CORRIGENDUM

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

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Fig. 4B incorrectly showed an image of an apGal4>UAS-hWIF1 wing disc instead of an apGal4>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.
The WIF domain of the human and *Drosophila* Wif-1 secreted factors confers specificity for Wnt or Hedgehog

David Sánchez-Hernández, Javier Sierra, João Ramalho Ortigão-Farias and Isabel Guerrero*

**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 glypicans 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 (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).

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 (WIFI) is downregulated in cancers (Mazieres et al., 2004; Kansara et al., 2009) and the mouse Wif-1 knockout accelerates the development of radiation-induced osteosarcomas in vivo (Kansara et al., 2009). Furthermore, overexpression of human WIFI1 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; Gorflinkiel 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; Gorflinkiel 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 (Gorflinkiel et al., 2005).

It has been reported that among the HSPGs the glypicans Dally-like (Dlp) have 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.
Development 139 (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<sub>Dm</sub>-WD<sub>Hs</sub>-EGF<sub>Dm</sub>, NT<sub>Dm</sub>-WD<sub>Hs</sub>-EGF<sub>Hs</sub> and NT<sub>Hs</sub>-WD<sub>Dm</sub>-EGF<sub>Hs</sub> 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 NotI/KpnI sites for cloning. Primers (5'-3') for the NT<sub>Dm</sub>-WD<sub>Dm</sub>-EGF<sub>Hs</sub> chimera: 5'NotIDmNT, GCCGGCGCATGACACATGCGCATGCG; 3'DmNT, GCATCGATCATAGCGAGTGCGCTCCTCC; 5'HsWD, GGAGAGCGGCATGCATTGTAGATGCG; 3'HsWD, CACACCTCGTGGGCGACATGTTTAAGAA; 5'DmEGF, CTTTCAGAACATGGCCCA-CAGAGGTTGATGTA; 3'KpnIDmEGF, GGATCCCTAGAACATTGGAGT-GAGTCCAG. For the NT<sub>Hs</sub>-WD<sub>Dm</sub>-EGF<sub>Hs</sub> chimera: 5'NotIHsNT, GCCGGCGCATGCGCCGCGGAGAGCGGC; 3'HsNT, CTCTTAGTCCACAAGTACGGCT-TCCCTCGTCG; 3'DmWD, GAGAGGAGGCTTACCTGTAGGTGATCGATAGATGCG; 3'DmWD2, GCACCTCGTGGTACCTGATGCG; 3'TGAATGGAGC; 5'HsEGF2. CTCTACAGAGGATGCAACTAACGAGGCAGCC; 3'KpnIHsEGF.

WIF<sub>Tg</sub>-WD<sub>Wt</sub>-EGF<sub>Wt</sub> 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 Wnt with the five EGF motifs (amino acids 262 to 456). The NT<sub>Dm</sub>-WD<sub>Dm</sub>-EGF<sub>Hs</sub> 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 WFI1 including the five EGF motifs (amino acids 178 to 379). The NT<sub>Hs</sub>-WD<sub>Dm</sub>-EGF<sub>Hs</sub> 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 WFI1 with the five EGF motifs (amino acids 178 to 379). All final PCR products were cloned and sequenced into pUAS vectors using the NotI and KpnI restriction sites and injected into embryos to obtain transgenic lines.

For UAS>NT<sub>Dm</sub>-WD<sub>Wt</sub>-EGF<sub>Wt</sub>-HA, UAS>NT<sub>Dm</sub>-WD<sub>Wt</sub>-EGF<sub>Wt</sub>-HA and UAS>WIF-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 (supplemental material Fig. S1). Protein extracts from third instar larvae of tubGal4/tubGal80<sup>33</sup>Shf<sup>V5</sup>, tubGal4/tubGal80<sup>33</sup>WIF<sup>1-HA</sup>, tubGal4/tubGal80<sup>33</sup>NT<sub>Dm</sub>-WD<sub>Dm</sub>-EGF<sub>Hs</sub> and tubGal4/tubGal80<sup>33</sup>NT<sub>Hs</sub>-WD<sub>Dm</sub>-EGF<sub>Hs</sub> genotypes were prepared in lyso 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): apGal4 (Callegaro et al., 1996) and hhGal4 (Tanimoto et al., 2000). We also used additional pUAS fly lines: UAS-Shf<sup>V5</sup> (Glise et al., 2005), UAS-Shf<sup>K44A</sup> (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 tubGal80<sup>80</sup> was achieved by maintaining the fly crosses at 18°C and then

**MATERIALS AND METHODS**

**Fly mutants**

Mutations, insertions and transgenes used are described in FlyBase. shf<sup>2</sup>, shf<sup>K44A</sup>, shf<sup>Wt</sup> (Gorfinkiel et al., 2005), dally<sup>2</sup>, dlp<sup>20</sup> (Franch-Marro et al., 2005) and 2005 and DallyTrap lines (http://www.flyprot.org/stock_report.php? stock_id=17071) were used in this work.
immunostaining of imaging 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 was performed using 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/interproscan/) were also used to predict appropriate domains and to generate the alignments with the hmm files. The phylogenetic analysis of the WD 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 (Pathy, 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; Gorfeinkiel 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 overexpressing the Drosophila and human Shf proteins. The spreading of Shf can be visualized more clearly by using anti-Wg or anti-V5 and untagged form. We also tested the possible effect of the N-terminal part (NT), as Shf has a much longer NT than WIF1, which is not rescued by WIF1. This suggests that Shf spreads further than WIF1.
disc was found mostly in its expression domain. However, NTDm-WDDm-EGFHs dispersed even further than Shf (Fig. 2D,D/H11032; supplementary material Fig. S3D,D/H11032), 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/H11032). 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/H11032, 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. 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 hhGal4>NTDm-WDh-EGFh discs, Wg targets were not activated (Fig. 3D,D’, arrows) and the Wg gradient was extended (Fig. 3I). NTDm-WDh-EGFh protein affected the Wg pathway in a manner similar to full-length WIF1 (Fig. 3C,C/H11032), although the effect was stronger. Thus, ectopic NTDm-WDh-EGFh 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
Specificity of Wif-1 for Wnt or Hh

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 on 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. daily 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 WIFI1 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 WIFI1 or WT Dm-WDH-EGFm proteins. However, the daily2 mutant background slightly alleviated the notched wing phenotype and repression of Wg signaling observed after expressing WIFI1 or WT Dm-WDH-EGFm proteins. This chimeric protein also 기타 다음의 항목

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
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 apGal4 driver) in the D compartment results in a slight enhancement of Dlp levels throughout the disc (Fig. 8A, A/H11032), 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/H11032). 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 NTdm-WDHs-EGFDm and NTdm-WDDm-EGFHs chimeras gave different results. Ectopic NTdm-WDHs-EGFDm expression caused a substantial accumulation of Dlp (Fig. 8C, C'), whereas ectopic NTdm-WDDm-EGFHs 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 WDfish-EGFfly 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 glypicans Dlp, possibly owing to the evolutionary divergence of the EGF repeats.
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 NTDm-WDDm-EGFHs 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 NTDm-WDDm-EGFHs protein, the retention of Wg caused by Dlp occurs at the basolateral level of the epithelium (supplementary material Fig. S7B). More importantly, decreasing the endogenous levels of Dlp by expressing WIF1 or NTDm-WDDm-EGFHs in a dlp 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.
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 and shf 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 and shf 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 NTm-WDm-EGFm 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 WIFWif-1-EGFShf 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. 8. Shf and Wifi1 interact with the glypican Dlp. (A–D') Dlp distribution in apGal4>Shf (A,A'), apGal4>hWifi1 (B,B'), apGal4>NTm-WDm-EGFm (C,C') and apGal4>NTm-WDm-EGFm (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 NTm-WDm-EGFm protein causes a strong accumulation of Dlp in the D compartment only (C,C'). However, in the case of ectopic NTm-WDm-EGFm, the ectopic WIFI1 does not affect Dlp protein levels (D,D'). (E) Overexpression of NTm-WDm-EGFm in a dlp heterozygous mutant background. Note the weaker effects of ectopic WIFI1 and NTm-WDm-EGFm 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.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.080028/-/DC1

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