

# Secreted frizzled-related proteins are required for Wnt/ $\beta$ -catenin signalling activation in the vertebrate optic cup

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## SUMMARY

Secreted frizzled-related proteins (Sfrps) are considered Wnt signalling antagonists but recent studies have shown that specific family members enhance Wnt diffusion and thus positively modulate Wnt signalling. Whether this is a general and physiological property of all Sfrps remains unexplored. It is equally unclear whether disruption of Sfrp expression interferes with developmental events mediated by Wnt signalling activation. Here, we have addressed these questions by investigating the functional consequences of Sfrp disruption in the canonical Wnt signalling-dependent specification of the mouse optic cup periphery. We show that compound genetic inactivation of *Sfrp1* and *Sfrp2* prevents Wnt/ $\beta$ -catenin signalling activation in this structure, which fails to be specified and acquires neural retina characteristics. Consistent with a positive role of Sfrps in signalling activation, Wnt spreading is impaired in the retina of *Sfrp1*<sup>-/-</sup>;*Sfrp2*<sup>-/-</sup> mice. Conversely, forced expression of *Sfrp1* in the wing imaginal disc of *Drosophila*, the only species in which the endogenous Wnt distribution can be detected, flattens the Wg gradient, suppresses the expression of high-Wg target genes but expands those typically activated by low Wg concentrations. Collectively, these data demonstrate that, in vivo, the levels of Wnt signalling activation strongly depend on the tissue distribution of Sfrps, which should be viewed as multifunctional regulators of Wnt signalling.

**KEY WORDS:** Ciliary margin, Wnt diffusion, Patterning, Mouse, *Drosophila*

## INTRODUCTION

Secreted frizzled-related proteins (Sfrps) are a family of secreted factors involved in embryonic development and tissue homeostasis. Owing to their homology to the extracellular portion of the Wnt receptor Frizzled (Fzd), Sfrps have been described as scavengers of Wnt signal activation. Indeed, gain of Sfrp function has proven especially useful to antagonise both canonical and non-canonical Wnt signalling in a variety of contexts (Bovolenta et al., 2008). However, single or compound genetic inactivation or knockdown of individual Sfrps in different vertebrates has provided only partial support for the idea that, in the absence of Sfrps, Wnt signalling is overactivated (Esteve et al., 2004; Joesting et al., 2008; Misra and Matise, 2010; Satoh et al., 2006; Satoh et al., 2008; Trevant et al., 2008).

This discrepancy might be explained by the demonstration that Sfrps can interact with different proteins, thereby interfering with molecular cascades other than those activated by Wnts (Esteve et al., 2011; He et al., 2010; Kobayashi et al., 2009; Lee et al., 2006; Muraoka et al., 2006). Furthermore, Sfrps can modulate Fzd-mediated signalling independently of Wnts, forming complexes with both Fzd (Bafico et al., 1999; Dufourcq et al., 2008; Rodriguez et al., 2005) and Wnts (Uren et al., 2000), possibly through differential domain binding (Lopez-Rios et al., 2008). This raised the hypothesis that Sfrps could promote Wnt-Fzd interaction, either by bringing ligand and receptor into proximity or by favouring ligand dispersion (Lopez-Rios et al., 2008). According to the latter possibility, FrzB/Sfrp3 and Crescent, two Sfrp family members, appear to

promote the diffusion and expand the signalling range of otherwise non-diffusible, exogenously added Wnts in gastrulating *Xenopus* embryos (Mii and Taira, 2009). These results provide a novel view of Sfrp function and raise the question of whether this is a common Sfrp property. Indeed, Crescent has no apparent homologue in mammals and FrzB structurally diverges from Sfrp1/2/5 (Bovolenta et al., 2008). Furthermore, genetic inactivation of FrzB causes articular cartilage loss and increases Wnt signalling (Lories et al., 2007), leaving open the question of whether Sfrp knockout has physiological consequences compatible with a positive function in Wnt signalling activation.

Here, we have addressed these questions by demonstrating that Sfrp1 promotes the spreading of endogenous and exogenously added Wnts and provide genetic evidence that Sfrp1 and Sfrp2 are required for the activation of canonical Wnt signalling implicated in the specification of the mouse eye periphery.

## MATERIALS AND METHODS

### Animals

*Sfrp1*<sup>-/-</sup>;*Sfrp2*<sup>+/-</sup> mice were generated and intercrossed to generate *Sfrp1*<sup>-/-</sup>;*Sfrp2*<sup>-/-</sup> double-mutant embryos as described (Satoh et al., 2006; Esteve et al., 2011).

### Antibodies

Antibodies used: mouse monoclonal BrdU (Boehringer Mannheim); Islet1, Wg, Cut, Myc; active  $\beta$ -catenin (ABC; Millipore); N-cadherin (Sigma); Myc (clone 9E10, DSHB); rat monoclonal Ci (Motzny and Holmgren, 1995); rabbit polyclonal Sfrp1, Otx2 (Abcam); P-Smad1,5,8, P-cJun, (Cell Signalling); cJun (Santa Cruz); calbindin (Swant); Pax6 (Covance); Par3 (Upstate); Pax2 (Invitrogen); Chx10 (a kind gift from R. McInnes, The Sick Kids Hospital, Toronto, Canada); Distal-less (Panganiban et al., 1994); PH-H3 (Roche Diagnostics); Hh (a kind gift from S. Eaton, MPI, Dresden, Germany); guinea-pig polyclonal Vestigial (Kim et al., 1996); and Sens (a kind gift from H. Bellen, Jan and Dan Duncan Neurological Research Institute, Houston, TX, USA). Rabbit Alexa 488 or 594 and mouse Alexa 488 (Molecular Probes) were used as secondary antibodies.

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### In situ hybridisation (ISH) and immunohistochemistry (IHC)

E9-16.5 mouse embryos were processed for ISH and IHC as described (Esteve et al., 2003). In some cases, tissues were processed for antigen retrieval with an uncloaking chamber (Biocare Medical) at 115°C for 2 minutes in 10 mM citrate buffer (pH 6). The employed digoxigenin-labelled antisense riboprobes were: *Otx1*, *Msx1*, *Axin2*, *Lef1*, *Wnt2b*, *Wnt5a*, *Wnt3a*, *Wnt7a* and *Wnt7b*, *Bmp4*, *Bmp7*, cyclin D1 (*Ccnd1*), *Chx10* and *Crx*. For BrdU analysis, pregnant dams were injected intraperitoneally with BrdU (50 µg/g), sacrificed 1 hour later and processed for IHC. IHC of *Drosophila* imaginal discs was performed as described (Torroja et al., 2004). Tissues were examined with a confocal laser-scanning microscope (LSM510 Vertical, Zeiss) or a DM500 microscope (Leica).

### Electroporation and detection of Wnts

Expression plasmids (pCAG) encoding Venus-tagged Wnt8 and Wnt11 [kindly provided by Dr M. Taira (Mii and Taira, 2009)] were electroporated into isolated retinas from E12.5 wild-type, *Sfrp1*<sup>-/-</sup> and *Sfrp1*<sup>-/-</sup>;*Sfrp2*<sup>-/-</sup> mouse embryos. Tissue was then cultured for 24 hours in DMEM/F12 medium supplemented with N2 (Gibco) with or without the addition of purified recombinant Sfrp1. Retinas were fixed in 4% PFA for 2 hours and flat mounted. The extent of fluorescent signal diffusion was analysed by confocal microscopy with the aid of ImageJ software.

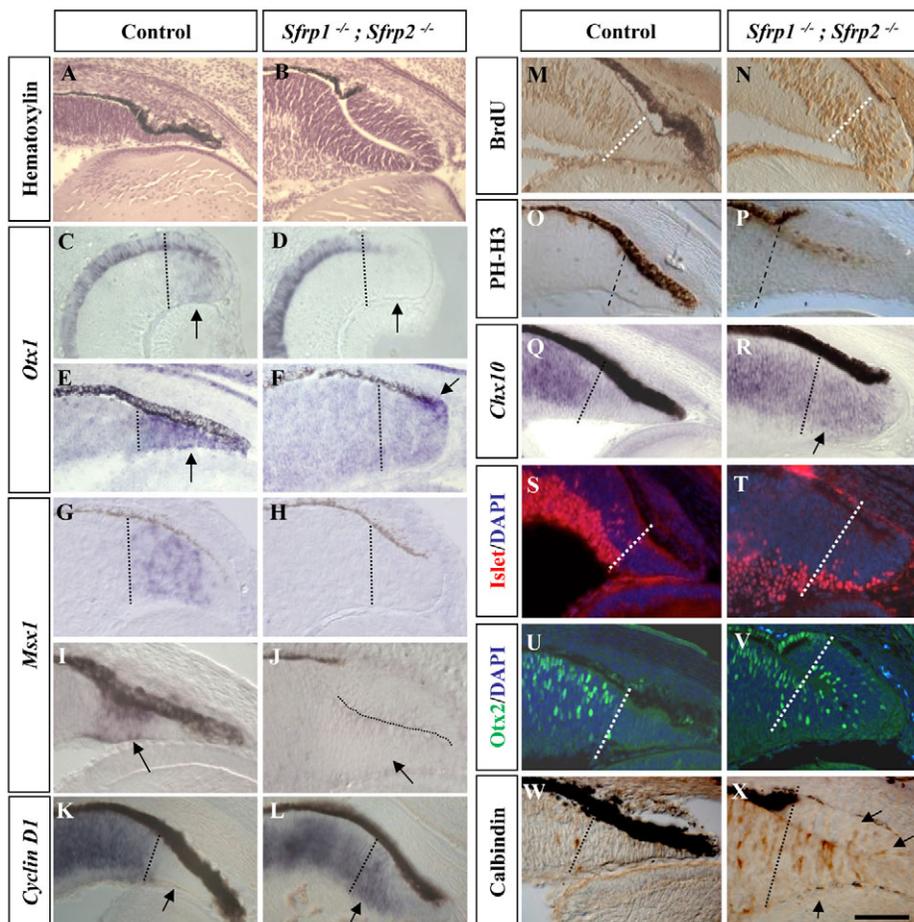
### Overexpression of Sfrp1 in *Drosophila*

*Sfrp1* cDNA was fused in frame to a C-terminal Myc tag and cloned into the pUAST vector to generate transgenic fly lines expressing Sfrp1 under the UAS promoter. The Hh-Gal4 (Tanimoto et al., 2000) and MD638-Gal4 (scalloped-Gal4) (Mullor et al., 1997) drivers were used to express Sfrp1 in the posterior compartment or the entire wing disc, respectively. As a positive control for planar cell polarity defects, *dachsous* function (Matakatsu and Blair, 2004) was knocked down by double-stranded (ds) RNAi expression using the same Gal4 driver.

### RESULTS AND DISCUSSION

The optic cup can be molecularly subdivided in a central region, which will differentiate into central neural retina (cNR) and retinal pigment epithelium (RPE), and a peripheral portion, from which the ciliary body and the iris are derived. The specification of the dorsal and peripheral optic cup (OCP) requires the activation of canonical Wnt signalling (Fuhrmann et al., 2009; Veien et al., 2008). Indeed, ectopic canonical signal activation in the cNR suffices to induce peripheral marker expression (Trimarchi et al., 2009). Conversely, OCP identity is lost after eye-specific conditional inactivation of  $\beta$ -catenin (Liu et al., 2007) or expression of a dominant-negative form of Lef1 (Cho and Cepko, 2006). However, the source of the Wnt ligand responsible for signalling activation remains undefined. The locally expressed *Wnt2b* is an unlikely possibility because its expression seems to be regulated by Wnt-mediated activation of Bmp signalling (Muller et al., 2007) and its ectopic expression does not induce peripheral characteristics (Liu et al., 2007). Lens-derived Wnts are also unlikely because genetic ablation of lens precursors does not affect initial OCP specification (Zhang et al., 2007). *Wnt3*, *Wnt5a*, *Wnt7a* and *Wnt7b* are transiently expressed in the cNR (Liu et al., 2003) and would require long-range distribution to qualify as candidates.

In this scenario, we asked whether *Sfrp1* and *Sfrp2*, which are abundantly expressed during murine eye development (Liu et al., 2003), are required for Wnt signalling activation. *Sfrp2* transcripts are predominant in the cNR, whereas *Sfrp1* localises to the OCP and RPE but its protein is also detected in the cNR (Esteve et al., 2011), probably because Sfrps efficiently diffuse in the extracellular space (Mii and Taira, 2009). According to this



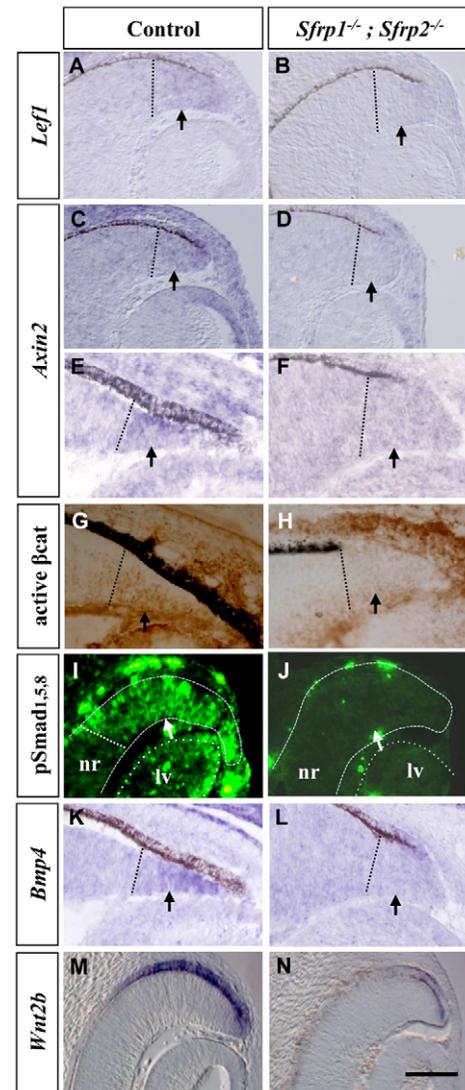
**Fig. 1. In *Sfrp1/2* double-mutant embryos the dorsal optic cup periphery (OCP) fails to be specified.** Frontal cryostat sections of E10.5 (C,D), E12.5 (G,H) and E16.5 (A,B,E,F,I-X) control and *Sfrp1/2* double-mutant mouse embryos stained with Hematoxylin, hybridised or immunostained as indicated. Dashed lines indicate the approximate boundary between central neural retina (cNR) and OCP. Arrows point to relevant differences in staining. Scale bar: 30 µm.

overlapping distribution and the proposed Sfrp functional redundancy (Misra and Matise, 2010; Satoh et al., 2006; Satoh et al., 2008), the eye of *Sfrp1* and *Sfrp2* single-null mouse embryos appeared histologically normal (data not shown). By contrast, at E16.5, the latest viable stage, the eyes of *Sfrp1<sup>-/-</sup>;Sfrp2<sup>-/-</sup>* compound mutants were smaller and grossly altered (see Fig. S1 in the supplementary material) (Esteve et al., 2011), with preponderant defects in the dorsal OCP (Fig. 1A,B). This phenotype was fully penetrant. According to morphological and molecular analyses, optic cup formation was initiated normally in mutant embryos and defects were first observed at ~E10.5 (see Figs S1 and S2 in the supplementary material), when the OCP begins to be specified.

The OCP is characterised by the expression of *Otx1* and *Msx1* (Trimarchi et al., 2009) (Fig. 1C,E,G,I), a low proliferation rate [few BrdU-positive or phospho-histone H3 (PH-H3)-positive cells] and by the absence of expression of the cell-cycle regulator *Ccnd1* (Fig. 1K,M,O), of cNR markers such as *Chx10* (*Vsx2* – Mouse Genome Informatics) or of retinal cell type-specific determinants, including *Islet1*, *Otx2* and calbindin (Fig. 1Q,S,U,W). If Sfrps were to directly antagonise Wnt/ $\beta$ -catenin signalling according to the proposed antagonist function (Leyns et al., 1997), the OCP, and thus the expression of its specific markers, would be expected to be expanded in *Sfrp1<sup>-/-</sup>;Sfrp2<sup>-/-</sup>* embryos. By contrast, from E10.5, the expression of *Otx1* and *Msx1* was nearly absent in the dorsal (Fig. 1D,F,H,J) and, occasionally, ventral OCP of the mutants, and was replaced by *Ccnd1*/BrdU/PH-H3/*Chx10*-positive proliferating cells (Fig. 1L,N,P,R). Thus, the OCP of *Sfrp1/2* compound mutants had lost its defining characteristics and acquired those of the cNR. Accordingly, at E16.5, when neurogenesis is ongoing, ectopic retina ganglion (RGC; *Islet1<sup>+</sup>*), amacrine (calbindin<sup>+</sup>), bipolar (*Otx2<sup>+</sup>*) and photoreceptor (*Crx<sup>+</sup>/Otx2<sup>+</sup>*) cells were ectopically observed in the mutant OCP (Fig. 1T,V,X; data not shown).

Thus, Sfrp1 and Sfrp2 cooperate to establish the border between the peripheral and central neural retina. In their absence, the dorsal and, to a minor extent, the ventral OCP fail to be specified, as observed after inactivation of canonical Wnt signalling (Cho and Cepko, 2006; Liu et al., 2007). Accordingly, and in contrast to what was observed in controls, expression of *Lef1* and *Axin2*, which are targets and readout of the Wnt/ $\beta$ -catenin pathway, was nearly absent in the dorsal OCP and in the periocular mesenchyme of *Sfrp1<sup>-/-</sup>;Sfrp2<sup>-/-</sup>* embryos (Fig. 2A-F). Similarly, the non-phosphorylated active form of nuclear  $\beta$ -catenin was almost absent (Fig. 2G,H). This lack of Wnt/ $\beta$ -catenin signalling activation was unlikely to be an aftermath of early changes in ligand expression because the mRNA distribution of *Wnt3*, *Wnt5a*, *Wnt7a* and *Wnt7b*, which are expressed at early stages of eye development (Liu et al., 2003), was unchanged in the mutant optic cup (see Fig. S3 in the supplementary material).

Bmp signalling acts downstream of the canonical Wnt pathway in the dorsal OCP (Veien et al., 2008) and its inhibition prevents the differentiation of the ciliary body, which is substituted by retinal ganglion-like cells (Zhao et al., 2002), thereby resembling a milder form of the *Sfrp1<sup>-/-</sup>;Sfrp2<sup>-/-</sup>* phenotype. Consistently, the levels of P-Smad1/5/8, which are Bmp signalling effectors, and the expression of *Msx1*, which is a direct Bmp target, and of *Bmp4* were strongly diminished in the dorsal OCP of E16.5 *Sfrp1/2* compound mutants (Fig. 1G-J; Fig. 2I-L), although *Bmp4* expression was initiated normally (see Fig. S1 in the supplementary material). Furthermore, *Wnt2b* expression, which is normally controlled by Bmp activity (Muller et al., 2007), was diminished in the mutant OCP (Fig. 2M,N).



**Fig. 2. Wnt/ $\beta$ -catenin and Bmp signalling are not active in the dorsal OCP of *Sfrp1<sup>-/-</sup>;Sfrp2<sup>-/-</sup>* embryos.** Frontal cryostat sections of E10.5 (I,J,M,N), E11.5 (A-D) and E16.5 (E-H,K,L) eye cups from control and *Sfrp1/2* double-mutant mouse embryos hybridised with probes against *Lef1*, *Axin2*, *Bmp4* and *Wnt2b* or immunostained with antibody against (non-phosphorylated) active- $\beta$ -catenin or pSmad1/5/8. Dashed lines indicate the approximate boundary between cNR and OCP. Arrows point to relevant differences in staining. In I,J, the lens vesicle (lv) and neural retina (nr) are outlined. Scale bar: 30  $\mu$ m.

Altogether, these data demonstrate that Sfrp1 and Sfrp2 cooperate to positively modulate Wnt/ $\beta$ -catenin signalling in the dorsal OCP. There are several possible scenarios to explain this finding. In a classical view, Sfrp1/2 could antagonise non-canonical Wnt signalling, which might normally prevent the activation of canonical activity in the OCP. This regulatory interaction has been proposed to explain the phenotypes obtained by altering the expression of Wnt/Fzd/Sfrp family members during eye field specification in fish (Cavodeassi et al., 2005; Esteve et al., 2004). To address this possibility we asked whether the levels of phospho-cJun, a readout of non-canonical signalling (Sokol, 2000), were different in wild-type and mutant retinas. Immunohistochemical and western blot analyses revealed no differences (see Fig. S2G-I in the supplementary material). Similarly, the distribution of Par3

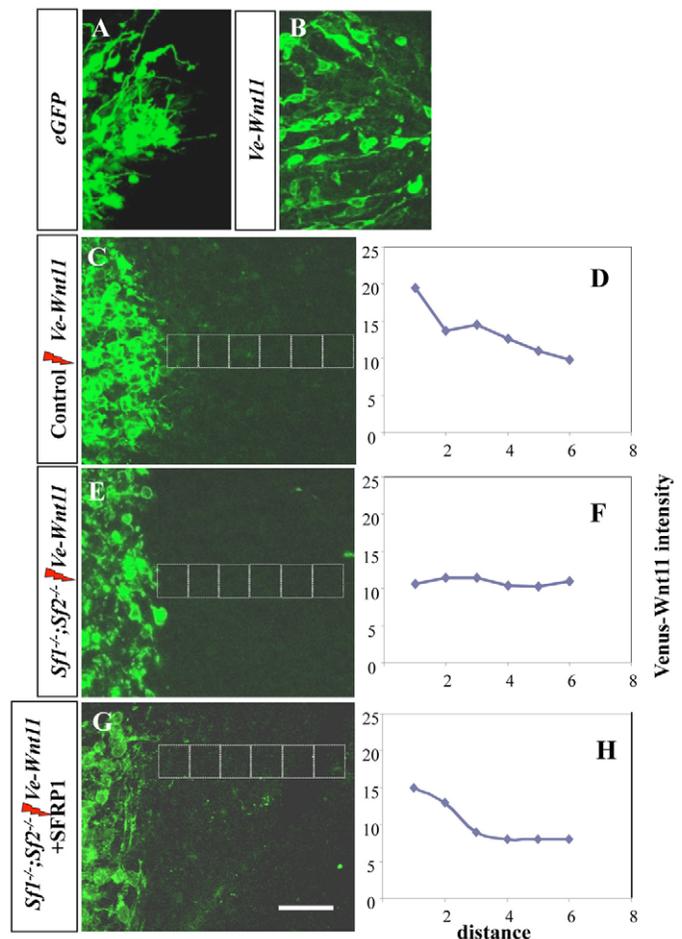
and aPKC, two cell polarity markers linked to non-canonical signalling (Sokol, 2000), was also unchanged (see Fig. S2C-F' in the supplementary material).

As a second possibility, Sfrp1/2 could directly activate Fzd, as shown in other contexts (Dufourcq et al., 2008; Rodriguez et al., 2005). Co-immunoprecipitation studies confirmed that Sfrp1 interacts with Fzd7 and Fzd4 (Dufourcq et al., 2008; Rodriguez et al., 2005) (data not shown), which are strongly expressed in the mouse OCP (Liu et al., 2003). However, Sfrp1 addition to Fzd-expressing cells has no effect on luciferase reporter expression coupled to  $\beta$ -catenin-responsive Tcf binding sites (Esteve et al., 2003; Lopez-Rios et al., 2008), suggesting that the Sfrp1-Fzd interaction is likely to influence only non-canonical components (Dufourcq et al., 2008; Rodriguez et al., 2005), unless additional, as yet unidentified, molecules participate in the interaction.

As a third possibility, Sfrp1 and Sfrp2 could favour Wnt spreading across the OCP, behaving similarly to Crescent and FrzB in *Xenopus* embryos (Mii and Taira, 2009). If this were the case, Wnt spreading should be impaired in the retina of *Sfrp1/2* compound mutants. In vertebrates, detection of endogenously expressed Wnts is severely hampered by the lack of appropriate antibodies. Therefore, we took advantage of the Venus-tagged versions of Wnt8 and Wnt11 (Mii and Taira, 2009). The choice of these ligands seemed appropriate because Wnt11 is transiently expressed in the retina, whereas Wnt8, to our knowledge, is not. The Venus-Wnt11 and Venus-Wnt8 expression constructs contain a signal peptide and are normally secreted into the culture medium (Mii and Taira, 2009). Consistently, when the constructs were electroporated into E13 wild-type retinas, Venus-Wnt11, but not Venus-Wnt8 (not shown) or a control cytoplasmic eGFP (Fig. 3A), was released into the parenchyma and detected as a weak but measurable signal under confocal microscopy (Fig. 3B,C). This punctuate signal was intense up to 30  $\mu$ m from the electroporation front and slowly decreased to background values beyond 80  $\mu$ m (Fig. 3D), indicating diffusion of the fluorescent protein. By contrast, the Venus-Wnt11 signal was detected only in close proximity to the electroporation front in *Sfrp1/2* mutant retina, despite similarity in the areas targeted (Fig. 3E,F). Notably, increased fluorescence levels distant from the electroporation front were restored when *Sfrp1/2* null retinas electroporated with Venus-Wnt11 were cultured for 22 hours in the presence of Sfrp1 protein (Fig. 3G,H).

These observations indicate that Sfrp1 (and likely Sfrp2) favours the spreading of Wnt11, supporting the proposal that, in vivo, Sfrp1/2 might promote Wnt-Fzd interaction by bringing ligand and receptor into proximity (Lopez-Rios et al., 2008). Alternatively, Sfrp1/2 might contribute to regulating the arrangement of the extracellular matrix, which, in turn, is fundamental for morphogen diffusion (Guerrero and Chiang, 2007). This last hypothesis would be consistent with the observation that Sfrps act as inhibitors of Adam10 (Esteve et al., 2011), a sheddase that proteolyzes several membrane proteins. In either case, in the absence of Sfrps the concentration of Wnts that reaches Fzd in the OCP would be insufficient to activate the canonical pathway.

To distinguish between these two possibilities and to further test whether Sfrp1/2 can modify Wnt diffusion and hence signalling activation, we turned to the *Drosophila* wing imaginal disc, which has considerable advantages. First, in contrast to vertebrates, *Drosophila* is the only species in which the endogenous Wingless (Wg; a Wnt homologue) gradient can be easily detected with specific antibodies. Second, Wg activity has been very well characterised and can be unequivocally followed (Neumann and Cohen, 1997; Zecca et al., 1996). Third, no apparent SFRPs

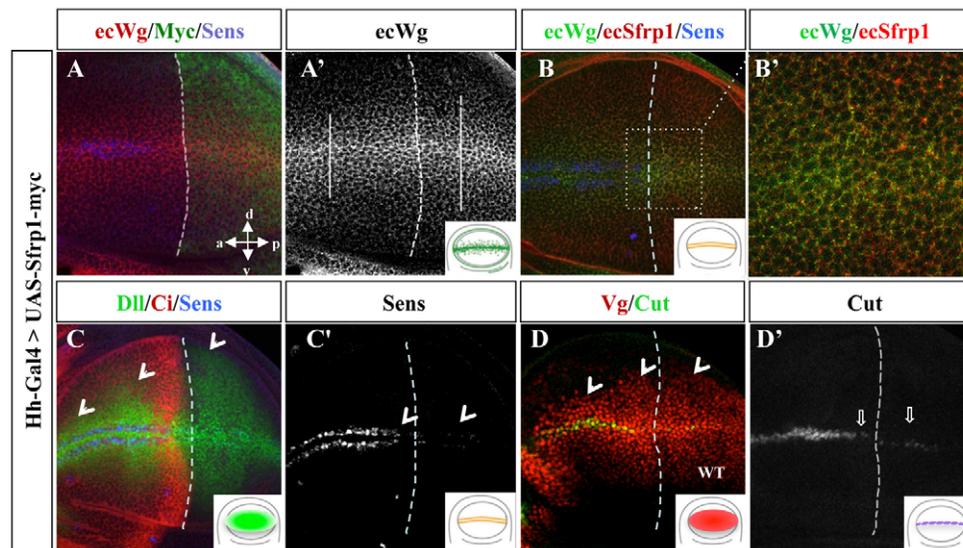


**Fig. 3. Sfrps favour the long-range distribution of Wnt11.**

(A-C,E,G) Confocal images of E12.5 wild-type (A-C) and *Sfrp1*<sup>-/-</sup>; *Sfrp2*<sup>-/-</sup> (E,G) mouse retinas electroporated with expression plasmids for eGFP or Venus-Wnt11 as indicated. Explants in G were cultured in the presence of SFRP1, which restores diffusion of Wnt11. (D,F,H) Quantification of fluorescence pixel intensity within the six boxed regions shown in the corresponding confocal image. Results of a typical experiment are shown; the analysis was repeated four times in duplicate, with similar results. Scale bar: 20  $\mu$ m.

homologues have been identified in the *Drosophila* genome (Bovolenta et al., 2008), effectively offering an Sfrp null background. Despite this absence, Wg efficiently binds to Sfrp1 (Uren et al., 2000), mimicking vertebrate Wnt1 or Wnt8 interaction with Sfrp1 (Lopez-Rios et al., 2008).

Wg is expressed in a stripe of cells at the dorsoventral (DV) boundary of the wing imaginal disc and forms a symmetrical gradient in dorsal and ventral compartments (Neumann and Cohen, 1997; Zecca et al., 1996). UAS-mediated expression of Sfrp1 in the posterior compartment of the wing disc using the Hh-Gal4 driver (HhGal4>UAS-Sfrp1-Myc) affected extracellular Wg protein localisation (Fig. 4A). In contrast to the anteriormost compartment, which served as control, high levels of extracellular Wg were no longer observed at the posterior DV boundary. Rather, the Wg signal was widely distributed across the entire posterior compartment, colocalising with Sfrp1 (Fig. 4B). Notably, a similar colocalisation was observed in an 8- to 10-cell body-wide fringe of the anterior compartment (Fig. 4B,B'), indicating that Sfrp1 diffuses from the producing cells to influence Wg distribution non-cell-autonomously.



**Fig. 4. Sfrp1 expression affects the extracellular distribution of Wg and interferes with its signalling in the *Drosophila* wing disc.** (A–D') Distribution of extracellular (ec) Wg, extracellular Sfrp1, Senseless, Distal-less, Vestigial (Vg), Cut and Cubitus interruptus (Ci) expression in an Hh-Gal4>UAS-Sfrp11-Myc wing imaginal disc expressing Sfrp1 in the posterior compartment, as indicated. The wild-type expression pattern of each gene is represented in the bottom right-hand corner of each panel. The region boxed in B is magnified in B'. Ci defines the border of the anterior compartment; dashed lines indicate the boundary between anterior and posterior compartments; and solid lines in A' indicate the extension of ecWg dispersion in the anterior and posterior compartments. Arrowheads in C–D indicate the dorsoventral limits of Dll and Vg expression in the anterior and posterior compartments. Arrows in D' indicate the repression of Cut in both the posterior and boundary regions of the anterior compartment due to the non-cell-autonomous effect of Sfrp1.

Confirming a flattening of the gradient, the expression of Senseless (Sens), a canonical target normally activated by high Wg levels in two discrete stripes at the DV boundary (Nolo et al., 2000), was no longer observed in the posterior compartment or at the anterior edge of the anterior-posterior border (Fig. 4C,C'). By contrast, the expression of Distal-less (Dll) and Vestigial (Vg), two Wg targets that are activated in response to low ligand concentrations (Neumann and Cohen, 1997; Zecca et al., 1996), was expanded at the anterior boundary and in the posterior compartment, but not in the anteriormost compartment (Fig. 4C,D), indicating that Sfrp1 flattens the Wg gradient without blocking pathway activation. Furthermore, the expression of Cut, a marker of the wing DV boundary that is directly activated by Notch signalling (Micchelli et al., 1997), was completely absent in the posterior compartment (Fig. 4D,D'), consistent with the notion that Sfrp1 modulates Notch signalling through Adam10 regulation (Esteve et al., 2011). We have shown that forced co-expression of Sfrp1 and Kuzbanian, the *Drosophila* Adam10 homologue (Rooke et al., 1996), completely rescues Cut but not Sens expression (Esteve et al., 2011), indicating that Sfrp1-mediated regulation of Adam10 and Wnt signalling are independent events. This last observation, together with Sfrp1/Wg colocalisation, favour the hypothesis that Sfrp1/2 positively modulate canonical Wnt signalling by bringing Wnts into proximity with their receptors (Lopez-Rios et al., 2008).

Collectively, our data suggest that the presence of Sfrp proteins might be crucial to determine the levels and distribution of Wnts and that they act as fine regulators of canonical signalling activation and target gene expression. Whether Sfrps have a similar regulatory function in non-canonical signalling remains unclear. In *Sfrp1/2* mutant eyes, developmental events linked to non-canonical signalling appeared normal. Similarly, scalloped-Gal4-mediated expression of Sfrp1 throughout the entire *Drosophila* wing primordium had no major effect on bristle or trichome orientation,

which are planar cell polarity-mediated events (Strutt and Strutt, 2007). Instead, scalloped-Gal4-Ds-RNAi-mediated knockdown of Dachous, a cadherin-like protein required for the establishment of planar cell polarity (Matakatsu and Blair, 2004), induced a characteristic phenotype (data not shown). Nevertheless, these data do not rule out the possibility that, in other contexts, Sfrp proteins might control the diffusion of Wnts responsible for the activation of non-canonical signalling.

Nonetheless, our findings, together with those of Mii and Taira (Mii and Taira, 2009), contrast with the general designation of Sfrp1/2 as Wnt inhibitors and provide an alternative mechanism by which these proteins modulate Wnt signalling. This alternative view offers a possible explanation for previous results showing that Sfrp1 is required for positive regulation of  $\beta$ -catenin in, for example, hematopoietic progenitors (Renstrom et al., 2009; Yokota et al., 2008).

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#### Competing interests statement

The authors declare no competing financial interests.

#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.065839/-/DC1>

## References

- Bafico, A., Gazit, A., Pramila, T., Finch, P. W., Yaniv, A. and Aaronson, S. A.** (1999). Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. *J. Biol. Chem.* **274**, 16180-16187.
- Bovolenta, P., Esteve, P., Ruiz, J. M., Cisneros, E. and Lopez-Rios, J.** (2008). Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. *J. Cell Sci.* **121**, 737-746.
- Cavodeassi, F., Carreira-Barbosa, F., Young, R. M., Concha, M. L., Allende, M. L., Houart, C., Tada, M. and Wilson, S. W.** (2005). Early stages of zebrafish eye formation require the coordinated activity of Wnt11, Fz5, and the Wnt/beta-catenin pathway. *Neuron* **47**, 43-56.
- Cho, S. H. and Cepko, C. L.** (2006). Wnt2b/beta-catenin-mediated canonical Wnt signaling determines the peripheral fates of the chick eye. *Development* **133**, 3167-3177.
- Dufourcq, P., Leroux, L., Ezan, J., Descamps, B., Lamaziere, J. M., Costet, P., Basoni, C., Moreau, C., Deutsch, U., Couffignal, T. et al.** (2008). Regulation of endothelial cell cytoskeletal reorganization by a secreted frizzled-related protein-1 and frizzled 4- and frizzled 7-dependent pathway: role in neovessel formation. *Am. J. Pathol.* **172**, 37-49.
- Esteve, P., Trousse, F., Rodriguez, J. and Bovolenta, P.** (2003). SFRP1 modulates retina cell differentiation through a beta-catenin-independent mechanism. *J. Cell Sci.* **116**, 2471-2481.
- Esteve, P., Lopez-Rios, J. and Bovolenta, P.** (2004). SFRP1 is required for the proper establishment of the eye field in the medaka fish. *Mech. Dev.* **121**, 687-701.
- Esteve, P., Sandonis, A., Cardozo, M., Malapeira, J., Ibanez, C., Crespo, I., Marcos, S., Gonzalez-Garcia, S., Toribio, M. L., Arribas, J. et al.** (2011). SFRPs act as negative modulators of ADAM10 to regulate retinal neurogenesis. *Nat. Neurosci.* **14**, 562-569.
- Fuhrmann, S., Riesenberger, A. N., Mathiesen, A. M., Brown, E. C., Vetter, M. L. and Brown, N. L.** (2009). Characterization of a transient TCF/LEF-responsive progenitor population in the embryonic mouse retina. *Invest. Ophthalmol. Vis. Sci.* **50**, 432-440.
- Guerrero, I. and Chiang, C.** (2007). A conserved mechanism of Hedgehog gradient formation by lipid modifications. *Trends Cell Biol.* **17**, 1-5.
- He, W., Zhang, L., Ni, A., Zhang, Z., Mirosou, M., Mao, L., Pratt, R. E. and Dzau, V. J.** (2010). Exogenously administered secreted frizzled related protein 2 (Sfrp2) reduces fibrosis and improves cardiac function in a rat model of myocardial infarction. *Proc. Natl. Acad. Sci. USA* **107**, 21110-21115.
- Joesting, M. S., Cheever, T. R., Volzing, K. G., Yamaguchi, T. P., Wolf, V., Naf, D., Rubin, J. S. and Marker, P. C.** (2008). Secreted frizzled related protein 1 is a paracrine modulator of epithelial branching morphogenesis, proliferation, and secretory gene expression in the prostate. *Dev. Biol.* **317**, 161-173.
- Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, K., Magee, J. and Carroll, S. B.** (1996). Integration of positional signals and regulation of wing formation and identity by *Drosophila* vestigial gene. *Nature* **382**, 133-138.
- Kobayashi, K., Luo, M., Zhang, Y., Wilkes, D. C., Ge, G., Grieskamp, T., Yamada, C., Liu, T. C., Huang, G., Basson, C. T. et al.** (2009). Secreted Frizzled-related protein 2 is a procollagen C proteinase enhancer with a role in fibrosis associated with myocardial infarction. *Nat. Cell Biol.* **11**, 46-55.
- Lee, H. X., Ambrosio, A. L., Reversade, B. and De Robertis, E. M.** (2006). Embryonic dorsal-ventral signaling: secreted frizzled-related proteins as inhibitors of tollid proteinases. *Cell* **124**, 147-159.
- Leyns, L., Bouwmeester, T., Kim, S. H., Piccolo, S. and De Robertis, E. M.** (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell* **88**, 747-756.
- Liu, H., Mohamed, O., Dufort, D. and Wallace, V. A.** (2003). Characterization of Wnt signaling components and activation of the Wnt canonical pathway in the murine retina. *Dev. Dyn.* **227**, 323-334.
- Liu, H., Xu, S., Wang, Y., Mazerolle, C., Thurig, S., Coles, B. L., Ren, J. C., Taketo, M. M., van der Kooy, D. and Wallace, V. A.** (2007). Ciliary margin transdifferentiation from neural retina is controlled by canonical Wnt signaling. *Dev. Biol.* **308**, 54-67.
- Lopez-Rios, J., Esteve, P., Ruiz, J. M. and Bovolenta, P.** (2008). The Netrin-related domain of Sfrp1 interacts with Wnt ligands and antagonizes their activity in the anterior neural plate. *Neural Dev.* **3**, 19.
- Lories, R. J., Peeters, J., Bakker, A., Tylzanowski, P., Derese, I., Schrooten, J., Thomas, J. T. and Luyten, F. P.** (2007). Articular cartilage and biomechanical properties of the long bones in Frzb-knockout mice. *Arthritis Rheum.* **56**, 4095-4103.
- Matakatsu, H. and Blair, S. S.** (2004). Interactions between Fat and Dachsous and the regulation of planar cell polarity in the *Drosophila* wing. *Development* **131**, 3785-3794.
- Micchelli, C. A., Rulifson, E. J. and Blair, S. S.** (1997). The function and regulation of cut expression on the wing margin of *Drosophila*: Notch, Wingless and a dominant negative role for Delta and Serrate. *Development* **124**, 1485-1495.
- Mii, Y. and Taira, M.** (2009). Secreted Frizzled-related proteins enhance the diffusion of Wnt ligands and expand their signalling range. *Development* **136**, 4083-4088.
- Misra, K. and Matise, M. P.** (2010). A critical role for sFRP proteins in maintaining caudal neural tube closure in mice via inhibition of BMP signaling. *Dev. Biol.* **337**, 74-83.
- Motzny, C. K. and Holmgren, R.** (1995). The *Drosophila* cubitus interruptus protein and its role in the wingless and hedgehog signal transduction pathways. *Mech. Dev.* **52**, 137-150.
- Muller, F., Rohrer, H. and Vogel-Hopker, A.** (2007). Bone morphogenetic proteins specify the retinal pigment epithelium in the chick embryo. *Development* **134**, 3483-3493.
- Mullor, J. L., Calleja, M., Capdevila, J. and Guerrero, I.** (1997). Hedgehog activity, independent of decapentaplegic, participates in wing disc patterning. *Development* **124**, 1227-1237.
- Muraoka, O., Shimizu, T., Yabe, T., Nojima, H., Bae, Y. K., Hashimoto, H. and Hibi, M.** (2006). Sizzled controls dorso-ventral polarity by repressing cleavage of the Chordin protein. *Nat. Cell Biol.* **8**, 329-338.
- Neumann, C. J. and Cohen, S. M.** (1997). Long-range action of Wingless organizes the dorsal-ventral axis of the *Drosophila* wing. *Development* **124**, 871-880.
- Nolo, R., Abbott, L. A. and Bellen, H. J.** (2000). Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. *Cell* **102**, 349-362.
- Panganiban, G., Nagy, L. and Carroll, S. B.** (1994). The role of the Distal-less gene in the development and evolution of insect limbs. *Curr. Biol.* **4**, 671-675.
- Renstrom, J., Istvanffy, R., Gauthier, K., Shimono, A., Mages, J., Jardon-Alvarez, A., Kroger, M., Schiemann, M., Busch, D. H., Esposito, I. et al.** (2009). Secreted frizzled-related protein 1 extrinsically regulates cycling activity and maintenance of hematopoietic stem cells. *Cell Stem Cell* **5**, 157-167.
- Rodriguez, J., Esteve, P., Weinel, C., Ruiz, J. M., Fermin, Y., Trousse, F., Dwivedy, A., Holt, C. and Bovolenta, P.** (2005). SFRP1 regulates the growth of retinal ganglion cell axons through the Fz2 receptor. *Nat. Neurosci.* **8**, 1301-1309.
- Rooke, J., Pan, D., Xu, T. and Rubin, G. M.** (1996). KUZ, a conserved metalloprotease-disintegrin protein with two roles in *Drosophila* neurogenesis. *Science* **273**, 1227-1231.
- Satoh, W., Gotoh, T., Tsunematsu, Y., Aizawa, S. and Shimono, A.** (2006). Sfrp1 and Sfrp2 regulate anteroposterior axis elongation and somite segmentation during mouse embryogenesis. *Development* **133**, 989-999.
- Satoh, W., Matsuyama, M., Takemura, H., Aizawa, S. and Shimono, A.** (2008). Sfrp1, Sfrp2, and Sfrp5 regulate the Wnt/beta-catenin and the planar cell polarity pathways during early trunk formation in mouse. *Genesis* **46**, 92-103.
- Sokol, S.** (2000). A role for Wnts in morphogenesis and tissue polarity. *Nat. Cell Biol.* **2**, E124-E125.
- Strutt, D. and Strutt, H.** (2007). Differential activities of the core planar polarity proteins during *Drosophila* wing patterning. *Dev. Biol.* **302**, 181-194.
- Tanimoto, H., Itoh, S., ten Dijke, P. and Tabata, T.** (2000). Hedgehog creates a gradient of DPP activity in *Drosophila* wing imaginal discs. *Mol. Cell* **5**, 59-71.
- Torres, C., Gorfinkel, N. and Guerrero, I.** (2004). Patched controls the Hedgehog gradient by endocytosis in a dynamin-dependent manner, but this internalization does not play a major role in signal transduction. *Development* **131**, 2395-2408.
- Trevent, B., Gaur, T., Hussain, S., Symons, J., Komm, B. S., Bodine, P. V., Stein, G. S. and Lian, J. B.** (2008). Expression of secreted frizzled related protein 1, a Wnt antagonist, in brain, kidney, and skeleton is dispensable for normal embryonic development. *J. Cell. Physiol.* **217**, 113-126.
- Trimarchi, J. M., Cho, S. H. and Cepko, C. L.** (2009). Identification of genes expressed preferentially in the developing peripheral margin of the optic cup. *Dev. Dyn.* **238**, 2327-2329.
- Uren, A., Reichsman, F., Anest, V., Taylor, W. G., Muraiso, K., Bottaro, D. P., Cumberledge, S. and Rubin, J. S.** (2000). Secreted frizzled-related protein-1 binds directly to Wingless and is a biphasic modulator of Wnt signaling. *J. Biol. Chem.* **275**, 4374-4382.
- Veien, E. S., Rosenthal, J. S., Kruse-Bend, R. C., Chien, C. B. and Dorsky, R. I.** (2008). Canonical Wnt signaling is required for the maintenance of dorsal retinal identity. *Development* **135**, 4101-4111.
- Yokota, T., Oritani, K., Garrett, K. P., Kouro, T., Nishida, M., Takahashi, I., Ichii, M., Satoh, Y., Kincaid, P. W. and Kanakura, Y.** (2008). Soluble frizzled-related protein 1 is estrogen inducible in bone marrow stromal cells and suppresses the earliest events in lymphopoiesis. *J. Immunol.* **181**, 6061-6072.
- Zecca, M., Basler, K. and Struhl, G.** (1996). Direct and long-range action of a wingless morphogen gradient. *Cell* **87**, 833-844.
- Zhang, Y., Overbeek, P. A. and Govindarajan, V.** (2007). Perinatal ablation of the mouse lens causes multiple anterior chamber defects. *Mol. Vis.* **13**, 2289-2300.
- Zhao, S., Chen, Q., Hung, F. C. and Overbeek, P. A.** (2002). BMP signaling is required for development of the ciliary body. *Development* **129**, 4435-4442.