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Secreted Frizzled-related proteins enhance the diffusion of Wnt ligands and expand their signalling range

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Secreted Frizzled-related proteins (sFRPs) are thought to negatively modulate Wnt signalling. Although Wnt proteins are thought to diffuse extracellularly and act as morphogens, little is known about the diffusibility of either Wnts or sFRPs. Here we show that Frzb and Crescent (Cres), which are members of the sFRP family, have the ability to regulate the diffusibility and signalling areas of the Wnt ligands Wnt8 and Wnt11. We found, using the *Xenopus* embryo, that Wnts do not diffuse effectively, whereas Frzb and Cres spread very widely. Interestingly, Frzb and Cres substantially promoted the diffusion of Wnt8 and Wnt11 through extracellular interactions. Importantly, we show that Wnt8 conveyed by sFRPs can activate canonical Wnt signalling despite the function of sFRPs as Wnt inhibitors, suggesting a novel regulatory system for Wnts by sFRPs.

KEY WORDS: *Xenopus laevis*, Diffusion, Morphogen, sFRP, Frzb, sFRP3, Crescent, Wnt8, Wnt11

INTRODUCTION

Wnts are a family of secreted signalling proteins that are present in eumetazoans ranging from the sea anemone *Nematostella vectensis* to vertebrates, and play crucial and versatile roles in embryonic development, organ regeneration, stem cell proliferation, carcinogenesis and many other biological processes (Veeman et al., 2003; Logan and Nusse, 2004; Clevers, 2006). Frizzled-related proteins (sFRPs) are a family of secreted proteins that bind to Wnts extracellularly and are so-called Wnt inhibitors (Kawano and Kypta, 2003; Bovolenta et al., 2008). Although *Drosophila* does not have sFRP genes, at least one has been found in the *Nematostella* genome (Guder et al., 2006), suggesting that the regulatory interactions between sFRP and Wnt existed in the ancestral eumetazoan.

Wnt signalling can be divided into the canonical and non-canonical pathways. In the *Xenopus* embryo, Wnt8 mainly activates the canonical pathway and is expressed in the marginal zone, excluding the dorsal mesoderm, whereas Wnt11, which is expressed in the dorsal lip, mainly activates non-canonical pathways. It has been shown that the canonical pathway controls anteroposterior (AP) patterning in the gastrula (Kiecker and Niehrs, 2001), whereas non-canonical pathways control convergent extension movements (CEMs) (Heisenberg et al., 2000; Keller, 2002; Veeman et al., 2003), which cause elongation of the trunk region. In the anterior region, Frzb [also known as Frzb1 and sFRP3 (Kawano and Kypta, 2003; Bovolenta et al., 2008)] and Crescent (Cres) are thought to negatively regulate Wnt signalling to protect the head region from posteriorisation and CEMs by Wnts by opposite concentration gradients of sFRPs against Wnts (Leyns et al., 1997; Wang et al., 1997; Pera and De Robertis, 2000; Shibata et al., 2005). Therefore, it is believed that these sFRPs and Wnts diffuse extracellularly to create anterior-to-posterior and posterior-to-anterior morphogen gradients, respectively (Tabata and Takei, 2004; Zhu and Scott, 2004), and that they interact with each other in between. However, there are no direct data concerning the diffusibility of Wnts and sFRPs in the embryo.

In a previous study, we noticed that knockdown of Cres by antisense morpholino oligonucleotides causes not only a reduction of the head region, but also a shortening of the trunk region, implying a long-range action of Cres, whereas ectopically expressed *wnt11* or *wnt8* only affects the region where the mRNA has been injected (Shibata et al., 2005) (M. Itoh and M.T., unpublished), implying a short-range action of Wnt8 and Wnt11. Using *Xenopus* embryos, we addressed the questions of how sFRPs and Wnts behave in the extracellular space and what happens when sFRPs and Wnts meet.

MATERIALS AND METHODS

Plasmid constructs, microinjection, microscopy and immunostaining

A new vector, pCSf107mT, which contains SP6/T7 terminator sequences (see Fig. S1 in the supplementary material), was used to prepare plasmid constructs for mRNA synthesis. Four-cell stage *Xenopus laevis* embryos were microinjected with mRNAs for Venus (a derivative of yellow fluorescent protein) or Myc-tagged constructs and for monomeric red fluorescent protein (mRFP) at 0.8-1.0 ng RNA/embryo (Campbell et al., 2002) or nuclear β-galactosidase (nβ-gal) at 50 pg RNA/embryo as a tracer. Gastrula embryos (stages 10.25-11) were observed directly, or fixed and stained by standard protocols (Brivanlou and Harland, 1989; Sive et al., 2000; Shibata et al., 2005).

Confocal and fluorescence microscopy analyses were carried out using a LSM5 PASCAL imaging system (63× 1.2 NA and 20× 0.60 NA; Carl Zeiss). Fluorescence intensity was measured using Image J (NIH). Myc-tagged proteins were visualised with anti-Myc monoclonal antibody (9E10, BIOMOL) and Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) or anti-mouse IgG conjugated with alkaline phosphatase (Promega) and BM Purple (Roche Diagnostics) for the chromogenic reaction. Endogenous β-catenin was visualised with rabbit anti-β-catenin antibody (C2206, Sigma) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (Molecular Probes). SYTOX Blue (Molecular Probes) was used for nuclear staining. Embryos were bleached before (β-catenin) or after (Myc tag in chromogenic reaction) immunostaining.

For oocyte experiments, manually isolated oocytes were injected with mRNA and then cultured in O-R2 medium supplemented with 1% lamb serum at 19°C for 3 days (Sive et al., 2000). Incubation media were pooled and lysates were prepared from 10-13 oocytes. One oocyte-equivalent of the

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lysates and a half oocyte-equivalent of the media were analysed for expressed proteins by western blotting with IRDye800-labelled anti-GFP antibodies (Rockland) or anti-β-tubulin (TUB2.1, Sigma) and Alexa Fluor 680-conjugated goat anti-mouse IgG (Molecular Probes). Bands were visualised and quantitated using the Odyssey image analyzer (Li-Cor).

Luciferase reporter assays

TOP-FLASH reporter DNA (van de Wetering et al., 1997) (200 pg/blastomere) with or without mRNAs was injected into the animal pole region of a ventral blastomere at the four-cell stage. The injected embryos were cultured at 14°C and collected at the mid-gastrula stage (stage 11). Five to ten independent pools of three embryos were assayed for each experimental condition. A representative result from reproducible experiments is presented, as luciferase activity varied in different batches of embryos as observed previously (Yamamoto et al., 2003). The statistical significance (*P*-value) was calculated using Student's or Welch's *t*-test after comparison of the variances of a set of data by *F*-test.

RESULTS AND DISCUSSION

We first examined the diffusibility of Wnts and sFRPs using Venus-tagged proteins (Fig. 1A), which have the signal peptide of TGFβ1 followed by Venus. mRNAs for Venus-tagged proteins and mRFP as a cell lineage tracer were microinjected into the animal pole region of a ventral blastomere of four-cell stage *Xenopus* embryos, and injected embryos were observed at the early gastrula stage. Confocal microscopy confirmed that Venus-Frzb and Venus-Cres were present in the extracellular space, between mRFP-negative cells (Fig. 1B; for Venus-Frzb see Fig. S2A,B in the supplementary material). Similarly, Venus-Wnt8 and Venus-Wnt11 were detected in the extracellular space (indicated by arrows) with punctate signals (indicated by arrowheads in Fig. 1B; see Fig. S2C in the supplementary material). Thus, all the Venus-tagged constructs are properly secreted. In addition, these constructs were biologically active as assayed by mRNA injection into *Xenopus* embryos (data not shown).

We next observed the diffusibility of Venus-tagged proteins at lower magnification (Fig. 1C). Interestingly, Venus-Frzb and Venus-Cres were widely detected in the embryos, whereas Venus-Wnt8 and Venus-Wnt11 were not, or barely, detected at any distance from the source (merged pictures shown; see Fig. S3A in the supplementary material). Confocal analysis showed that Venus-Frzb and Venus-Cres were also observed in the extracellular space in a region distant from the source (see Fig. S2B in the supplementary material; data for Venus-Cres not shown). By contrast, Venus-Wnt8 and Venus-Wnt11 were only detected a short distance from the source cells (see Fig. S2C in the supplementary material; data for Venus-Wnt11 not shown). To compare distributions of mRFP and Venus-tagged proteins, fluorescence intensities (of the regions indicated by blue windows in Fig. 1C) were measured along the longitudinal direction and plotted after normalisation. In Fig. 1C (see graphs), the distributions of Venus-Frzb and Venus-Cres are clearly plotted above those of mRFP, indicating that these sFRPs had diffused away from the source cells. Notably, plots for Venus-Wnt8 and Venus-Wnt11 mostly overlapped with those of mRFP, indicating short-range distributions outside of the source cells. We conclude that Frzb and Cres diffuse much more efficiently than Wnt8 and Wnt11, a finding supported by the results of our previous study, which showed that Cres, but not Wnt11, can affect cell movements over a long distance (Shibata et al., 2005). The much lower diffusibility of Wnt8 and Wnt11 might be due to lipid modifications and strong interactions of Wnts with proteoglycans (Logan and Nusse, 2004).

The substantial difference in diffusibility between sFRPs and Wnts prompted us to examine whether the diffusion areas of Wnts change when coexpressed with sFRPs. Surprisingly, we found that Venus-Wnt8 coexpressed with Frzb or Cres, and Venus-Wnt11 coexpressed with Cres, spread widely throughout the entire embryo, as indicated by the distribution plots of Venus, which are above those of mRFP (Fig. 1D, first, third and fourth graphs from the left; for separate pictures, see Fig. S3B in the supplementary material). The wide distribution of Wnts by sFRPs was probably not due to protein stabilisation because the amount of Venus-Wnt8 was not affected by coexpression with Cres, as assayed by western blotting (see Fig. S3C in the supplementary material). These data suggest that Frzb and Cres can enhance the diffusion of Wnt8 and Wnt11, but not Frzb of Wnt11 (Fig. 1D, second graph, where the distribution plot of Venus is similar to that of mRFP). These specificities are in good agreement with our previous data showing that Frzb forms a complex less efficiently with Wnt11 than with Wnt8, whereas Cres efficiently forms a complex with Wnt8 or Wnt11 (Shibata et al., 2005). We conclude that complex formation between sFRPs and Wnts enhances Wnt diffusion.

To assess the secretion of signal peptide-Venus constructs from the cell and also to examine whether Cres promotes release of Wnts from the cell surface, we performed oocyte injection experiments. All Venus constructs, but not EGFP (negative control), were detected in the culture medium by western blotting (Fig. 1E), indicating that the signal peptide functions properly. Comparing the amounts of protein in the medium with those in the corresponding oocytes showed that only a small amount of Venus-Wnt8 was released from the oocytes, as compared with Venus-Cres. Interestingly, when oocytes expressing Venus-Wnt8 were co-cultured with those expressing Cres, the amount of Venus-Wnt8 released into the medium increased (Fig. 1E, right-most panel), suggesting that Cres can reduce the affinity of Venus-Wnt8 for the oocyte cell surface.

Because direct observation of Venus-tagged protein by fluorescence is not sensitive enough to detect low levels of proteins, we further examined diffusibility by immunostaining with C-terminally Myc-tagged Wnt8 and Wnt11, in which the original signal peptides were retained (Fig. 2A). In addition, six Myc tags (99 residues, including a linker sequence), which are smaller than Venus (239 residues), might exhibit fewer side effects, if any, on the behaviour of Wnts, and with immunostaining it is also possible to process a number of embryos at once. We also assessed whether Wnt8-Myc and Wnt11-Myc are biologically active (data not shown). Confocal analysis confirmed that Wnt-Myc proteins were secreted into the extracellular space (Fig. 2B).

We classified immunostained embryos into three groups according to the distribution of blue-stained Wnt-Myc proteins (Fig. 2C). When expressed alone, Wnt8-Myc and Wnt11-Myc exhibited low diffusibility (Fig. 2D, 1 and 2), consistent with the fluorescence observations (Fig. 1C). By contrast, when coexpressed with Frzb or Cres, the diffusion of Wnt8-Myc was greatly enhanced (Fig. 2D, 3 and 4). Similarly, the diffusion of Wnt11-Myc was also enhanced by Cres, but weakly by Frzb (Fig. 2D, 6 and 5, respectively). These data are consistent with the binding specificities of these sFRPs for Wnts (Shibata et al., 2005). Thus, using Myc-tagged constructs and immunostaining, we reached basically the same conclusions as with fluorescence: that sFRPs can enhance Wnt diffusion and that enhanced diffusion of Wnts by sFRPs is mediated by complex formation. To examine whether extracellular interactions with sFRPs mediate the enhanced diffusion of Wnts, mRNAs for Wnts and sFRPs were separately injected into different blastomeres (Fig. 2D, 7-10). Under these conditions, although less efficient than in the co-injection experiments, sFRPs were able to enhance the diffusion of Wnts. Furthermore, the efficiencies of enhanced Wnt diffusion by

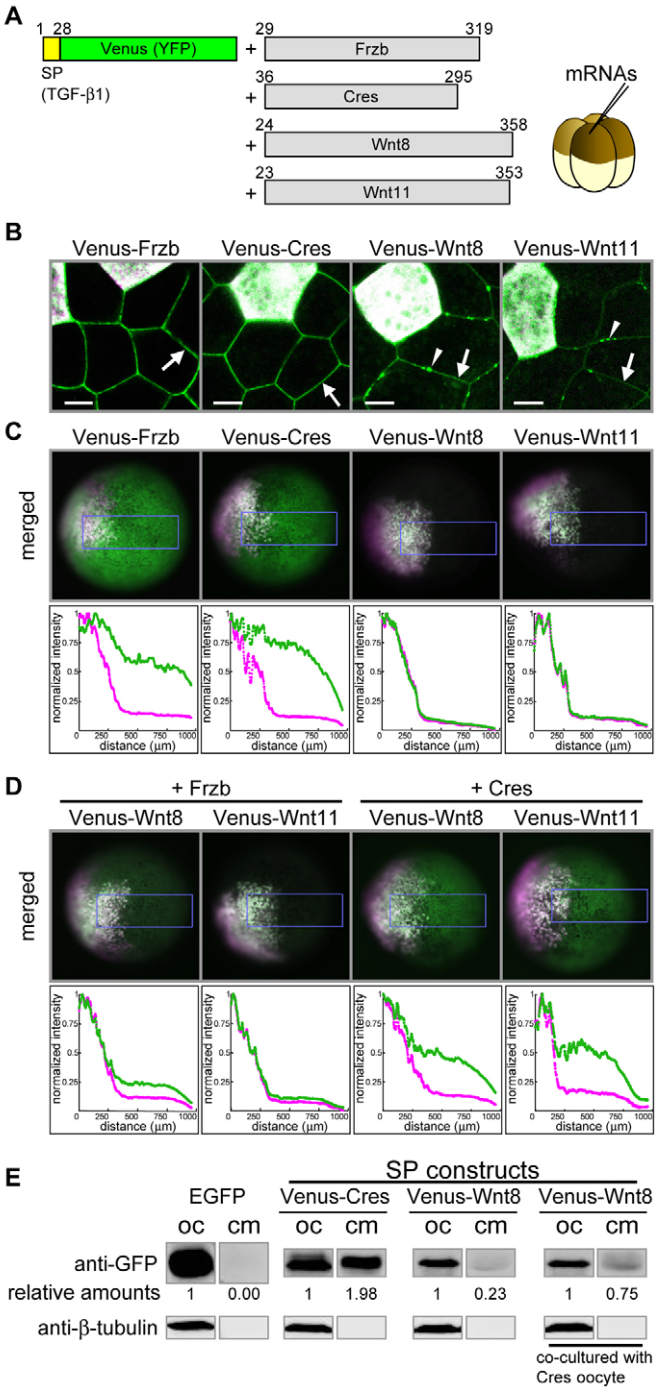

Fig. 1. Differential diffusibility of sFRPs and Wnts in *Xenopus* embryos. (**A**) The Venus-tagged Frzb, Cres, Wnt8 and Wnt11 proteins. The signal peptide (SP) of TGFβ1 followed by Venus was used throughout. Amino acid positions are numbered (accession numbers: TGFβ1, AAA35369; Frzb, AAC60114; Cres, NP_001082025; Wnt8, NP_001081637; Wnt11, NP_001084327). mRNAs for Venus-tagged protein (2.5 ng/embryo) and mRFP as a cell lineage tracer were co-injected into the animal pole region of a single ventral blastomere at the four-cell stage as shown on the right. Injected embryos were observed at the early gastrula stage (stages 10.25-10.5). (**B**) Confocal analysis. The Venus-tagged proteins (green) all localise in the extracellular space (arrows) beside the source cells expressing mRFP (magenta, or white when merged with green). Punctate signals were observed in the extracellular space for Venus-Wnt8 and Venus -Wnt11 (arrowheads; see Fig. S2 in the supplementary material). Scale bar: 10 μm. (**C**) Fluorescence observation of whole embryos. Upper row are merged pictures of mRFP (magenta) and Venus (green). The lower row are graphs of normalised fluorescence intensity of mRFP (magenta) or Venus (green) in regions indicated by the blue windows in the merged images. (**D**) Enhanced diffusion of Wnts by Frzb and Cres. *frzb* or *cres* mRNA (0.5 ng/embryo) was co-injected with mRNAs for Venus-Wnts and mRFP. Merged pictures are presented. Quantitation of fluorescent signals was as in C. (**E**) Oocyte injection analysis of SP-Venus constructs. Oocytes were injected with mRNA as indicated. Amounts of mRNAs (ng/oocyte) were: EGFP (non-secreted control), 19; SP-Venus-Cres, 35; SP-Venus-Wnt8, 40 (these three are equimolar); Cres, 40. oc, oocyte lysate; cm, culture medium.

sFRPs were again consistent with their binding specificities, suggesting that Frzb and Cres enhance diffusion of Wnts through extracellular complex formation.

Because sFRPs have long been thought to be Wnt inhibitors, although a small amount of human sFRP1 reportedly promotes Wnt signalling in cultured cells (Uren et al., 2000), the crucial question arises as to the role of this enhanced diffusion of Wnts by sFRPs. We speculated that Wnt ligands conveyed by sFRPs might retain some activity, because protein-protein interactions are generally reversible. We examined the effect of Cres on the signalling activity of Wnt8 in luciferase reporter assays using the TOP-FLASH construct, which responds to canonical Wnt signalling (van de Wetering et al., 1997). We performed two sets of experiments (Fig. 3A,B). In the first set, we examined the effect of Cres when reporter DNA and mRNAs for Wnt8 and Cres were all co-injected into the same blastomere (Fig. 3A). This approach revealed that the greater the amount of Cres, the greater the inhibition of luciferase activity (Fig. 3A, 4-6), demonstrating that Cres functions as an inhibitor of Wnt8, as expected, although a concentration-dependent biphasic effect was not observed, in contrast to the reported data (Uren et al., 2000). In the second set of experiments, the reporter DNA and *wnt8* mRNA were separately injected into different blastomeres, and *cres* mRNA was injected into the third blastomere (Fig. 3B), or co-injected with *wnt8* mRNA or the reporter DNA (see Fig. S4A,B in the supplementary material). Under these conditions, although not as strong as in the first set, Wnt8 was able to activate the reporter gene (compare 2 in Fig. 3A with 2 in Fig. 3B). This is consistent with the low diffusibility of Wnt8 shown above (see Fig. 1C, Fig. 2D). However, it is somewhat surprising and very important that activation of luciferase activity by Wnt8 was further enhanced by coexpression of lower amounts of Cres, but decreased with higher amounts of Cres (Fig. 3B; see Fig. S4A,B in the supplementary material). These data suggest that Wnt8, when conveyed by suitable amounts of Cres in the extracellular space, can activate the reporter gene.

To confirm that diffusion of Cres is actually required for the positive regulatory effect, we used a glycosylphosphatidylinositol-anchored construct (Cres-GPI), which should be anchored to the plasma membrane. This construct still inhibited Wnt signalling when co-injected into one blastomere (Fig. 3C, 1-3), but no longer enhanced Wnt signalling when expressed in a different blastomere (Fig. 3C, 4-9). These results indicated that diffusion is required for Cres to enhance reporter activation by Wnt8.

We next examined the effects of Wnt8 and sFRPs on the expression of an endogenous anterior marker, *otx2*, which is expressed in the anterior neuroectoderm of the gastrula. DNA constructs for Wnt8 and

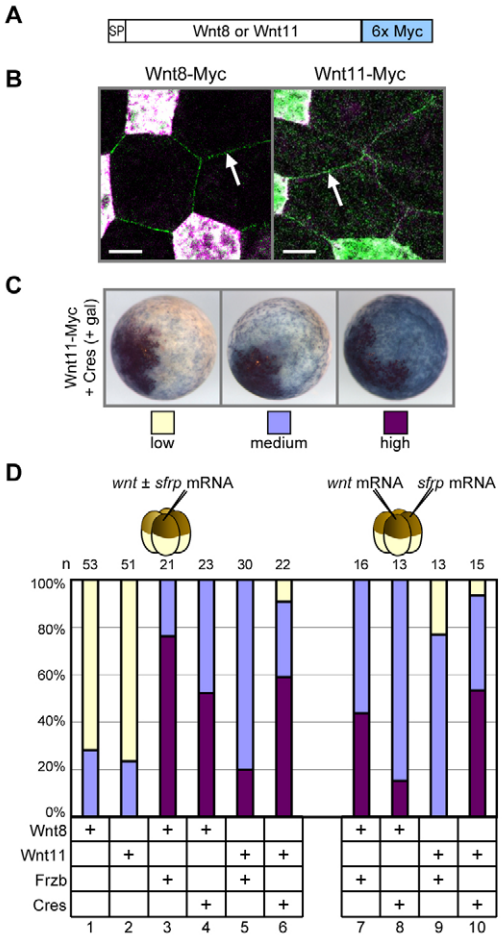

Fig. 2. sFRPs enhance the diffusion of Wnts. (**A**) *Xenopus* Wnt-Myc proteins. Full-length Wnt8 or Wnt11, including the original signal peptide (SP), was fused to 6× Myc tags at the C-terminus. (**B**) Confocal analysis. Wnt-Myc proteins were detected in the extracellular space (arrows) beside the source cells expressing mRFP (magenta, or white when merged with green). Scale bar: 10 μm. (**C**) Diffusion of Wnt-Myc proteins visualised by immunostaining. Blue, Wnt11-Myc protein; red, source cells expressing nβ-gal as a tracer. Distribution levels of Wnt-Myc proteins were categorised into 'low', 'medium' and 'high'. Representative examples are shown of the distribution of Wnt11-Myc coexpressed with Cres. (**D**) Summary of Wnt-Myc diffusion results. Combinations of injected mRNAs (marked +) are indicated at the bottom. Left (samples 1-6): mRNAs for Wnt-Myc, sFRP (Frzb or Cres), and nβ-gal were co-injected into the same blastomere. Right (samples 7-10): *sfrp* mRNA was injected into a blastomere next to the one that had been injected with mRNAs for Wnt-Myc and nβ-gal. Amounts of injected mRNAs (pg/embryo) were: Wnt8-Myc and Wnt11-Myc, 250 (B) or 100 (D); Frzb and Cres, 400 (D, 1-6) or 2,500 (D, 7-10).

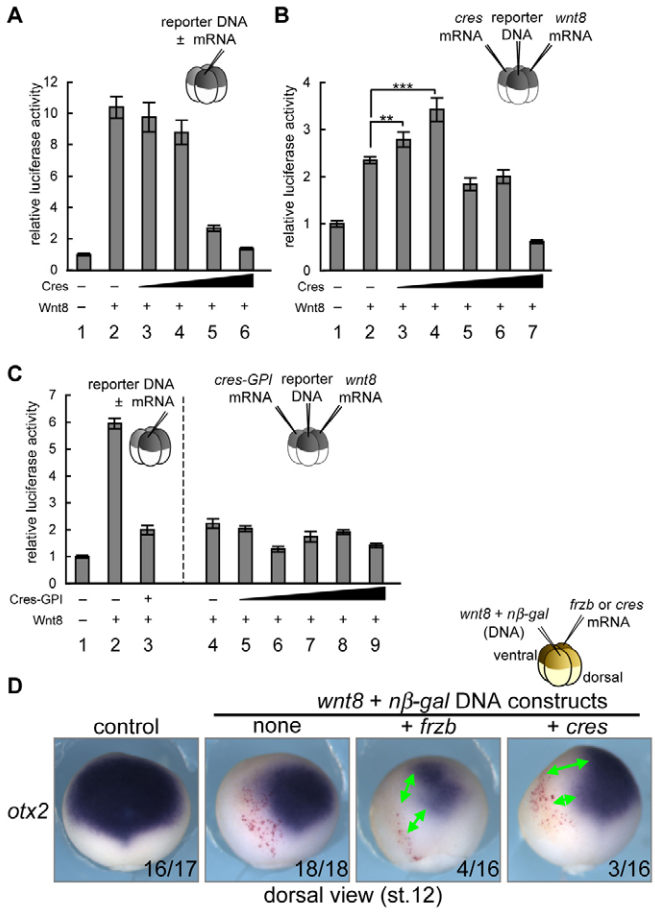

Fig. 3. Cres expands the signalling area of Wnt8 through extracellular interactions. (**A-C**) TOP-FLASH reporter analysis. The TOP-FLASH reporter was co-injected with mRNAs for Wnt8 and Cres into one blastomere (A), or separately injected into different blastomeres (B) of four-cell stage *Xenopus* embryos as indicated. GPI-anchored Cres was used as an immobilised form (C). Luciferase (luc) activity was assayed at the gastrula stage. Combinations of injected mRNAs are indicated (by +). Amounts of injected mRNAs (pg/embryo) were: Wnt8, 25 in A,C; Wnt8-Myc, 33 in B; *cres* mRNA, 0.33, 1.0, 3.3 and 10 in A and 0.10, 0.33, 1.0, 3.3 and 10 in B,C). $^{**}P<0.01$, $^{***}P<0.001$ (*t*-test); error bars, s.e.m.; $n=10$ samples, except 4 ($n=9$) in C. (**D**) Expansion of Wnt8 signalling as assayed by *otx2* expression. Wnt8 was expressed by injecting a DNA expression construct (pCS2+Xwnt8, 25 pg/embryo) together with nβ-gal (pCS2+nβ-gal, 100 pg/embryo; red cells). *frzb* or *cres* mRNA (1.0 pg/embryo) was injected into the blastomere diagonal to that expressing Wnt8. *otx2* expression was visualised by whole-mount in situ hybridisation. The green double-headed arrows indicate the limits of the regions of *otx2* inhibition.

the cell lineage tracer nβ-gal were co-injected into the future neural plate region. As expected, *otx2* expression was inhibited in the Wnt8-expressing area, but not distant from it (Fig. 3D, second panel). By contrast, when *frzb* or *cres* mRNA was injected at low dose into a blastomere diagonal to that expressing Wnt8, the region downregulating *otx2* expression expanded (Fig. 3D, third and fourth panels). Without exogenous Wnt8, this low dose of *frzb* or *cres* did not obviously affect *otx2* expression (data not shown). Thus, these data again suggest that sFRPs can expand the signalling area of Wnt8 as assayed by *otx2* expression.

To examine whether Frzb and Cres function as Wnt conveyers in vivo, we performed a loss-of-function analysis by injecting the antisense morpholino oligonucleotides *cres*MO (Shibata et al., 2005) and *frzb*MO, which was designed for the translation initiation site of *frzb* mRNA (for the specificity of *frzb*MO, see Fig. S5 in the supplementary material). In the early *Xenopus* gastrula (stage 10.25), *wnt8*, which is thought to be the predominant canonical Wnt ligand, is expressed in the marginal zone excluding the dorsal lip, where *frzb* and *cres* mRNAs are expressed. Because Frzb and Cres are highly diffusible, they

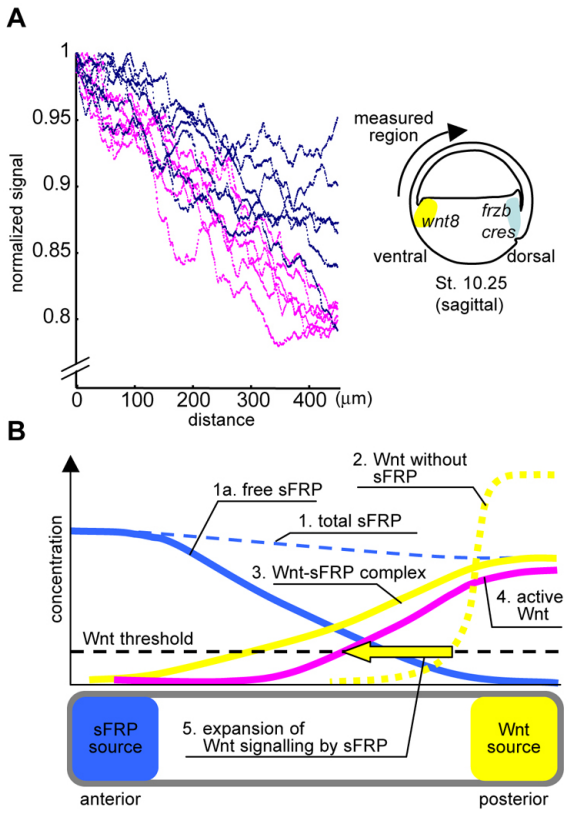

Fig. 4. Formation of an active gradient of Wnt by sFRP in vivo. (**A**) Knockdown of both Frzb and Cres by MOs alters the distribution of nuclear β-catenin. Plots show normalised signals of nuclear β-catenin visualised by immunostaining on the outside of gastrula embryos (stage 10.25), from the marginal zone to the animal pole region (arrow). The diagram illustrates the expression patterns of *wnt8*, *frzb* and *cres* on a sagittal section of a gastrula embryo (for details, see Fig. S6 in the supplementary material). Plots in blue, control embryos; magenta, embryos co-injected with *frzb*MO and *cres*MO. Note that the declining curves of nuclear β-catenin staining are steeper in the morphants than in the controls. (**B**) A model for AP patterning in the *Xenopus* embryo. Line 1 (dashed, blue), total sFRP secreted from the head organiser region; line 1a (blue), free sFRP; line 2 (dotted, yellow), Wnt in the absence of sFRP; line 3 (yellow), a Wnt-sFRP complex; line 4 (magenta), an active gradient of Wnt; line 5 (thick yellow arrow), expansion of Wnt signalling by sFRP. See text for details.

could affect Wnt activity in the ventral region of the embryo, and because Frzb and Cres MOs affected gastrulation movements, the ventral side is more appropriate than the dorsal side for comparing active gradients of Wnt. We examined the distribution of nuclear β-catenin, which reflects the canonical Wnt pathway. In control embryos, gradients of nuclear β-catenin were observed from the marginal zone to the animal pole region. Remarkably, when both Cres and Frzb were depleted, the gradients of nuclear β-catenin became steeper than those in the controls (Fig. 4A). These in vivo data further support our hypothesis that sFRPs act as conveyers to expand the Wnt signalling area.

Based on the present data, we propose a working model for the formation of the active gradient of Wnts in AP patterning (Fig. 4B). sFRPs expressed in the anterior region diffuse towards the posterior to generate concentration gradients (Fig. 4B, line 1), whereas Wnts in the posterior tend to remain there in the absence of sFRPs (line 2). However, when Wnts in the posterior region interact extracellularly with sFRPs travelling from the anterior region, the resultant Wnt-sFRP complex diffuses in a posterior-to-anterior direction, thereby creating its posterior-to-anterior gradient (line 3). This is not merely an inhibitory complex of Wnt but can transduce Wnt signalling, as suggested by our data (Fig. 3B,D, Fig. 4A; see Fig. S4 in the supplementary material). As a result, the opposed gradients of Wnt-sFRP complexes and free sFRP (Fig. 4B, line 3 versus line 1a) create an 'active gradient' of Wnt (line 4). This sFRP-mediated active gradient defines a signalling area of Wnt, where a level of 'active Wnt' is above the threshold (dashed black line), thereby expanding Wnt signalling by sFRP (yellow arrow, line 5).

Among known sFRPs, human sFRP1 reportedly has a concentration-dependent biphasic activity in a cell culture system (Uren et al., 2000). As suggested in our oocyte injection experiments (Fig. 1E), Wnt is likely to become more soluble when complexed with sFRP. Therefore, a small amount of sFRP1 might prevent Wnt from becoming trapped in the extracellular matrix, or in the dish substrate in a culture system, and show a biphasic effect. Nevertheless, Cres does not show such a biphasic effect in the embryo (Fig. 3A), but has the ability to expand the signalling area of Wnt8 (Fig. 3B,D). Therefore, although sFRPs might have some positive effects on Wnt receptors, as postulated (Shibata et al., 2005; Bovolenta et al., 2008), our data strongly support the idea that sFRPs effectively expand the signalling area of Wnt signalling.

Our suggested role for Wnt-sFRP interactions is reminiscent of that of BMP-Chordin (Chd) (or Dpp-Sog in *Drosophila*) interactions in determining signalling range, in which diffusibility of BMP ligands is promoted by binding to Chd, a well-known BMP inhibitor (Eldar et al., 2002; De Robertis and Kuroda, 2004; Ben-Zvi et al., 2008). In the BMP-Chd complex, Chd is cleaved by Tolloid to reactivate BMP ligands, suggesting by analogy that sFRPs might be processed to release Wnts from their complex. However, this is unlikely because we have not detected processed forms of Cres or Frzb in the presence of Wnts, as assayed by western blotting (data not shown). Instead, we propose that, as reversible interactions occur between Wnts and sFRPs (Wawrzak et al., 2007), Wnts can be released from the complex to bind to Frizzled receptor, which exists locally, to transduce signalling.

In *Drosophila*, Frizzled receptors and glypicans reportedly regulate the Wingless gradient on the surface of segmented body walls and imaginal discs (Cadigan et al., 1998; Lecourtois et al., 2001; Han et al., 2004), and Wnt ligands need to be retained on the surface, otherwise they would diffuse out of those tissues (Lecourtois et al., 2001). This situation differs from that in the *Xenopus* embryo, in which secreted Wnt and sFRP proteins are observed in the intercellular spaces sealed from the outside by the tight junctions (see Fig. S2A, arrow in inset). Although Frizzled and proteoglycans could affect the diffusion of Wnts in *Xenopus*, sFRPs are likely to release Wnts from the plasma membrane, as suggested by oocyte experiments (Fig. 1E), to diffuse into the intercellular space inside the embryo. It should be noted that *Drosophila* has reportedly lost sFRP genes (Guder et al., 2006; Bovolenta et al., 2008), probably by adopting other regulatory mechanisms of Wnt diffusion, such as transcytosis (Entchev et al., 2000) and via argosomes (Greco et al., 2001) and lipoprotein particles (Panakova et al., 2005). Although these mechanisms might exist in other eumetazoans, it is possible that eumetazoans possessing both sFRP and Wnt genes use sFRPs for the regulation of Wnt ligand diffusion and activity. Neighbouring expression domains of Wnts and sFRPs

have been observed in many developmental processes in vertebrates and perhaps in other organisms as well. This raises the possibility that sFRPs might be a conveyer of Wnts to the tissue where the Wnt ligand functions.

In summary, our results suggest that sFRPs can act as extracellular transporters of Wnt ligands. This provides new insight into the mechanisms underlying AP patterning and CEMs in vertebrate embryos, and, more generally, into the regulation of the range of Wnt signalling in diverse developmental processes.

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Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/24/4083/DC1

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