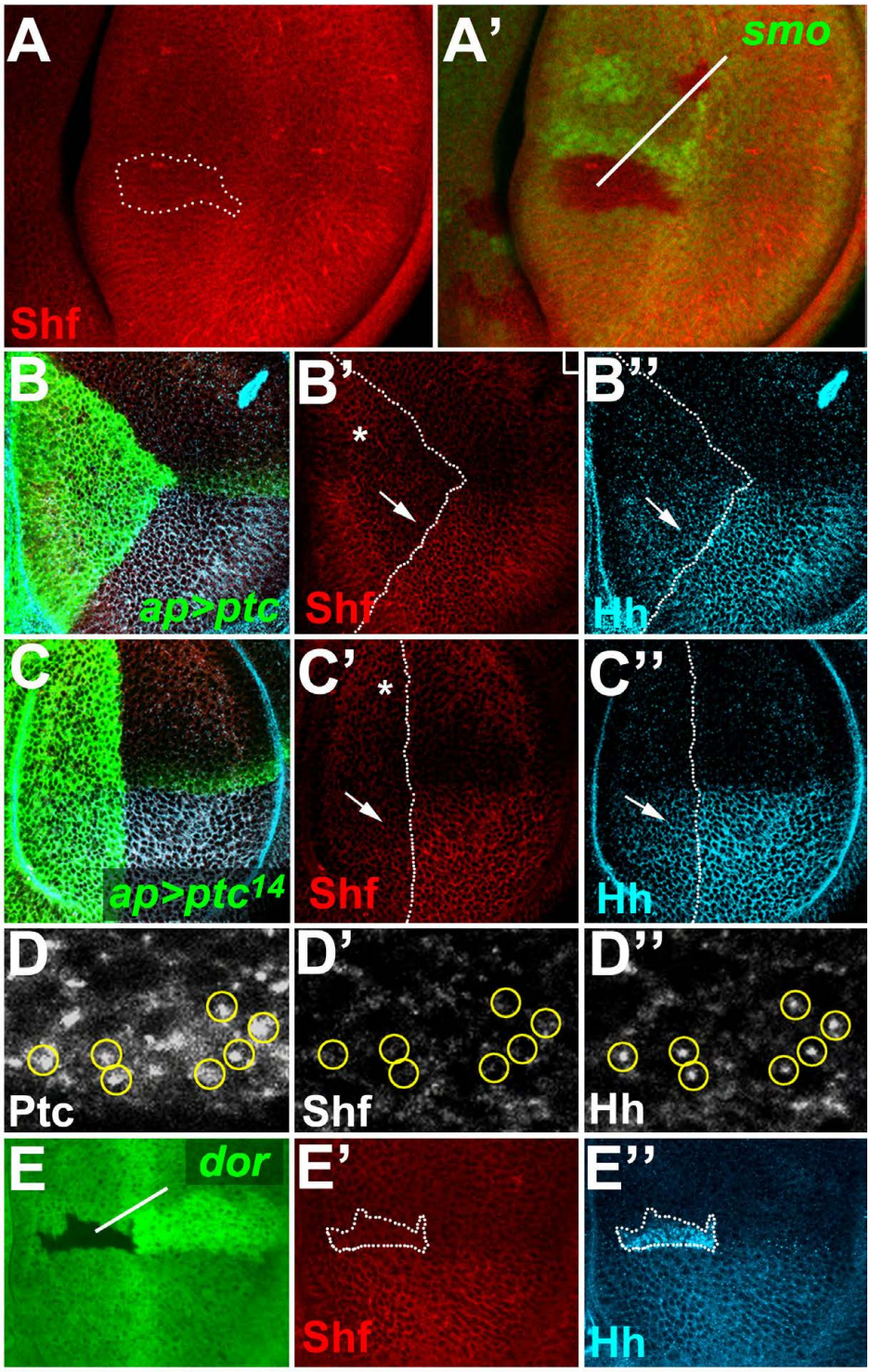


Fig. S5. Effects of Hh receptors and Hh signal transduction on Shf accumulation. (**A**,**A**′) Anti-Shf staining (red) does not change in *smoothened* (*smo*) mutant clone (identified by the absence of GFP, green) of anterior origin along the anterior-posterior compartment boundary, despite the loss of all Hh signaling within the clone. (**B-B**″) Dorsal *ap-Gal4*-driven overexpression of *UAS-ptc.* Discs were stained for Ptc (green), Shf (red) and Hh (blue). Owing to dominant-negative effects of the wild-type receptor on Hh signaling and Hh targets, this decreases growth of the dorsal compartment. Anti-Shf staining (red) is not increased by the excess Ptc, and in the posterior is reduced (arrows), along with anti-Hh staining (blue). Shf levels are not changed by *UAS-ptc* in the far anterior compartment (asterisk in B′), where Hh levels are low. (**C-C**″) Dorsal *ap-Gal4*-driven expression of the internalization defective Ptc^{14} allele (marked with anti-Ptc, green). This reduces anti-Shf (red) and anti-Hh (blue) staining in the posterior compartment (arrows in C′ and C″). Anti-Shf staining is not changed by Ptc^{14} expression in the far anterior compartment (asterisk in C′). (**D-D**″) Anterior cells at the anterior-posterior boundary of a wild-type disc show distinct Ptc- and Hh-positive puncta that do not include Shf. (**E-E**″) dor^{8} mutant clones, identified by the absence of green GFP, increase anti-Hh staining (blue) and anti-Ptc staining (not shown), but do not affect anti-Shf staining (red).

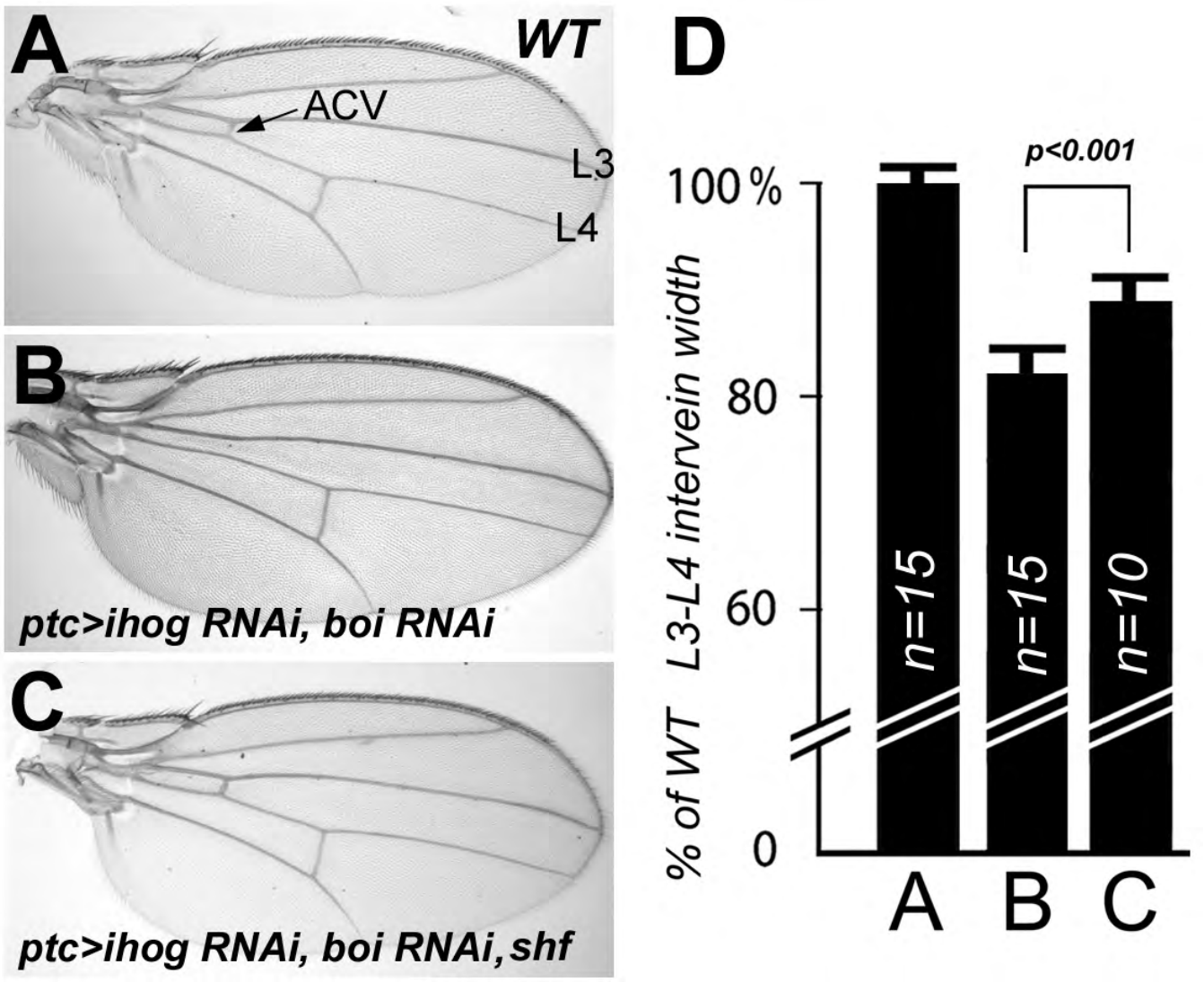


Fig. S6. Influence of *shf* overexpression on the effects of *ihog/boi* knockdown on L3-L4 spacing. (**A-C**) Adult wings from wild type (A), and after anterior *ptc-Gal4*-driven knockdown of *ihog* and *boi* levels using *UAS-ihog-RNAi* and *UAS-boi-RNAi*. *ihog* and *boi* knockdown reduces L3-L4 spacing (B), but this is partially rescued by overexpression of *UAS-shf* (C). (**D**) Quantification of changes in L3-L4 spacing in the genotypes of A-C. L3-L4 distances were normalized as in Fig. S3. Error bars show s.d. The improvement in L3-L4 in C compared with B was highly significant using Student's *t*-test. *ptc-Gal4* wings expressing *UAS-shf* alone had average L3-L4 distances that were slightly lower than wild type, although not significantly so (not shown, P=0.28).

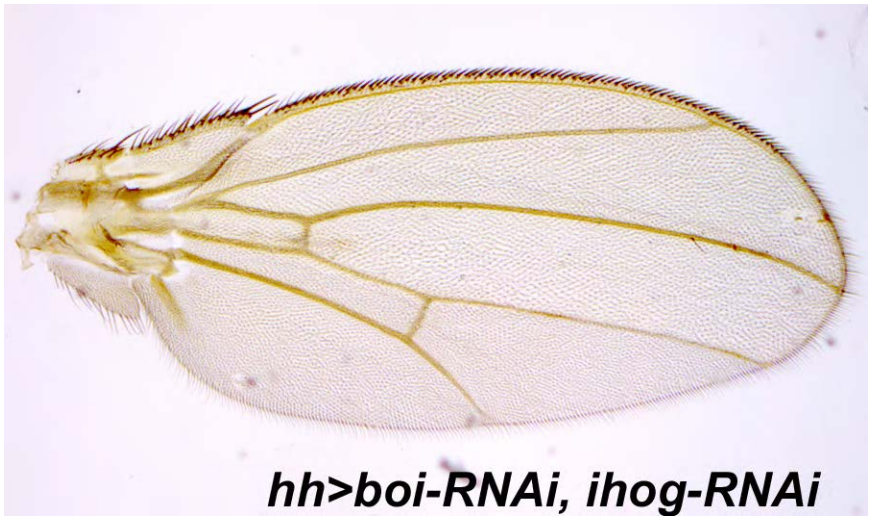


Fig. S7. Adult wing after posterior knockdown of *ihog* and *boi* expression. Posterior-specific knockdown of *ihog* and *boi* expression using *hh-Gal4*-driven expression of *UAS-ihog-RNAi* and *UAS-boi-RNAi* does not reduce the distance between the third and fourth longitudinal veins, an indicator of Hh signaling, despite the reduced size of the posterior compartment.