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CORRIGENDUM

The WIF domain of the human and *Drosophila* Wif-1 secreted factors confers specificity for Wnt or Hedgehog

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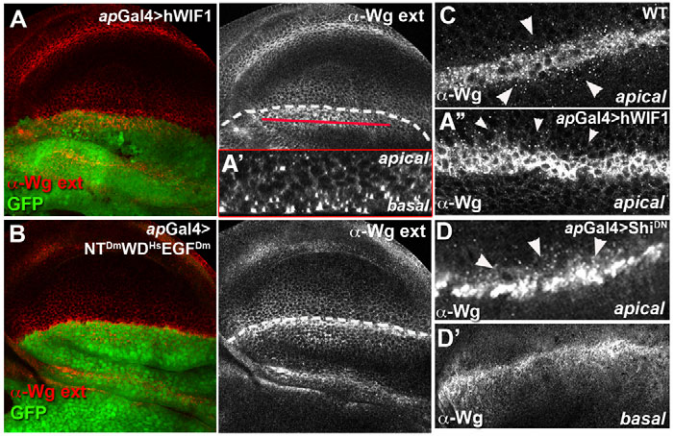
There was an error published in *Development* **139**, 3849-3858.

Fig. 4B incorrectly showed an image of an *ap*Gal4>UAS-hWIF1 wing disc instead of an *ap*Gal4>NT^{Dm}-WD^{Hs}-EGF^{Dm} wing disc. The correct figure is shown below.



As both genotypes display a very similar disc phenotype, this error does not change the conclusions of the paper.

The authors apologise to readers for this mistake.

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The WIF domain of the human and *Drosophila* Wif-1 secreted factors confers specificity for Wnt or Hedgehog

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SUMMARY

The Hedgehog (Hh) and Wnt signaling pathways are crucial for development as well as for adult stem cell maintenance in all organisms from *Drosophila* to humans. Aberrant activation of these pathways has been implicated in many types of human cancer. During evolution, organisms have developed numerous ways to fine-tune Wnt and Hh signaling. One way is through extracellular modulators that directly interact with Wnt or Hh, such as the Wnt inhibitory factor (Wif-1) family of secreted factors. Interestingly, Wif-1 family members have divergent functions in the Wnt and Hh pathways in different organisms. Whereas vertebrate Wif-1 blocks Wnt signaling, *Drosophila* Wif-1 [Shifted (Shf)] regulates only Hh distribution and spreading through the extracellular matrix. Here, we investigate which parts of the Shf and human Wif-1 (WIF1) proteins are responsible for functional divergence. We analyze the behavior of domain-swap (the *Drosophila* and human WIF domain and EGF repeats) chimeric constructs during wing development. We demonstrate that the WIF domain confers the specificity for Hh or Wg morphogen. The EGF repeats are important for the interaction of Wif-1 proteins with the extracellular matrix; *Drosophila* EGF repeats preferentially interact with the glypican Dally-like (Dlp) when the WIF domain belongs to human WIF1 and with Dally when the WIF domain comes from Shf. These results are important both from the evolutionary perspective and for understanding the mechanisms of morphogen distribution in a morphogenetic field.

KEY WORDS: Wif-1, Shifted, Hedgehog, Wnt

INTRODUCTION

Secreted signaling proteins of the Wnt and Hedgehog (Hh) families have important and conserved roles in metazoan development. These molecules also function postembryonically in homeostatic processes, such as stem cell maintenance. Alterations in these pathways during development cause a variety of congenital disorders and their aberrant activation has been implicated in proliferative disorders leading to many types of human cancer (Logan and Nusse, 2004; Moon et al., 2004). Hh and Wnt signals have been identified as morphogens in various systems. Morphogens are produced from a localized source and spread in the epithelium to form concentration gradients that organize patterning and control growth during development (Tabata and Takei, 2004). The mechanisms of morphogen distribution and the interpretation of morphogen gradients are of fundamental interest.

During evolution, organisms have developed many ways to fine-tune Wnt and Hh signaling. One way of controlling this process is through extracellular modulators that directly interact with Wnt or Hh. It is important to consider how these modulators contribute to the robustness of the gradients and the ability of the cells to measure different morphogen levels. Recently, increasing numbers of cell surface and extracellular modulators have been shown to bind morphogens and to regulate their distribution and signaling. In vertebrates, there are several extracellular modulators of Wnt, including the secreted Frizzled-related protein (SFRP) family (Uren et al., 2000), Cerberus (Willert et al., 2003) and the Wnt inhibitory factor 1 (Wif-1) family (Hsieh et al., 1999).

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Wif-1 has been described as a secreted antagonist of Wnt signaling (Hsieh et al., 1999; Hunter et al., 2004; Surmann-Schmitt et al., 2009). Hsieh and colleagues have proposed that Wif-1 might sequester Wnt ligands, preventing binding to the receptor Frizzled (Frl) (Hsieh et al., 1999). During development, Wif-1 expression is detectable at early and late stages (Hsieh et al., 1999; Hunter et al., 2004; Surmann-Schmitt et al., 2009). Wif-1 is also expressed in adults in the nervous system, lungs, heart, and cartilage-mesenchyme interfaces of various organisms (Hsieh et al., 1999; Surmann-Schmitt et al., 2009). A relationship between Wif-1 and cancer has also been reported. Thus, human Wif-1 (WIF1) is downregulated in cancers (Mazieres et al., 2004; Kansara et al., 2009) and the mouse *Wif1* knockout accelerates the development of radiation-induced osteosarcomas in vivo (Kansara et al., 2009). Furthermore, overexpression of human WIF1 inhibits the growth of cells from lung and bladder cancers (Lin et al., 2006; Tang et al., 2009).

In *Drosophila*, the extracellular matrix (ECM) component Shifted (Shf), which is the ortholog of vertebrate Wif-1, is implicated in Hh signaling (Glise et al., 2005; Gorfinkiel et al., 2005). In the absence of Shf there is no Hh gradient formation, the expression of Hh target genes is reduced and the levels of extracellular Hh are much reduced. It has been proposed that Shf mediates the interaction between Hh and heparan sulfate proteoglycans (HSPGs) (Glise et al., 2005; Gorfinkiel et al., 2005). Blocking synthesis of heparan sulfate glycosaminoglycan side chains of HSPGs reduces the extracellular accumulation of Shf (Glise et al., 2005). The genetic interaction between Hh and the HSPGs appears to be debilitated by the loss of Shf, indicating that HSPG function depends in large part on the presence of Shf (Gorfinkiel et al., 2005).

It has been reported that among the HSPGs the glypican Dally-like (Dlp) has opposite effects on Hh and Wnt signaling in *Drosophila*. Dlp is required for Hh signaling but can inhibit high-threshold Wingless (Wg) signaling when overexpressed in discs

(Yan et al., 2009). Similarly, vertebrate glypican 3 directly promotes Wnt signaling in cancer cells, but inhibits sonic hedgehog (Shh) signaling during development (Capurro et al., 2005; Capurro et al., 2008). Therefore, the strong parallels between the mechanisms of Wg and Hh signaling are implied by the dual roles of the glypican proteins in both pathways. Interestingly, *Drosophila* Shf and vertebrate Wif-1 functions also exhibit similarities in Wg and Hh signaling. Shf has no detectable role in Wnt signaling; overexpression of Shf does not generate Wnt-related defects, and neither the misexpression of various Wnts nor of the Wg signaling component Dishevelled (Dsh) can reproduce the *shf* phenotype (Glise et al., 2005). However, expression of the human WIF1 protein in the *Drosophila* wing disc blocks Wg signaling but does not rescue the *shf* mutant phenotype (Gorfinkiel et al., 2005). This observation is in agreement with the reported activity of vertebrate Wif-1 in Wnt signaling (Hsieh et al., 1999), and suggests that Wif family members might have divergent functions in each pathway.

Despite the functional divergence between *Drosophila* Shf and vertebrate Wif-1, the structure of these proteins is very similar, showing 30% sequence identity. An intriguing question is why Shf and Wif-1 participate in different signaling pathways in *Drosophila* and vertebrates. Wif protein consists of an N-terminal secretion signal sequence (NT), the WIF domain (WD), epidermal growth factor-like repeats (EGFs) and a hydrophilic C-terminus. Human WIF1 binds through its WD to *Drosophila* Wg, to zebrafish and *Xenopus* Wnt8 (Hsieh et al., 1999; Kawano and Kypta, 2003), to eight Wnts (including 3a, 4, 5a, 7a, 9a, 11) (Surmann-Schmitt et al., 2009), and to a protein involved in neuronal differentiation, olfactomedin 1 (Nakaya et al., 2008). However, a recent report has shown that human WIF1 binds to Wnts both through the WD and the EGF-like domains (Malinauskas et al., 2011).

The *Drosophila* wing imaginal disc is particularly suitable for studies of morphogen gradient formation. The disc forms a sac with a pseudostratified columnar epithelium (proper disc) and a squamous epithelium (peripodial membrane); both epithelia are formed from a cell monolayer with apicobasal polarity, and are separated by the disc lumen. Disc proper epithelium contains several distinct cell populations: the anterior (A), posterior (P), dorsal (D) and ventral (V) compartments. Hh is produced and secreted in the P compartment cells and spreads to the A compartment through the basolateral side to signal close to the A/P border in a concentration-dependent manner, within a range of 10-12 cell diameters (Callejo et al., 2011). By contrast, Wg is secreted from a strip of cells straddling the D/V boundary and undergoes long-range spreading in the wing pouch, inducing Wg target genes in the D and V disc cells in a concentration-dependent manner. Therefore, Wg and Hh spread along perpendicular axes in the same morphogenetic field (Kornberg and Guha, 2007).

In this work, we investigate how the specificity of human WIF1 and Shf for Wnt or Hh signal is conferred. To determine which domain of these two proteins is responsible for the functional divergence, we analyze the behavior of chimeric constructs in which the WD and EGFs repeats are exchanged. We conclude that the WD is responsible for the divergence between the *Drosophila* and human proteins, channeling the recognition either toward Hh or Wg signal, whereas the EGF repeats give the protein the ability to interact with glypicans.

MATERIALS AND METHODS

Fly mutants

Mutations, insertions and transgenes used are described in FlyBase. *shf*^{3}, *shf*^{919}, *shf*^{EY} (Gorfinkiel et al., 2005), *dally*^{32}, *dlp*^{20} (Franch-Marro et al., 2005) and DallyTrap lines (http://www.flyprot.org/stock_report.php?stock_id=17071) were used in this work.

Construction of *shf* and *WIF1* chimeric genes

The NT^{Dm}-WD^{Hs}-EGF^{Dm}, NT^{Dm}-WD^{Dm}-EGF^{Hs} and NT^{Hs}-WD^{Dm}-EGF^{Hs} chimeric genes were constructed using splicing by overlapping extension PCR (SOE-PCR) (Ho et al., 1989; Warrens et al., 1997; Povelones and Nusse, 2005). Primers for the second PCR include *Not*I/*Kpn*I sites for cloning. Primers (5′-3′) for the NT^{Dm}-WD^{Hs}-EGF^{Dm} chimera: 5′Not1DmNT, GCGGCCGCATGACACATCAGGGCATCGGC; 3′DmNT, GCATCGATCCATAGCGAGATGCCGCTCTCC; 5′HsWD, GGAGAGCGGCATCTCGCTATGGATCGATGCT; 3′HsWD, CACACCTCTGTGGGCACATGTTTTAAAGAA; 5′DmEGF, CTTTAAAACATGTGCCCACAGAGGTGTGTATGA; 3′Kpn1DmEGF, GGTACCCTAGAACTTGGAGTCATCG. For the NT^{Dm}-WD^{Dm}-EGF^{Hs} chimera: 5′Not1DmNT; 3′DmWD, GCACTCAGCTTGTTGTGTTAGTGAAGTAGGGTTGG; 5′HsEGF, CCTACTTCACTAACACAACAAGCTGAGTGCCCAGGC; 3′Kpn1HsEGF, GGTACCTCACCAGATGTAATTGGATTCAGG. For the NT^{Hs}-WD^{Dm}-EGF^{Hs} chimera: 5′Not1HsNT, GCGGCCGCATGGCCCGGAGGAGCGCC; 3′HsNT, CTCATTGATCCACAAGTACAGGCTCTCCTCCTGCG; 5′DmWD, GAGGAGAGCCTGTACTTGTGGATCAATGAGCAGCAGC; 3′DmWD2, GCACTCAGCTTGTTGGCATTCCTTTTTGAAGTTGAGGCG; 5′HsEGF2. CTTCAAAAAGGAATGCCAACAAGCTGAGTGCCCAGGC; 3′Kpn1HsEGF.

NT^{Dm}-WD^{Hs}-EGF^{Dm} encodes a 453 amino acid product that includes the N-terminal region of Shf (amino acids 1 to 118), the WD of WIF1 (amino acids 38 to 177) and the C-terminal region of Shf with the five EGF motifs (amino acids 262 to 456). The NT^{Dm}-WD^{Dm}-EGF^{Hs} product of 480 amino acids includes the N-terminal region and the WD of Shf (amino acids 1 to 278) and the C-terminal region of WIF1 including the five EGF motifs (amino acids 178 to 379). The NT^{Hs}-WD^{Dm}-EGF^{Hs} product of 382 amino acids includes the N-terminal region of WIF1 (amino acids 1 to 37), the WD of Shf (amino acids 119 to 261) and the C-terminal region of WIF1 with the five EGF motifs (amino acids 178 to 379). All final PCR products were sequenced and cloned into pUAS vector using the *Not*I and *Kpn*I restriction sites and injected into embryos to obtain transgenic lines.

For UAS>NT^{Dm}-WD^{Hs}-EGF^{Dm}-HA, UAS>NT^{Dm}-WD^{Dm}-EGF^{Hs}-HA and UAS>hWIF-1-HA, cDNAs were amplified by PCR, cloned into the entry vector pENTR/D-TOPO by directional TOPO cloning (Gateway System, Invitrogen) and introduced by recombination into the destination vector pTWHA (pUAST-HA). We generated several UAS lines for each construct and tested their effect in the wing disc. We also generated transgenic lines expressing the same UAS constructs without the HA tag and analyzed their morphogenetic effect. The effects of HA-tagged and untagged proteins were similar.

Western blot analysis

The expression levels of the proteins induced by the UAS constructs were analyzed by western blotting (supplementary material Fig. S1). Protein extracts from third instar larvae of *tub*Gal4/*tub*Gal80^{ts}>Shf-V5, *tubGal4*/*tub*Gal80^{ts}>hWIF-1-HA, *tub*Gal4/*tub*Gal80^{ts}>NT^{Dm}-WD^{Hs}-EGF^{Hs} and *tub*Gal4/*tub*Gal80^{ts}>NT^{Dm}-WD^{Dm}-EGF^{Hs} genotypes were prepared in lysis buffer containing protease inhibitors. Samples were resolved by SDS-PAGE, immunoblotted, and incubated with rabbit anti-HA 1:1000 (Sigma), mouse anti-V5 1:5000 (Invitrogen) or mouse anti-Actin 1:1000 (Developmental Studies Hybridoma Bank) antibodies. Horseradish peroxidase-conjugated secondary antibodies were used to develop the signal using the ECL System (Amersham Pharmacia).

Overexpression experiments

The following Gal4 drivers were used for ectopic expression experiments using the Gal4/UAS system (Brand and Perrimon, 1993): *ap*Gal4 (Calleja et al., 1996) and *hh*Gal4 (Tanimoto et al., 2000). We also used additional pUAS fly lines: UAS>Shf-V5 (Glise et al., 2005), UAS>Shi^{K44A} (Moline et al., 1999), UAS>Dally-GFP (Eugster et al., 2007) and UAS>Dlp-GFP (Baeg et al., 2004). UAS>Dlp-RNAi was obtained from the Vienna *Drosophila* RNAi Center.

Transient expression of the UAS constructs using Gal4 drivers with *tub*Gal80^{ts} was achieved by maintaining the fly crosses at 18°C and then

inactivating Gal80^{ts} for 24-48 hours at the restrictive temperature (29°C). After overexpression of the constructs, various wing discs were examined and at least two independent experiments were performed for each genotype.

Immunostaining of imaginal discs

Immunostaining was performed according to standard protocols (Capdevila and Guerrero, 1994). Antibodies were used at the following dilutions: rat monoclonal anti-Ci (Motzny and Holmgren, 1995) 1:5; mouse monoclonal anti-Ptc (Apa 1.3) (Capdevila and Guerrero, 1994) 1:50; mouse monoclonal anti-Dlp (Lum et al., 2003) 1:30; mouse monoclonal anti-Wg (Brook and Cohen, 1996) 1:20; guinea pig monoclonal anti-Sens (Nolo et al., 2000) 1:1000; rabbit polyclonal anti-Vg (Williams et al., 1991) 1:300; mouse monoclonal anti-Dll (Duncan et al., 1998) 1:400; mouse monoclonal anti-V5 (Invitrogen) 1:150; rabbit polyclonal anti-HA (Sigma) 1:50; mouse monoclonal anti-HA (Sigma) 1:100; rabbit polyclonal anti-β-Gal (ICN Biomed-Cappel) 1:1000; rat monoclonal anti-Shf (Glise et al., 2005) 1:1000. Extracellular labeling using anti-Wg or anti-V5 was performed as described (Torroja et al., 2004).

Microscopy and image processing

Bright-field imaging was performed using an Axioskop 2 Plus (Zeiss) microscope coupled to a CCD camera, and confocal fluorescence imaging used an LSM510 vertical laser-scanning confocal microscope (Zeiss). ImageJ software was employed for image processing and for the determination of fluorescence levels.

Multiple sequence alignment, domain architecture and phylogenetic analysis

Multiple sequence alignments were performed using Clustal Omega (Sievers et al., 2011) (http://www.ebi.ac.uk/Tools/msa/clustalo/). To investigate the evolutionary relationship between Shf and Wif-1, we selected from the RefSeq collection (NCBI) the protein sequences with high similarity to the Pfam WIF domain (accession PF02019) and to the Pfam EGF-like domain (accession PF07974) (supplementary material Table S1A). To analyze the phylogeny of domains we extracted the parts of the proteins that aligned with the Pfam files. We included the sequences of the Ryk/Derailed (Drl) family of tyrosine kinase-related receptors in the phylogenetic analysis of the WD (supplementary material Table S1B). Predicted sequences without experimental data proving their function were considered as Wif-1-like and receptor-like. The HMMER 3.0 package (http://hmmer.janelia.org/) was used to search for proteins containing the appropriate domains and to generate the alignments with the hmm files. The phylogenetic trees were constructed using MEGA 5.0 (Tamura et al., 2011). SMART modular architecture analysis programs (Schultz et al., 1998) (http://smart.embl-heidelberg.de/) and InterProScan (Hunter et al., 2012) (http://www.ebi.ac.uk/Tools/pfa/iprscan/) were also used to predict the domain architecture of the sequences used in the phylogenetic analysis.

RESULTS

Evolutionary divergence between human WIF1 and *Drosophila* Shf

A single-copy gene encodes the Wif-1 protein family in vertebrates and invertebrates. Phylogenetic analysis of the family members shows that Shf shares conserved sequence with its orthologs in arthropods, whereas Wif-1 has closely related orthologs in chordates (Fig. 1A). The WD is also present in the Ryk/Derailed (Drl) family of tyrosine kinase-related receptors, which function as Wnt receptors (Patthy, 2000; Yoshikawa et al., 2003). To investigate the phylogenetic relationships among WD-containing proteins, we used WD sequences from the species shown in Fig. 1A. The WD sequence from Wif-1 is more similar to that from Shf than to the domains found in the characterized Ryk/Drl receptors and uncharacterized putative receptors that contain the WD from chordates and arthropods (Fig. 1B,C).

Despite the sequence similarities between chordate and arthropod Wif-1 proteins, there is an extreme functional divergence between human and *Drosophila* Wif. Therefore, we undertook a detailed functional analysis of these two proteins as examples of arthropod and chordate sequences. It has been reported previously that both the WD and the EGFs of Shf are crucial for its function, as the expression of just the WD or EGF repeats in *Drosophila* does not rescue the *shf* phenotype (Glise et al., 2005; Gorfinkiel et al., 2005; Avanesov et al., 2012). We generated chimeric constructs by exchanging domains between the *Drosophila* and human proteins (Fig. 1D). These constructs were obtained by splicing and overlapping extension PCR (SOE-PCR) (Ho et al., 1989; Warrens et al., 1997; Povelones and Nusse, 2005) and were tagged with an HA tail at the C-terminus. These constructs, containing the WD or EGF repeats of Shf and WIF1 (NT^{Dm}-WD^{Hs}-EGF^{Dm}, NT^{Dm}-WD^{Dm}-EGF^{Hs}; Fig. 1D), were tested in the wing disc, both in HA-tagged and untagged form. We also tested the possible effect of the N-terminal part (NT), as Shf has a much longer NT than WIF1 (NT^{Hs}-WD^{Dm}-EGF^{Hs}; Fig. 1D).

The spreading properties of WIF1, Shf and chimeric Shf/WIF1 proteins

It has been proposed that Wif proteins are secreted factors that spread through the ECM and interact with HSPGs (Glise et al., 2005; Gorfinkiel et al., 2005; Avanesov et al., 2012). Shf is present throughout the wing primordium, with higher levels in the anteriormost part of the A compartment, lower levels near the A/P border (supplementary material Fig. S2A), and uniformly high levels in the P compartment due to its interaction with Hh (Gorfinkiel et al., 2005; Glise et al., 2005). Shf plays a major role in Hh stability in the basolateral part of the ECM (Glise et al., 2005), where Shf protein is located (supplementary material Fig. S2A′) and the Hh gradient is formed (Callejo et al., 2011). It has been proposed that HSPGs modulate the movement of lipid-modified Hh in a manner similar to Shf. Furthermore, Hh and Shf levels decrease in cells that are mutant for enzymes that synthesize glycosaminoglycans (GAGs) from HSPG (Glise et al., 2005). However, it has recently been reported that human WIF1 binds to the highly sulfated, negatively charged GAGs via the EGF-like domains. Among EGFs II-V, the conserved cluster of lysines and arginines on EGF IV provides the probable focus for the HSPG binding of Wif-1 (Malinauskas et al., 2011).

We propose that the ability of Shf and WIF1 to bind the GAGs of HSPGs via interactions with EGFs provides a mechanism to maintain these proteins near the target cell surface. To investigate the interaction of Shf or WIF1 with the ECM, we first analyzed the distribution of these two proteins when expressed in a particular domain of the wing imaginal disc, such as the D or P compartment. We observed differences in their distribution: Shf is found at high levels not only in the compartment where the protein is induced but also in the non-expressing compartment (Fig. 2A,A′; supplementary material Fig. S3A,A′), whereas WIF1 is found mostly in the expressing cells (Fig. 2B,B′; supplementary material Fig. S3B,B′). This suggests that Shf spreads further than WIF1. The spreading of Shf can be visualized more clearly by extracellular staining; Shf is found throughout the entire disc despite being expressed in a specific compartment (supplementary material Fig. S2B,B′). The long-range dispersion of Shf is in agreement with its reported dependence on Hh movement through the ECM in the wing disc (Glise et al., 2005; Gorfinkiel et al., 2005; Avanesov et al., 2012).

Next, we investigated the distribution of the chimeric proteins. NT^{Dm}-WD^{Hs}-EGF^{Dm} expressed in the D (Fig. 2C,C′) or P (supplementary material Fig. S3C,C′) compartment of the wing

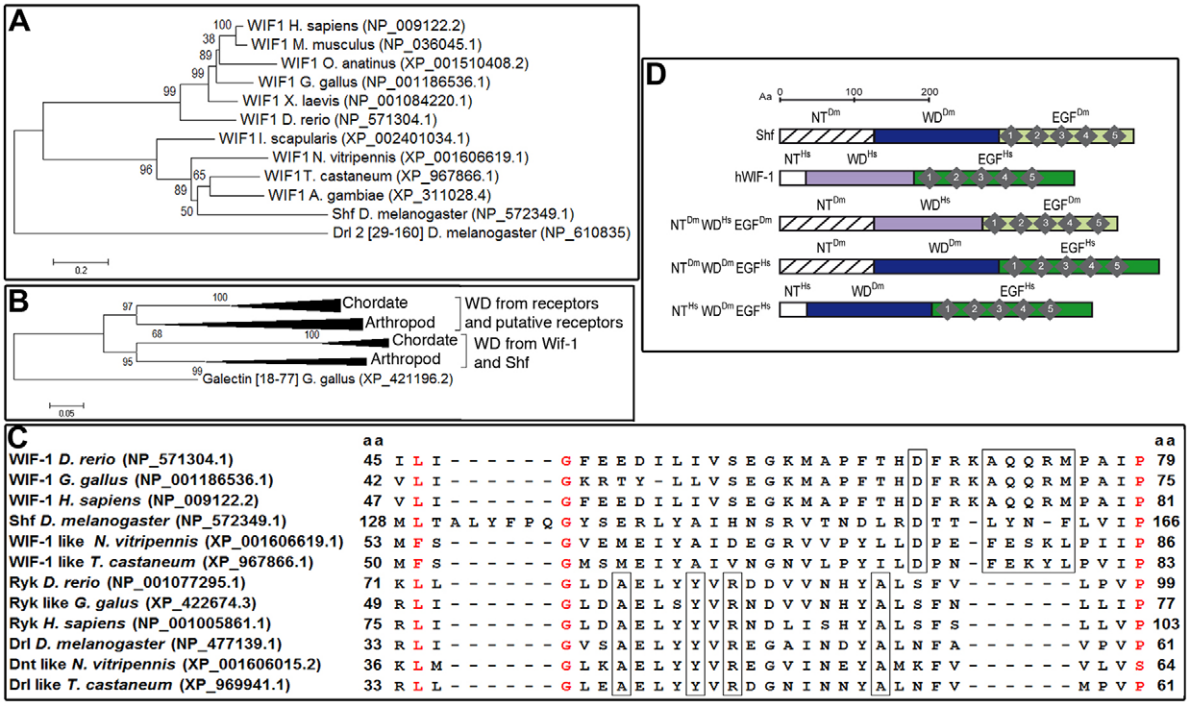

Fig. 1. Wif-1 protein domains and their phylogenetic conservation. (**A**) Phylogenetic tree of Shf and Wif-1 proteins from different species of chordates and arthropods. To compute the evolutionary distances, we used the Minimum Evolution method with 5000 bootstrap replicates and a Dayhoff matrix-based method. The representativeness of each cluster is shown as a percentage, near to the branches. The tree is rooted with the sequence of the WIF domain (WD) in Drl-2 from *Drosophila melanogaster*. The arthropod (Shf) proteins show greater similarity to each other than to their chordate orthologs (Wif-1). (**B**) Phylogenetic tree of the WD. The sequences of the WD were extracted from Shf, Wif-1 and from tyrosine kinase-related receptors. To obtain the evolutionary distances, we used the Minimum Evolution method with 5000 bootstrap replicates and the p-distance method. The tree is rooted with part of a galectin sequence from *G. gallus*, which has some similarity to the WD. The subtrees are compressed and represented by triangles. The length of each triangle is proportional to the divergence between sequences and the height is related to the number of taxa in the cluster. The WDs of vertebrate Wif-1 proteins are closer to those of Shf than to those of receptors that bind to Wnt in chordates and in arthropods. (**C**) Part of the alignment of the WD extracted from sequences of Wif-1/Shf and tyrosine kinase-related receptors. The most conserved positions are marked in red and the amino acids that best mark the differences between the WD from Wif-1/Shf and the receptors are boxed. (**D**) Chimeric proteins constructed by swapping human WIF1 and *Drosophila* Shf protein domains. NT^{Dm}-WD^{Hs}-EGF^{Dm}, N-terminal [fly]-WD [Human]-EGFs [Fly]; NT^{Dm}-WD^{Dm}-EGF^{Hs}, N-terminal [fly]-WD [fly]-EGFs [human]; NT^{Hs}-WD^{Dm}-EGF^{Dm}, N-terminal [human]-WD [fly]-EGFs [fly]. NT, N-terminal secretion signal sequence; EGF, epidermal growth factor-like repeat (1-5).

disc was found mostly in its expression domain. However, NT^{Dm}-WD^{Dm}-EGF^{Hs} dispersed even further than Shf (Fig. 2D,D′; supplementary material Fig. S3D,D′), indicating that the spreading of Shf and Wif-1 proteins might be due to their specificity for a morphogen.

The WD is responsible for the functional divergence between Shf and WIF1

It has been reported that, in the absence of Shf, there is no Hh gradient formation and expression of Hh target genes is restricted to the first row of cells of the A compartment (Glise et al., 2005; Gorfinkiel et al., 2005). In agreement with these data, overexpression of Shf in the P compartment of the wing disc results in an extension of the Hh gradient (see Fig. 6A; supplementary material Table S2) without affecting Wg signaling (Glise et al., 2005; Gorfinkiel et al., 2005; Avanesov et al., 2012) (Fig. 3B,B′). However, as we have previously reported, the expression of human WIF1 in the wing disc does not alter Hh levels or Hh signaling but causes a *wg* mutant phenotype in the wing (Gorfinkiel et al., 2005) (supplementary material Fig. S4G). Wg can induce the expression of its target genes in a concentration-dependent manner to activate Sensless (Sens) expression at short range and to activate Distal-less (Dll) or Vestigial (Vg) at long range (Zecca et al., 1996; Neumann and Cohen, 1997; Nolo et al., 2000). We observed that expression of WIF1 repressed the short-range Wg target Sens (Fig. 3C). The long-range targets also showed reduced expression levels, although their expression domain was slightly wider (Fig. 3C′, arrowheads), indicating that ectopic WIF1 compromises the response to Wg but expands the Wg gradient (Fig. 3H). Similarly, in the wing disc, the ectopic expression of mouse secreted frizzled-related protein 1 (SFRP1), which is known to bind directly to Wnts, produces analogous alterations in the Wg gradient (Esteve et al., 2011). These data suggest that WIF1 can interact with Wg, affecting its reception and/or spreading.

We expressed chimeric proteins in the wing disc to find out which protein domain is responsible for the functional divergence between Shf and WIF1. In *hh*Gal4>NT^{Dm}-WD^{Hs}-EGF^{Dm} discs, Wg targets were not activated (Fig. 3D,D′, arrows) and the Wg gradient was extended (Fig. 3I). NT^{Dm}-WD^{Hs}-EGF^{Dm} protein affected the Wg pathway in a manner similar to full-length WIF1 (Fig. 3C,C′), although the effect was stronger. Thus, ectopic NT^{Dm}-WD^{Hs}-EGF^{Dm} protein not only repressed Sens but also inhibited the expression of the low-threshold Wg targets Dll and Vg (Fig. 3D′); this phenomenon is discussed further below. We also observed a

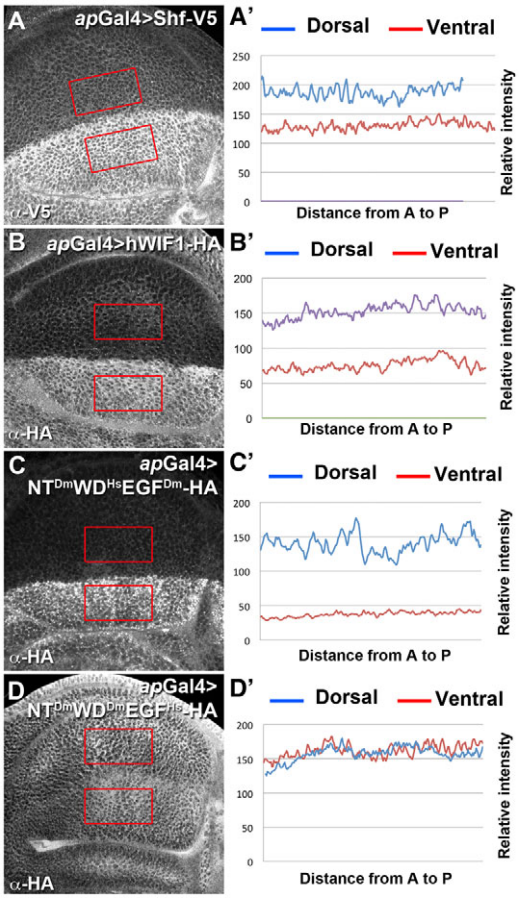

Fig. 2. The WD specifies the diffusion properties of the Shf/Wif-1 proteins. (**A-B′**) *ap*Gal4>UAS-Shf-V5 (A) and *ap*Gal4>UAS-hWIF-1-HA (B) *Drosophila* wing imaginal discs stained with anti-V5 and anti-HA, respectively. Note that Shf and human WIF1 show different diffusion behavior: Shf secreted from the D compartment is distributed in both the V and D compartment cells (A), whereas WIF1 is restricted mainly to the D compartment (B). (**C-D′**) *ap*Gal4>NT^{Dm}-WD^{Hs}-EGF^{Dm}-HA (C) and *ap*Gal4>NT^{Dm}-WD^{Dm}-EGF^{Hs}-HA (D) wing discs. Note that NT^{Dm}-WD^{Hs}-EGF^{Dm} (C) diffuses similarly to WIF1 (B) and that NT^{Dm}-WD^{Dm}-EGF^{Hs} (D) spreads like Shf (A), indicating that the WD confers diffusion characteristics to Shf and WIF1. (A′-D′) Quantification analyses of proteins in the D and V compartments (boxed regions) were performed using ten discs for each genotype. A, anterior; D, dorsal; P, posterior; V, ventral.

wg mutant phenotype in the wing upon expression of this chimera (supplementary material Fig. S4H). The most likely explanation for the repression of the Wg pathway by ectopic WIF1 or NT^{Dm}-WD^{Hs}-EGF^{Dm} proteins is inhibition of Wg reception by sequestration of Wg in the ECM and therefore competition with the Wg receptor Frizzled 2 (Fz2) for ligand binding. We then analyzed the distribution of extracellular Wg upon expression of ectopic WIF1 or NT^{Dm}-WD^{Hs}-EGF^{Dm}. Wg accumulated in the basolateral part of the disc epithelium where WIF1 or NT^{Dm}-WD^{Hs}-EGF^{Dm} is expressed (Fig. 4A,A′,B). To determine in which part of the disc epithelium Wg ligand is normally internalized by its receptors, we 'froze' endocytosis by expressing a dominant-negative form of Dynamin (Shi^{K44A}) in the D compartment, and examined the accumulation of Wg on the disc epithelium surface. We observed a strong reduction in subapical endocytic vesicles (arrows in Fig. 4D) in the D compartment and a basolateral, but not apical, Wg accumulation (Fig. 4D,D′). The similar basolateral Wg accumulation and inhibition of endocytic vesicles (Fig. 4A″) caused by expression of WIF1 or NT^{Dm}-WD^{Hs}-EGF^{Dm} (Fig. 4A-B) indicates that blocking Wg internalization is probably the mechanism of WIF1 inhibition of Wnt signaling.

We then tested the effect of the NT^{Dm}-WD^{Hs}-EGF^{Dm} chimera on the Hh pathway. This chimera, when expressed in the D compartment, does not rescue the *shf* mutant phenotype (Fig. 5C; supplementary material Fig. S4D). However, expression of NT^{Dm}-WD^{Dm}-EGF^{Hs} during development has no effect on the Wg pathway (Fig. 3E,E′), but rescues the *shf* mutant phenotype (Fig. 5D; supplementary material Fig. S4E). This chimeric protein also enhances the Hh gradient (Fig. 6D; supplementary material Table S2), similarly to the Shf protein (Fig. 6A; supplementary material Table S2), but, like WIF1 protein, ectopic NT^{Dm}-WD^{Hs}-EGF^{Dm} does not affect the Hh gradient (Fig. 6B,C; supplementary material Table S2). Furthermore, expression of NT^{Hs}-WD^{Dm}-EGF^{Hs} has the same effect as NT^{Dm}-WD^{Dm}-EGF^{Hs} (supplementary material Fig. S4F and Fig. S5B). From these findings we conclude that the Shf/WIF1 targeting of the Hh or Wg pathways is due to the WD and not to the EGF repeats.

Both the EGF repeats and WD of Shf and WIF1 interact with glypicans

It is possible that the specificity of Wif-1 proteins for a morphogen depends of its interaction with a particular HSPG. Among the *Drosophila* HSPGs, the glypicans Dally and Dlp are needed for the effective distribution and reception of Wg and Hh (Mikels and Nusse, 2006; Jiang and Hui, 2008; Yan et al., 2009). It has been shown that Shf mediates the interaction between Hh and the glypicans; Shf protein is stabilized in the extracellular space by glypicans (Glise et al., 2005; Avanesov et al., 2012). More specifically, the levels of endogenous Shf are increased in cells overexpressing Dally (Fig. 7A) and are reduced in clones that lack it (Fig. 7B) (Avanesov et al., 2012). It has also been proposed that Dally is required for the stability and long-range distribution of Hh and Wg. *dally* mRNA expression is strong at the A/P and D/V compartment borders and uniform in the notum (Fujise et al., 2001). A Dally-YFP reporter construct shows a similar expression pattern and most likely reproduces Dally protein distribution in the wing disc (Fig. 7D). We have not observed changes in the Dally protein expression domain after overexpressing WIF1 or any of the chimeric Wif proteins (supplementary material Fig. S6A-C). These data suggest a more specific interaction of Dally with Shf than with the WIF1 or NT^{Dm}-WD^{Hs}-EGF^{Dm} proteins. However, the $dally^{32}$ mutant background slightly alleviated the notched wing phenotype and repression of Wg signaling observed after expressing WIF1 or NT^{Dm}-WD^{Hs}-EGF^{Dm} in the wing disc (Fig. 7C; supplementary material Fig. S4I). This suggests a possible interaction of these proteins with Dally, as has been observed upon expressing zebrafish Wif1 or WD^{fish}-EGF^{Dm} in the wing disc (Avanesov et al., 2012).

Next, we tested a possible interaction with Dlp. Dlp protein distribution also reflects its requirement for the Hh and Wg pathways in the wing imaginal disc, with higher levels in the A compartment and reduced levels in the P compartment, and downregulation at the D/V border (Kreuger et al., 2004). In a transverse section, Dlp is located mainly in the basal part of the epithelium (Baeg et al., 2004). Dlp acts as a co-receptor of Hh (Desbordes and Sanson, 2003; Lum et al., 2003; Lin, 2004; Yan et al., 2010) and, whereas high concentrations of Dlp block Wg signaling (supplementary material Fig. S7A), low Dlp concentrations enhance it (Yan et al., 2009). Knocking down Dlp

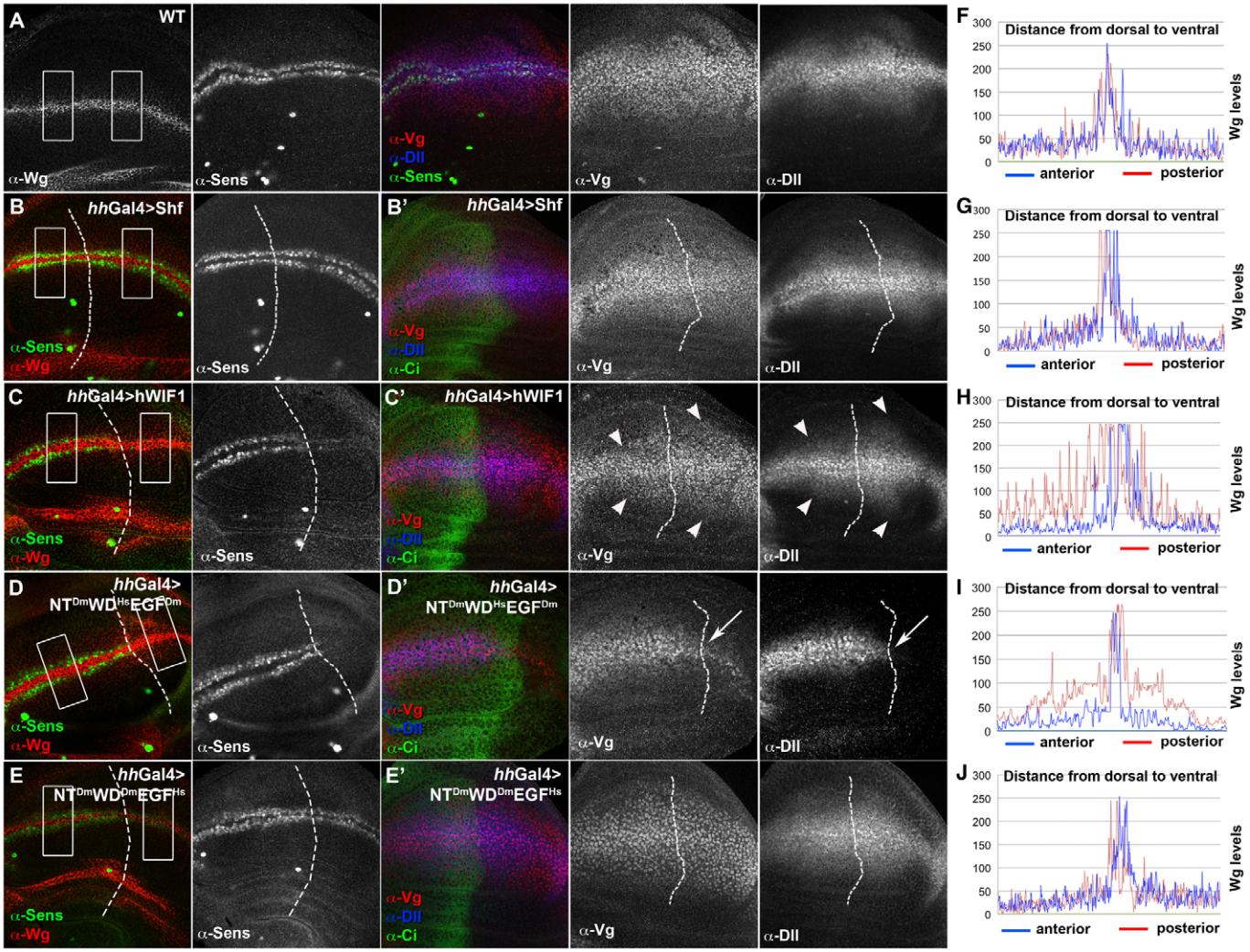

Fig. 3. The WD of WIF1 confers specificity for Wnt. (**A**) Wg, Sens, Vg and Dll expression patterns in a wild-type (WT) *Drosophila* wing disc. (**B**,**B′**) Ectopic expression of Shf in the P compartment does not affect Wg targets. (**C**,**C′**) Ectopic WIF1 in the P compartment affects Wg targets. We observe repression of the high-threshold Wg target Sens (C) and an expansion of the low-threshold targets Vg and Dll (C′, arrowheads). Note the non-autonomous effect shown by the repression of Sens, not only in the P compartment but also in some cell rows of the A compartment. (**D**,**D′**) Sens, Vg and Dll expression in an *hh*Gal4>NT^{Dm}-WD^{Hs}-EGF^{Dm} wing disc. The effect of ectopic NT^{Dm}-WD^{Hs}-EGF^{Dm} on the Wg pathway is much stronger than that of ectopic WIF1. We observe not only a repression of Sens (D) but also of Vg and Dll (D′, arrows). (**E**,**E′**) Sens, Vg and Dll expression in an *hh*Gal4>NT^{Dm}-WD^{Dm}-EGF^{Hs} wing disc is unaffected. In all cases, the P compartment is marked by the absence of Ci expression. The dashed line indicates the A/P border. (**F-J**) Fluorescence levels of Wg protein in wild type and under different experimental conditions (boxed regions in A-E). Wg expression is restricted mainly to the D/V border of the wing disc in the wild type (F) and after the overexpression of Shf (G) and NT^{Dm}-WD^{Dm}-EGF^{Hs} (J). The Wg gradient extends only with ectopic WIF1 (H) or NT^{Dm}-WD^{Hs}-EGF^{Dm} (I). Quantification was performed using ten discs of each genotype.

by specific RNAi did not modify Shf levels (supplementary material Fig. S7B; compare with supplementary material Fig. S2A). However, the ectopic expression of Shf (using the *ap*Gal4 driver) in the D compartment results in a slight enhancement of Dlp levels throughout the disc (Fig. 8A,A′), which is caused by the non-autonomous effect of the diffusible Shf. Interestingly, expressing WIF1 in the wing disc does not affect the Dlp distribution (Fig. 8B,B′). These results suggest that Shf EGFs, but not WIF1, have a stabilizing effect on Dlp.

We also performed a series of experiments using chimeric constructs to establish which of the Shf protein domains are involved in its interaction with Dlp. Overexpression of NT^{Dm}-WD^{Hs}-EGF^{Dm} and NT^{Dm}-WD^{Dm}-EGF^{Hs} chimeras gave different results. Ectopic NT^{Dm}-WD^{Hs}-EGF^{Dm} expression caused a substantial accumulation of Dlp (Fig. 8C,C′), whereas ectopic NT^{Dm}-WD^{Dm}-EGF^{Hs} had no effect (Fig. 8D,D′). Therefore, we can conclude that the interaction between Shf and Dlp occurs mainly through the *Drosophila* EGFs repeats. Supporting this conclusion, expression of the WD^{fish}-EGF^{fly} construct made using the zebrafish WD has been shown to rescue the *shf* phenotype and block Wg signaling (Avanesov et al., 2012), although expression of full-length zebrafish Wif1 protein does not have much effect in *Drosophila* (Glise et al., 2005). These data also indicate that the vertebrate EGFs (fish or human) might inefficiently recognize the *Drosophila* glypican Dlp, possibly owing to the evolutionary divergence of the EGF repeats.

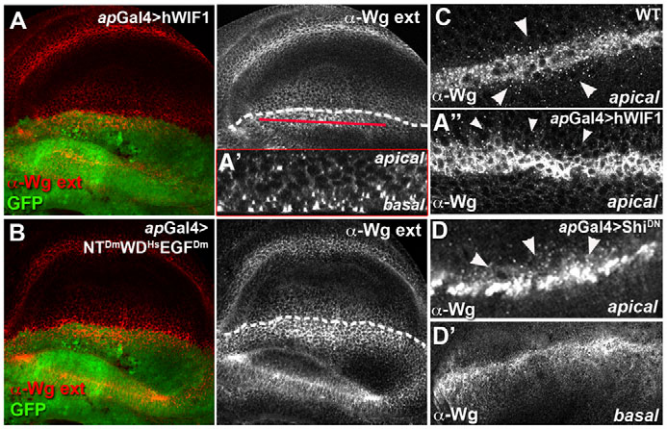

Fig. 4. WIF1 and NT^{Dm}-WD^{Hs}-EGF^{Dm} proteins block Wg internalization. (**A-A″**) Extracellular Wg accumulation in an *ap*Gal4>hWIF-1-HA *Drosophila* wing disc. Transverse section (red line in A marks the location of the section) of the same disc shows Wg accumulation in the basolateral part of the disc epithelium (A′). The number of endocytic vesicles (arrowheads) is strongly reduced in the D compartment where WIF1 is overexpressed (A″). (**B**) Extracellular Wg accumulation in *ap*Gal4>NT^{Dm}-WD^{Hs}-EGF^{Dm} wing disc. Note that the basolateral accumulation of Wg is similar to the effect of ectopic WIF1 expression. The GFP in A and B labels the ectopic expression domain. (**C**) Endocytic vesicles (arrowheads) in an apical view of a wild-type disc. (**D**,**D′**) Wg accumulation in an *ap*Gal4>Shi^{K44A}; *tub*Gal80^{ts} wing disc after 12 hours at the restrictive temperature. In the basal section, Wg accumulates in the D compartment of the disc epithelium (D′). In the apical confocal section (D) of the same disc, we observe a substantial decrease in the number of endocytic vesicles (arrowheads, compare with wild-type wing disc in C) in the D compartment, but no Wg accumulation.

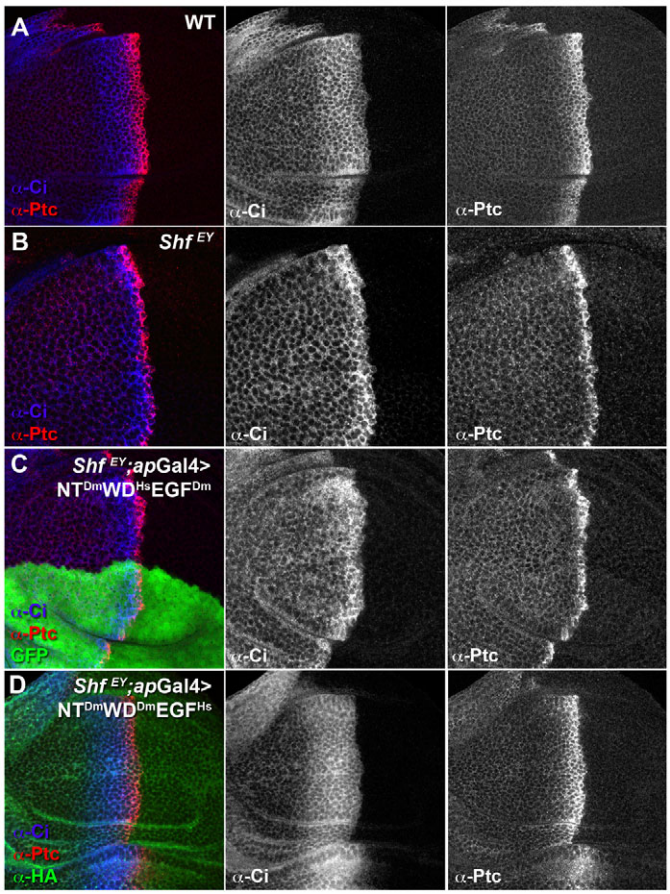

Fig. 5. Rescue of the *shf* mutant phenotype by ectopic expression of Wif-1 chimeric proteins. (**A**) Ptc (red) and Ci (blue) expression patterns in a wild-type *Drosophila* wing disc. (**B**) Ptc and Ci expression in an *shf* mutant disc. In *shf* mutants, Ptc is expressed only in the first row of cells of the A compartment adjacent to the A/P border, and the cytoplasmic accumulation of Ci is restricted to a few cells in the A compartment abutting the A/P compartment boundary. (**C**,**D**) Normalized Ptc and Ci expression in a shf^{EY}; *ap*Gal4>NT^{Dm}-WD^{Dm}-EGF^{Hs} (D) but not in a shf^{EY};*ap*Gal4>NT^{Dm}-WD^{Hs}-EGF^{Dm} (C) wing disc.

It has been reported that high levels of Dlp block Wg reception in *Drosophila* by increasing Wg retention in the ECM (Franch-Marro et al., 2005; Yan et al., 2009). The ability of Dlp to affect Wnt signaling is 'biphasic' (concentration dependent): low Dlp levels promote and high levels inhibit the signaling (Kreuger et al., 2004; Baeg et al., 2004; Yan et al., 2009). Dlp might compete with or provide ligands for the receptor (Marois et al., 2006; Yan et al., 2009). Therefore, the upregulation of Dlp by the *Drosophila* EGFs but not the human EGFs would explain why ectopic NT^{Dm}-WD^{Hs}-EGF^{Dm} has a stronger effect than WIF1, both on Wg retention in the ECM (Fig. 4B) and on blocking the Wg pathway (Fig. 3C-D′). Significantly, as in the case of expressing NT^{Dm}-WD^{Hs}-EGF^{Dm} protein, the retention of Wg caused by Dlp occurs at the basal level of the epithelium (supplementary material Fig. S7B). More importantly, decreasing the endogenous levels of Dlp by expressing WIF1 or NT^{Dm}-WD^{Hs}-EGF^{Dm} in a dlp^{20} heterozygous background reduces the repression of the Wg pathway (Fig. 8E; supplementary material Fig. S5J). Altogether, these results suggest that the *Drosophila* EGF repeats confer to Shf the ability to interact with glypicans and, more specifically, with Dlp when the WD derives from WIF1 and the ability to interact with Dally when the WD derives from Shf.

DISCUSSION

We conclude here that the WD is responsible for the functional divergence between *Drosophila* Shf and human WIF1, conferring the specificity for Hh or Wnt, whereas the EGF repeats are needed for the interaction of the Wif-1 proteins with ECM components. Although both Dally and Dlp have an influence on Shf, WIF1 and NT^{Dm}-WD^{Hs}-EGF^{Dm} behavior in the wing disc, we show that the *Drosophila* EGF repeats interact mainly with the glypican Dlp when the WD derives from WIF1 and with Dally when the WD derives from Shf. This suggests that both the EGF repeats and WD confer structural characteristics to Shf and WIF1 necessary to recognize glypicans.

Specificity of Wif-1 for a morphogen

We have shown here that the ectopic expression of either WIF1 or the NT^{Dm}-WD^{Hs}-EGF^{Dm} chimera blocks Wg signaling but does not rescue the *shf* phenotype. However, ectopic NT^{Dm}-WD^{Dm}-EGF^{Hs} and Shf does not block Wg signaling but rescues the *shf* phenotype. These data strongly suggest that the WD confers the functional divergence between Shf and WIF1. In addition, we have shown that both the WD and EGFs bind to a morphogen in a synergistic manner. It has been reported that the WD of WIF1, on its own, binds Wnt and blocks Wnt signaling, although not as effectively as the complete protein, suggesting that EGFs I-V are essential for the full activity of Wif-1 (Hsieh et al., 1999; Malinauskas et al., 2011).

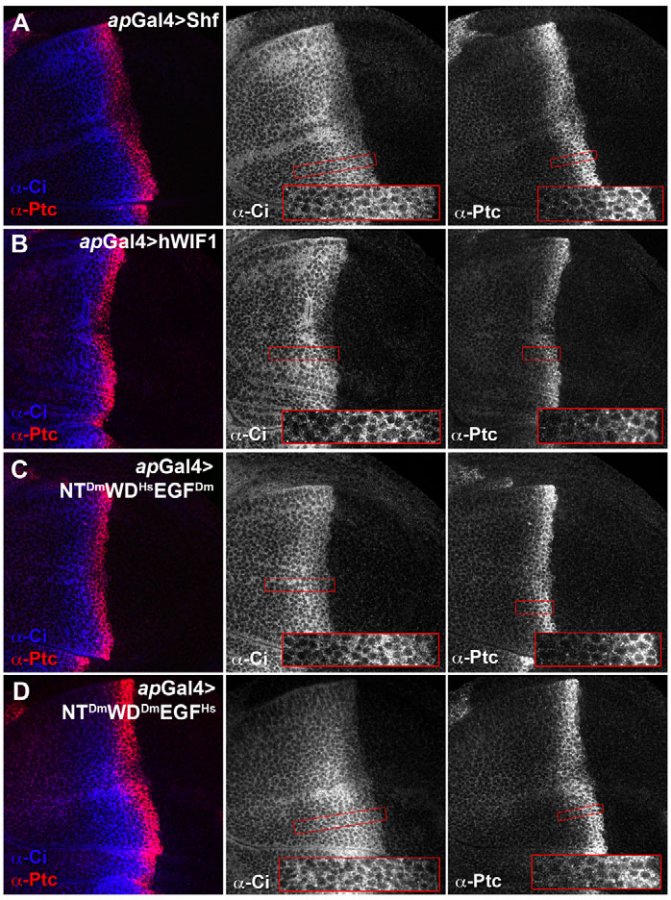

Fig. 6. The effect of ectopic expression of Wif-1 chimeric proteins in the Hh pathway. Ptc (red) and Ci (blue) expression in (**A**) *ap*Gal4>Shf, (**B**) *ap*Gal4>hWIF-1, (**C**) *ap*Gal4>NT^{Dm}-WD^{Hs}-EGF^{Dm} and (**D**) *ap*Gal4>NT^{Dm}-WD^{Dm}-EGF^{Hs} wing discs. The *Drosophila* Shf protein enhances the Hh gradient, whereas human WIF1 does not affect Hh targets. In the case of chimeric constructs, ectopic NT^{Dm}-WD^{Hs}-EGF^{Dm} has no effect on the Hh gradient (the behavior is similar to that of ectopic WIF1), whereas ectopic NT^{Dm}-WD^{Dm}-EGF^{Hs}, like ectopic Shf, enhances the expression of Hh targets. Insets show the boxed regions at higher magnification.

Structural analysis and site-directed mutagenesis in combination with cellular and biophysical assays have shown that Wnt binds both to the WD and to the EGF-like domains (Malinauskas et al., 2011). The structure of Wif-1 allows the WD and EGFs to bind Wnt in a synergistic manner. The EGF-like (EGFs I-V) domain adopts a specific (wrapped-back) position relative to the WD, interfacing with WD at the EGF III region. Interestingly, point mutations in conserved Cys residues of the EGF III repeat of Shf have been identified in the hypomorphic shf^{2} and shf^{919} alleles (Glise et al., 2005; Gorfinkiel et al., 2005). These Shf mutant proteins have lost their ability to interact with Hh (Glise et al., 2005). We can speculate that the proteins encoded by shf^{2} and shf^{919} have lost their ability to interact with Hh because the mutant EGF domains do not adopt the correct positions relative to the WD. Therefore, we believe that both the WD and EGFs of Shf are crucial for Shf function in *Drosophila*.

Phylogenetic analysis of these domains shows that EGFs I, II, IV and V are conserved among chordates and arthropods and that EGF III is divergent (supplementary material Fig. S8). Despite this divergence the chimeric protein NT^{Dm}-WD^{Hs}-EGF^{Dm} still blocks Wg signaling, and even more strongly than WIF1. These data support our conclusion that the specificity of Shf or WIF1 for Hh or Wg, respectively, depends on the WD type and not on the EGF repeats. However, both domains are important for Shf binding to Hh, as has been previously proposed for the binding of WIF1 to Wnt (Malinauskas et al., 2011). This synergism of the WD and EGFs would explain why expression of the WD or EGF repeats alone does not rescue the *shf* phenotype in *Drosophila* (Glise et al., 2005; Avanesov et al., 2012).

Although we conclude that Shf/WIF1 targeting of the Hh or Wg pathways is due to the WD and not to the EGF repeats, the activity of WDs in Hh signaling may also vary between different vertebrates. Thus, the $WIF^{Wif\text{-}1}$-EGF^{Shf} construct made using the zebrafish WD can rescue the *shf* phenotype but also blocks Wg signaling in *Drosophila*, indicating that the fish WD is able to recognize both morphogens (Avanesov et al., 2012). Curiously, the WD sequences of zebrafish are more divergent from those of its chordate equivalents, and zebrafish Wif1 protein is distant from WIF1 in the phylogenetic tree (see Fig. 1A).

Both the EGF repeats and WD interact with glypicans

Despite the interaction of Dlp with the *Drosophila* EGF repeats, they do not provide Shf/WIF1 with the specificity for Wg or Hh morphogen or for a preferential interaction with a specific glypican. Moreover, as Dlp acts in both the Hh and Wg signaling pathways in *Drosophila* (Desbordes and Sanson, 2003; Lum et al., 2003; Lin, 2004; Yan et al., 2010), specificity for each morphogen based on the Dlp-EGF domain interaction is unlikely. Our data suggest that both the EGF repeats and WD confer structural characteristics to Shf and WIF1 necessary to recognize glypicans, which is in agreement with recent functional and structural analyses of Shf,

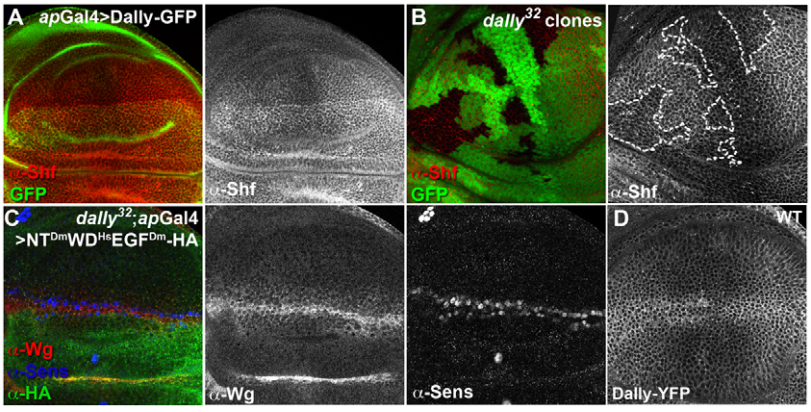

Fig. 7. Shf interaction with Dally. (**A**) Shf expression in an *ap*Gal4>UAS-Dally-GFP *Drosophila* wing disc. Overexpression of Dally in the D compartment causes an accumulation of Shf protein. (**B**) Shf expression in *dally* loss-of-function clones (marked by the absence of GFP, outlined). Shf levels decrease in *dally* mutant cells. (**C**) Overexpression of NT^{Dm}-WD^{Hs}-EGF^{Dm} in a *dally* mutant background. Note the weaker effects of ectopic NT^{Dm}-WD^{Hs}-EGF^{Dm} on Wg accumulation and Sens repression in a *dally* mutant background (C, compare with Fig. 3C,D). (**D**) Dally distribution in a wild-type wing imaginal disc using a DallyTrap-YFP line.

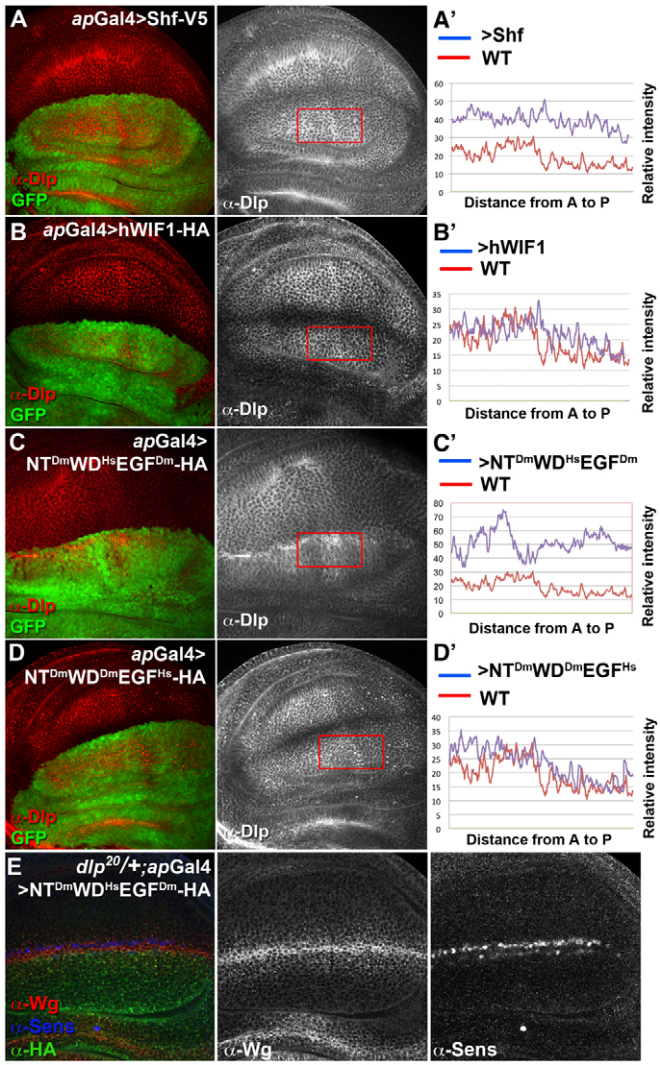

Fig. 8. Shf and WIF1 interact with the glypican Dlp. (**A-D'**) Dlp distribution in *ap*Gal4>Shf (A,A'), *ap*Gal4>hWIF-1 (B,B'), *ap*Gal4>NT^{Dm}-WD^{Hs}-EGF^{Dm} (C,C') and *ap*Gal4>NT^{Dm}-WD^{Dm}-EGF^{Hs} (D,D') *Drosophila* wing discs. Boxed regions were quantified for Dlp protein levels in wild type and under different experimental conditions (A'-D'). Ectopic Shf, owing to its diffusible character, slightly enhances Dlp levels throughout the disc (A,A'). This increase is more pronounced at the D/V compartment border where Dlp protein levels are very low in a wild-type disc (E). Overexpression of NT^{Dm}-WD^{Hs}-EGF^{Dm} protein causes a strong accumulation of Dlp in the D compartment only (C,C'). However, as in the case of ectopic NT^{Dm}-WD^{Dm}-EGF^{Hs}, the ectopic WIF1 does not affect Dlp protein levels (D,D'). (**E**) Overexpression of NT^{Dm}-WD^{Hs}-EGF^{Dm} in a *dlp* heterozygous mutant background. Note the weaker effects of ectopic WIF1 and NT^{Dm}-WD^{Hs}-EGF^{Dm} on Wg accumulation and Sens repression in the *dlp* mutant background (E, compare with Fig. 3C,D). Quantification was performed using ten discs of each genotype. The red line in the plots corresponds to Dlp fluorescence in a wild-type disc and the blue line corresponds to Dlp fluorescence after the ectopic expression of the indicated constructs.

fish Wif1 and human WIF1 proteins (Avanesov et al., 2012; Malinauskas et al., 2011). We predict that both the WD and EGFs are important for WIF1 function in blocking Wnt internalization and reception in humans.

Which glypican contributes to the Wnt-inhibiting functions of vertebrate Wif-1? Several vertebrate glypicans have been implicated in Wnt signaling and might be involved in interactions with Wif-1 (Capurro et al., 2005; Filmus and Capurro, 2008). The results presented here will help us to understand the effect of vertebrate Wif-1 on Wnt distribution and signaling during development. The silencing of human WIF1 described in several types of cancer could increase the dispersion and reception of Wnt, which favors the proliferation of tumor cells. Likewise, SFRP1 is silenced in some types of cancer and probably blocks Wnt signaling using a similar mechanism (Esteve et al., 2011).

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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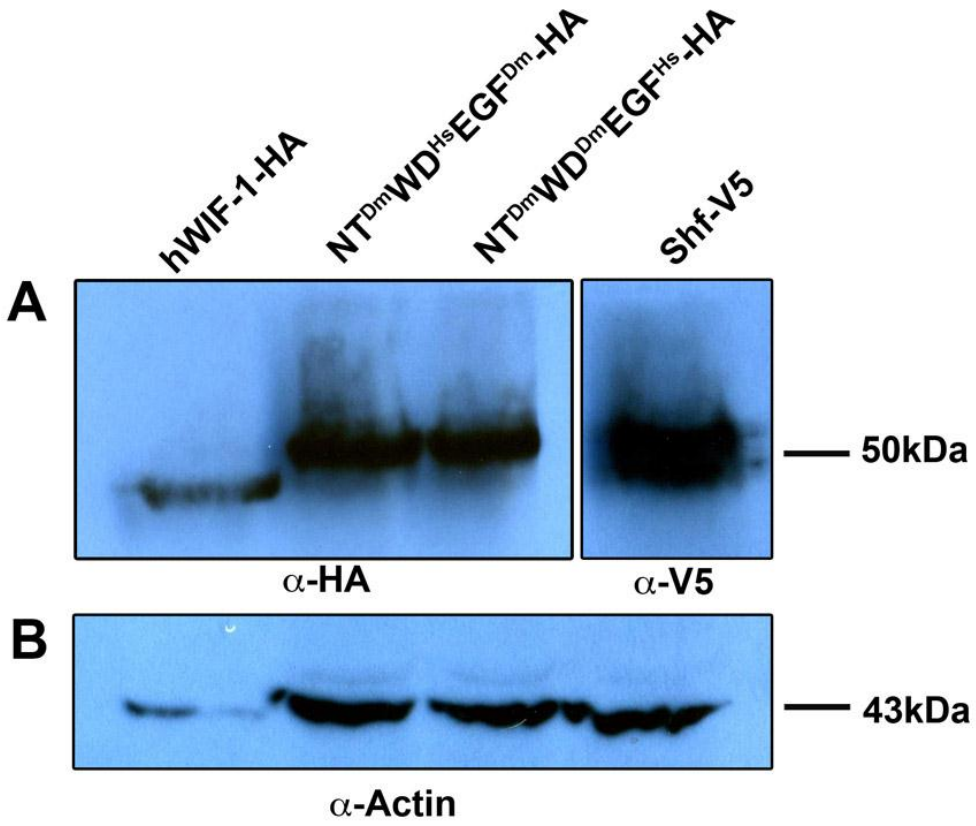

Fig. S1. Expression levels of the UAS constructs analyzed by western blotting. (**A**) hWIF-1-HA, NT^{Dm}-WD^{Hs}-EGF^{Dm}-HA, NT^{Dm}-WD^{Dm}-EGF^{Hs}-HA and Shf-V5 third instar larvae extracts stained with anti-HA or anti-V5 antibodies after their induction by the tub-gal4/tub-$gal80^{ts}$ system for 24 hours. (**B**) Endogenous actin protein levels were used as a control in all extracts.

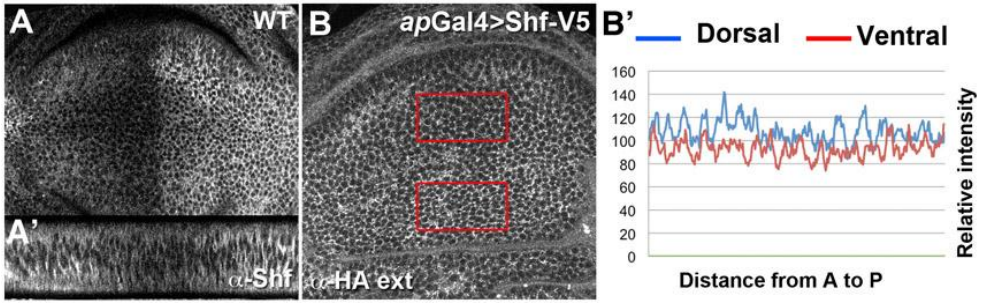

Fig. S2. Extracellular localization of Shf protein. (**A**,**A9**) Wild-type distribution of Shf in a wing disc. Note that Shf levels are higher in the entire P compartment and in the most anterior part of the anterior compartment, and lower at the A/P border. (**B**,**B9**) Extracellular staining using anti-V5 antibody of a wing disc expressing Shf-V5 in the dorsal compartment (*ap*Gal4>UAS-Shf-V5 wing imaginal disc). The extracellular Shf protein is homogenously distributed in both D and V compartments (B9). Quantification of proteins in dorsal and ventral compartments was performed using 12 discs for each genotype (B9).

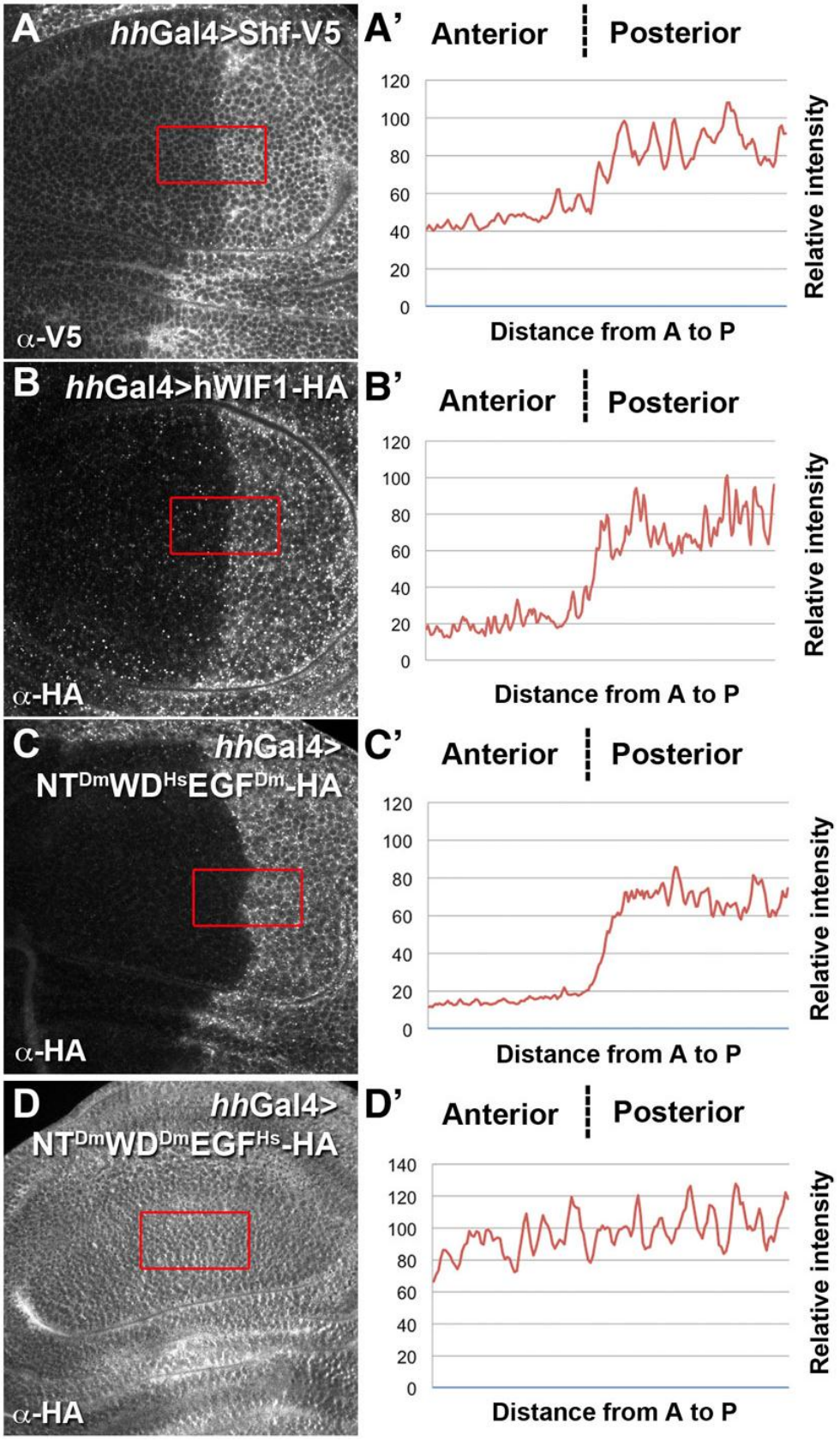

Fig. S3. Spreading properties of Shf, WIF1 and the hybrid Shf/WIF1 proteins. (**A-B9**) *hh*Gal4>Shf-V5 (A,A9) and *hh*Gal4>hWIF-1-HA (B,B9) wing imaginal discs stained with anti-V5 and anti-HA, respectively. Despite Shf only being induced in the P compartment, it is also found in the A compartment (A,A9). However, WIF1 is mostly restricted to its expression domain (B,B9). **(C-D9)** *hh*Gal4>NT^{Dm}-WD^{Hs}-EGF^{Dm}-HA (C,C9) and *hh*Gal4>NT^{Dm}-WD^{Dm}-EGF^{Hs}-HA (D,D9) wing discs. Note that NT^{Dm}-WD^{Hs}-EGF^{Dm}-HA protein is restricted to its expression domain (C,C9), while the distribution of NT^{Dm}-

WD^{Dm}-EGF^{Hs}-HA is similar to that of Shf (D,D9). Quantification of proteins in the A and P compartments was performed using an average of 13 discs for each genotype (A9-D9).

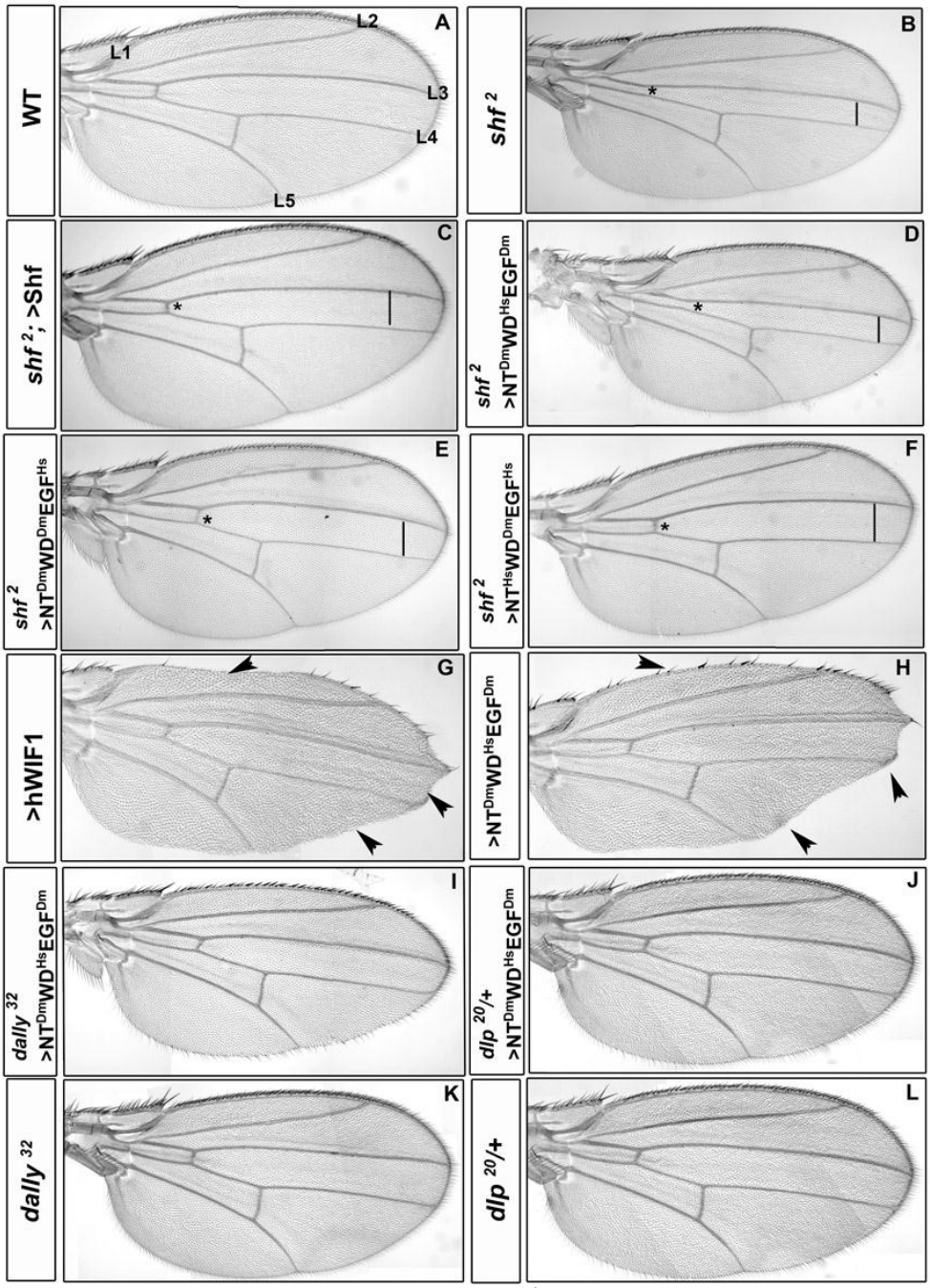

Fig. S4. Wing phenotypes. (**A**) Wild-type adult wing. (**B**) *shf*^{2} mutant wing. Note that the distance between L3-L4 veins is reduced (bar) and the anterior crossvein is absent (asterisk). (**C-F**) *shf*^{2}; *ap*Gal4>UAS-Shf-V5 (C), *shf*^{2};*ap*Gal4>NT^{Dm}-WD^{Hs}-EGF^{Hs} (D), *shf*^{2}; *ap*Gal4>UAS-NT^{Dm}-WD^{Dm}-EGF^{Hs} (E) and *shf*^{2}; *ap*Gal4>UAS-NT^{Hs}-WD^{Dm}-EGF^{Hs} (F) adult wings. Note that the L3-L4 distance reduction and the anterior crossvein of *shf* wings are fully rescued in C, E and F but not in D. At least ten flies were analyzed for each genotype. (**G**,**H**) *ap*Gal4>UAS-hWIF-1 (G) and apGal4>UAS-NT^{Dm}-WD^{Hs}-EGF^{Dm} (H) adult wings. Note the characteristic *wg* mutant phenotype with nicks in the wing margin (arrows). (**I**,**J**) Overexpression of NT^{Dm}-WD^{Hs}-EGF^{Dm} in a *dally* (I) or a *dlp* (J) mutant background. The *wg* mutant adult phenotype is partially rescued in both mutant backgrounds (compare with H). (**K**,**L**) *dally*^{32} and *dlp*^{20} mutant wings.

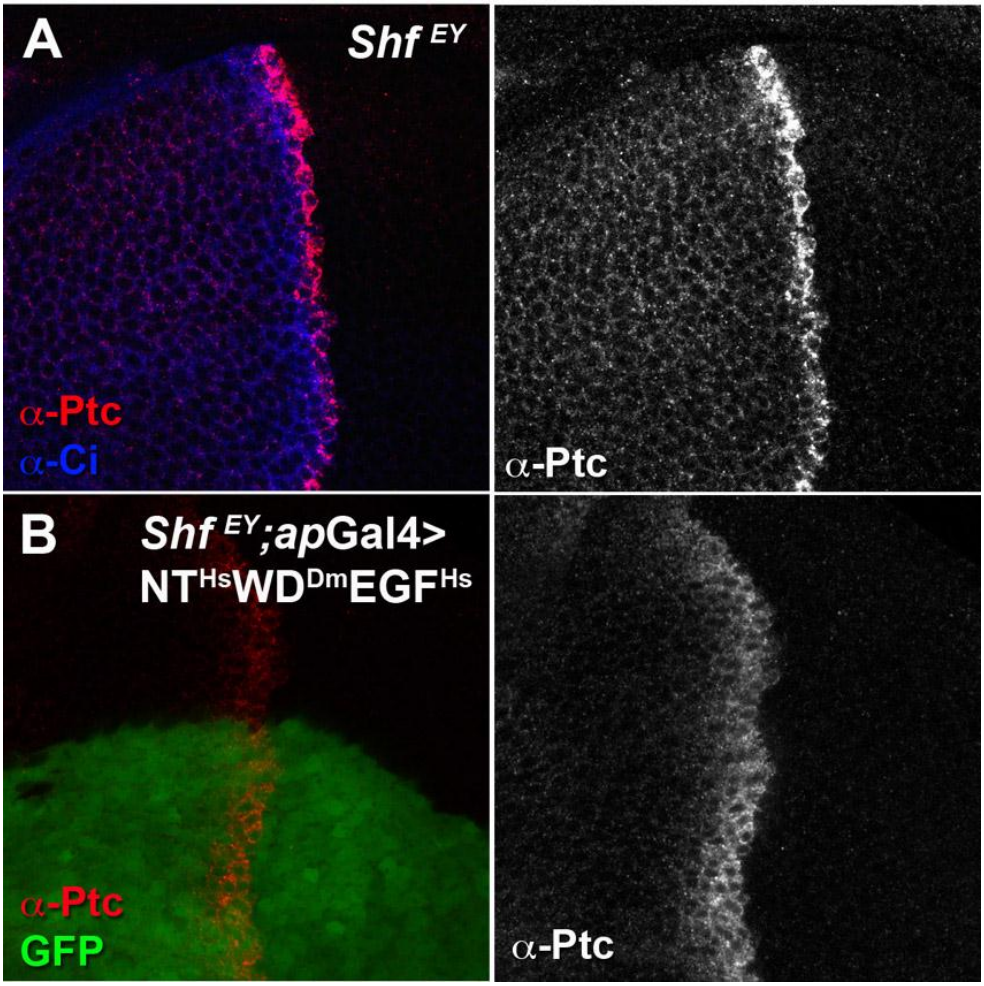

Fig. S5. Ectopic expression of NT^{Hs}-WD^{Dm}-EGF^{Hs} rescues the *shf* mutant disc phenotype. (**A**) Ptc expression in a *shf* mutant disc. In *shf* mutants, Ptc is expressed only in the first row of cells of the A compartment adjacent to the A/P border. (**B**) Normalized Ptc expression in shf^{EY}; *ap*Gal4>$NT^{Hs}WD^{Dm}$-EGF^{Hs}.

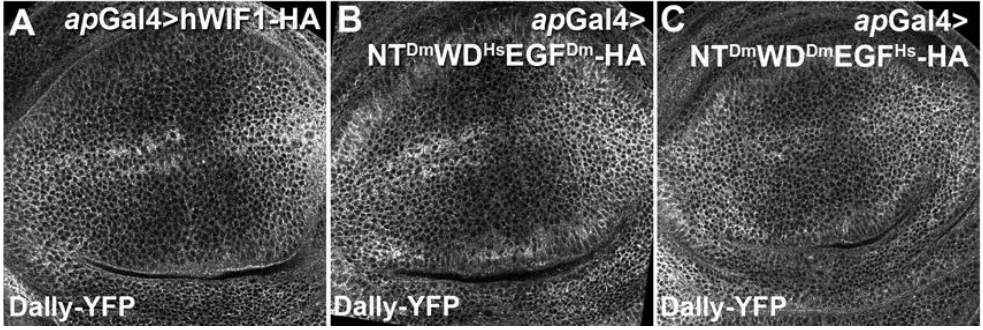

Fig. S6. Ectopic expression of WIF1 or chimeras has no effect on Dally. Overexpression of (**A**) WIF1, (**B**) NT^{Dm}-WD^{Hs}-EGF^{Dm} or (**C**) NT^{Dm}-WD^{Dm}-EGF^{Hs} in the dorsal compartment of the wing disc using the *ap*Gal4 driver did not have any effect on the distribution of the glypican Dally.

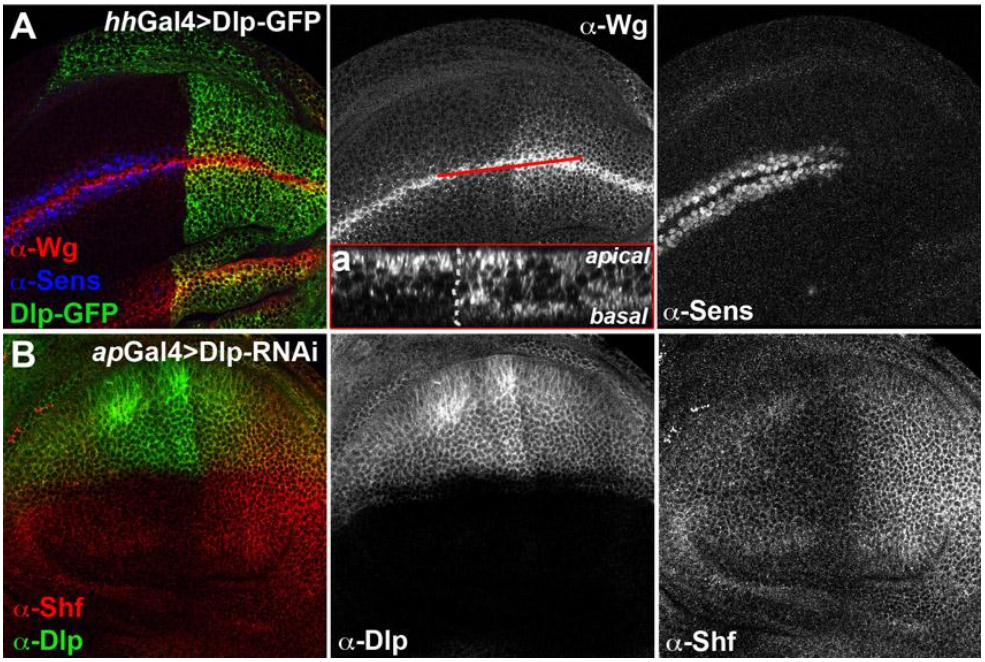

Fig. S7. Dlp attaches to Wg in the basolateral part of the disc epithelium. (**A**) Wg and Sens expression in *hh*Gal4>UAS-Dlp wing disc. Note the accumulation of Wg and the repression of Sens. (**B**) A transverse section (red line marks the location of the section) of the same disc to show the accumulation of Dlp and Wg mainly in the basal part of the epithelium. (**C**) Endogenous Shf protein levels in an *ap*Gal4>UAS-Dlp-RNAi wing disc. Diminution of Dlp expression does not affect Shf protein levels.

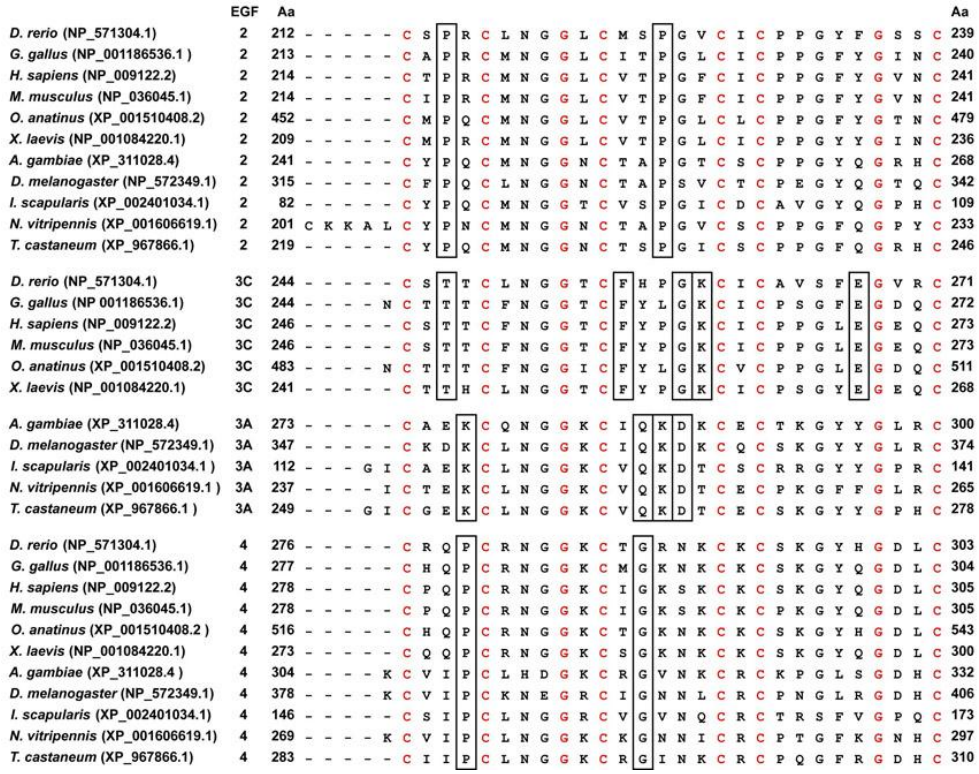

Fig. S8. Alignment of EGF domains 2, 3 and 4, extracted from sequences of Wif-1 and Shf. EGF 3 of chordates (3C) does not share conserved positions with EGF 3 of arthropods (3A), unlike EGFs 2 and 4. Positions conserved in all EGFs are marked in red and amino acids conserved in each EGF are boxed.

Table S1A. Sequences of Wif-1 and Shf used in the phylogenetic analysis.
WIF domain corresponds to Pfam PF02019 and EGF domains correspond to Pfam PF07974

Species	NCBI accession	WD	EGF 1	EGF 2	EGF 3	EGF 4	EGF 5
Chordates		Position of each domain in the amino acid sequence					
Danio rerio	NP_571304.1	36-171	180-207	212-239	244-271	276-303	308-335
Gallus gallus	NP_001186536.1	33-172	181-208	213-240	244-272	277-304	309-336
Homo sapiens	NP_009122.2	38-173	182-209	214-241	246-273	278-305	310-337
Mus musculus	NP_036045.1	38-173	182-209	214-241	246-273	278-305	310-337
Ornithorhynchus anatinus	XP_001510408.2	280-411	420-447	452-479	483-511	516-543	548-575
Xenopus laevis	NP_001084220.1	33-168	172-204	209-236	241-268	273-300	305-332
Arthropods		Position of each domain in the amino acid sequence					
Anopheles gambiae	XP_311028.4	49-186	207-236	241-268	273-300	304-332	339-368
Drosophila melanogaster	NP_572349.1	119-261	283-310	315-342	347-374	378-406	411-440
Ixodes scapularis	XP_002401034.1	1-87	absent	82-109	112-141	146-173	184-216
Nasonia vitripennis	XP_001606619.1	44-181	absent	201-233	237-265	269-297	304-335
Tribolium castaneum	XP_967866.1	41-178	178-214	219-246	249-278	283-310	319-347

Table S1B. Sequences of receptors and putative receptors that contain WD used in the phylogenetic analys
WIF domain corresponds to Pfam PF02019

Species	NCBI accession	WD
Chordates		Position of domain in the amino acid sequence
Danio rerio	NP_001077295.1	62-188
Gallus gallus	XP_422674.3	40-168
Homo sapiens	NP_001005861.1	66-194
Mus musculus	NP_038677.3	50-178
Ornithorhynchus anatinus	XP_001506678.2	1-76
Xenopus laevis	NP_001086278.1	55-182
Xenopus laevis	NP_001089445.1	56-184
Arthropods		Position of domain in the amino acid sequence
Anopheles gambiae	XP_316438.5	1-119
Anopheles gambiae	XP_317454.4	1-119
Drosophila melanogaster	NP_610835.2	29-160
Drosophila melanogaster	NP_477341.2	49-180
Drosophila melanogaster	NP_477139.1	24-155
Nasonia vitripennis	XP_001606015.2	27-155
Nasonia vitripennis	XP_001605949.1	5-144
Tribolium castaneum	XP_969941.1	24-153

Table S2. Expression domains of Hh targets after overexpression of Shf/WIF1 chimeric proteins in the posterior compartment of the wing disc

Genotype	Ptc	Ci
WT	7	12
*hh*Gal4>Shf	10.7*	16.7*
*hh*Gal4>hWIF1	7.4	11.6
*hh*Gal4>NT^{Dm}WD^{Hs}EGF^{Dm}	6.7	12.2
*hh*Gal4>NT^{Dm}WD^{Dm}EGF^{Hs}	10.6*	18.1*

Shown is the mean number of cells expressing the Hh targets, assessed as an average of two regions and in at least ten different discs for each genotype.

*P<0.05, Student's t-test, comparison with wild-type (WT) genotype.