Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease

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Summary

The secreted Frizzled-related proteins (SFRPs) are a family of soluble proteins that are structurally related to Frizzled (Fz) proteins, the serpentine receptors that mediate the extensively used cell-cell communication pathway involving Wnt signalling. Because of their homology with the Wnt-binding domain on the Fz receptors, SFRPs were immediately characterised as antagonists that bind to Wnt proteins to prevent signal activation. Since these initial studies, interest in the family of SFRPs has grown progressively, offering new perspectives on their function and mechanism of action in both development and disease. These studies indicate that SFRPs are not merely Wnt-binding proteins, but can also antagonise one another's activity, bind to Fz receptors and influence axon guidance, interfere with BMP signalling by acting as proteinase inhibitors, and interact with other receptors or matrix molecules. Furthermore, their expression is altered in different types of cancers, bone pathologies, retinal degeneration and hypophosphatemic diseases, indicating that their activity is fundamental for tissue homeostasis. Here we review some of the debated aspects of SFRP-Wnt interactions and discuss the new and emerging roles of SFRPs.

Key words: Bmp, Signalling antagonists, Cancer, Diseases

Introduction

The specification, differentiation and coordinated behaviour of tissues in multicellular organisms all require tight control of cell-to-cell communication. Intensive research in recent decades has determined that many such processes in vertebrates and invertebrates are orchestrated by secreted signalling molecules that belong to a small number of gene families. Among these, Hedgehog (Hh), Wingless (Wnt), transforming growth factor β (TGFβ), bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) protein families are of particular importance because of their pleiotropic activities during both development and maintenance of most tissues (Bovolenta and Marti, 2005). Members of these families commonly bind to specific cell-surface receptors and activate complex intracellular cascades that ultimately regulate gene transcription and modify cell behaviour according to the specific needs of the target cells. Because cells must change progressively, especially during embryonic development, it is crucial for the cell to receive the appropriate inputs at the correct time. To ensure that these inputs are adequately controlled, cell signalling pathways are tightly regulated at different levels. The Wnt signalling pathway provides a good example of these multiple controls.

The Wnt proteins are a large family of palmitoylated secreted glycoproteins that activate at least three different signalling pathways: the canonical or Wnt–β-catenin, the planar cell polarity (PCP; also known as non-canonical) and the Wnt-Ca^{2+} pathways (Bovolenta et al., 2006). Wnt activity is regulated at the cell surface by different transmembrane proteins and the secretion of Wnt proteins from the cell is promoted by the recently discovered protein Wntless (Wls) (Banziger et al., 2006; Bartscherer et al., 2006). Once secreted, Wnt proteins interact with glycosaminoglycans in the extracellular matrix, and these modulate their distribution, diffusion and signal transduction (Logan and Nusse, 2004). Frizzled (Fz) receptors on the receiving cells – seven-pass transmembrane proteins with the characteristics of G-protein-coupled receptors – are the main proteins responsible for binding to Wnt proteins on the plasma membrane. However, additional molecules are implicated in the activation of the signalling pathway. These comprise low-density lipoprotein-receptor-related proteins [LRP5 and LRP6 in vertebrates and Arrow (Arr) in *Drosophila*] that function as Fz co-receptors; the tyrosine kinase receptor Ror2, which contains a cysteine-rich domain similar to that of Fz; and the atypical tyrosine kinase receptor Derailed (Drl) and the related to tyrosine kinase protein (Ryk), which can interact with Fz (Bovolenta et al., 2006). Activation of Wnt signalling is further controlled by different antagonists, including Wnt inhibitory factor 1 (WIF1), Cerberus, Sclerostin, and members of the Dickkopf and secreted Frizzled-related protein (SFRP) families. Sclerostin and Dickkopf proteins interfere with Wnt activity and antagonise canonical signalling by binding to LRP5 or LRP6, whereas WIF1, Cerberus and SFRPs can interact directly with Wnt proteins (Kawano and Kypta, 2003; Li et al., 2005). Notably, WIF1 is characterised by the so called WIF domain, which is present also in the extracellular domain of Ryk and seems to be responsible for Wnt binding (Hsieh et al., 1999).

The SFRPs are the largest family of Wnt inhibitors. The founding member Frzb was initially identified through its sequence similarity with the Fz receptors (Hoang et al., 1996; Leyns et al., 1997), and immediately associated with Wnt signalling because of its ability to bind to Wnt8 and block its signalling in *Xenopus*, strongly supporting its role as a Wnt antagonist (Leyns et al., 1997; Wang et al., 1997). Concurrently, additional members of the family

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were isolated either through sequence homology with Fz receptors (Rattner et al., 1997) or, independently of Wnt activity, through their involvement in apoptosis (Melkonyan et al., 1997), or their co-purification with the heparin-binding factor hepatocyte growth factor/scattered factor (Finch et al., 1997).

Since their discovery, interest in this family of molecules has grown progressively, particularly because recent observations have offered a new perspective on their functions and mechanisms of action in both development and disease. These studies indicate that SFRPs are not merely Wnt-binding proteins but can also antagonise one another's activity (Yoshino et al., 2001), bind to Fz receptors (Bafico et al., 1999; Rodriguez et al., 2005) and provide axon-guidance information (Rodriguez et al., 2005). Moreover, they can interact with other receptors or matrix molecules (Chuman et al., 2004; Hausler et al., 2004; Lee, J. et al., 2004) and interfere with BMP signalling (Lee, H. et al., 2006; Muraoka et al., 2006; Yabe et al., 2003) by acting as proteinase inhibitors (Lee, H. et al., 2006). Furthermore, their expression is altered in different types of cancers (Rubin et al., 2006), in bone pathologies (Bodine and Komm, 2006), retinal degeneration (Jones et al., 2000) and hypophosphatemic diseases (Berndt and Kumar, 2007), which indicates that their activity is fundamental for tissue homeostasis. Reviews that centre on Wnt antagonism by SFRPs have recently been published elsewhere (Cadigan and Liu, 2006; Jones and Jomary, 2002; Kawano and Kypta, 2003); here, we discuss new aspects of SFRP activity, and review SFRP structure, expression and interactions with Wnt proteins.

The family of SFRPs

The SFRP family comprises five members in humans, SFRP1 to SFRP5, in which SFRP3 is the orthologue of the founding member Frzb. Sequence comparison and phylogenetic analysis show that SFRP1, SFRP2 and SFRP5 are closely related, and cluster together in a subgroup that diverges from the one formed by the related SFRP3 and SFRP4 (Fig. 1). This clustering also reflects a different genomic organisation. SFRP1, SFRP2 and SFRP5 are encoded by three exons on chromosome 8p12-p11.1, 4q31.3 and 10q24.1, respectively (Garcia-Hoyos et al., 2004), whereas SFRP3 and SFRP4 are both encoded by six exons – on chromosome 2q31-q33 and 7p14-p13, respectively. Orthologues of the five human genes have been found in all vertebrate species analysed so far (Fig. 1). Notably, a third subgroup, apparently not present in mammals, has been identified in *Xenopus*, zebrafish and chick. The components of this subgroup, named Sizzled, Crescent and Tlc, share sequence similarities with the SFRP1-SFRP2-SFRP5 subgroup (Fig. 1), and are characterised by a very restricted and anterior expression in gastrulating embryos (Bradley et al., 2000; Collavin and Kirschner, 2003; Chapman et al., 2004; Houart et al., 2002; Pfeffer et al., 1997; Salic et al., 1997; Yabe et al., 2003).

To date, SFRP homologues have not been identified in the *Drosophila* genome but family members that contain both a cysteine-rich domain (CRD) and a netrin (NTR) domain (see below) have been found in other invertebrates (Fig. 1), including the purple sea urchin (Lapraz et al., 2006), the nematode *Caenorhabditis elegans* (AC084197), the sea squirt *Ciona intestinalis* (Hino et al., 2003) and even in the sponge *Lubomirskia baicalensis* (Adell et al., 2007), which highlights the ancient origin of SFRPs. In addition, a protein related to SFRPs is expressed in sea urchin in a developmentally regulated pattern. This protein contains a putative signal sequence, four CRDs and a single Ig domain but lacks the NTR domain (Illies et al., 2002).

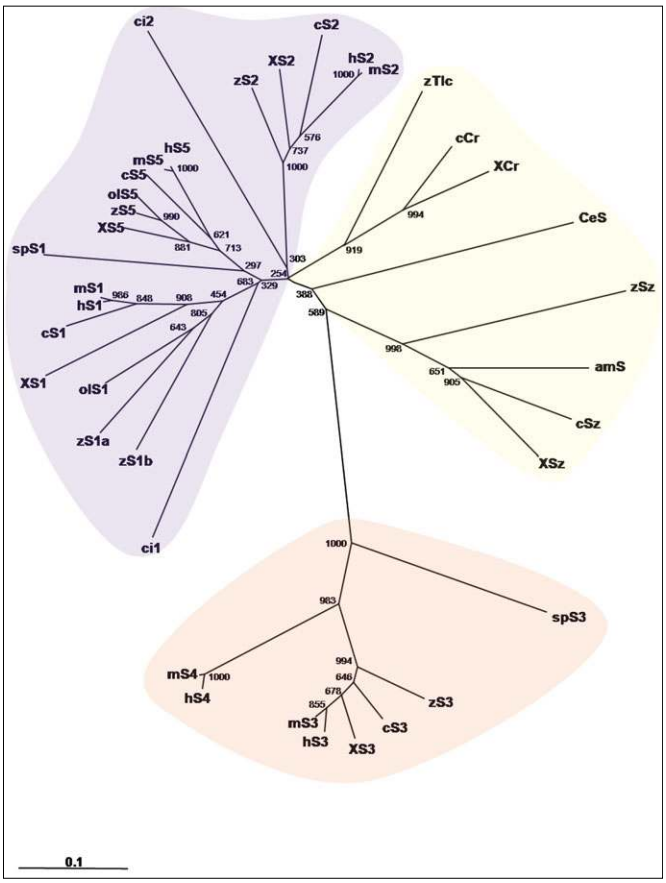

Fig. 1. Phylogenetic analysis of the SFRP family obtained by comparison of the CRD amino-acid sequences. Members displaying the most similarities in amino acid sequences cluster together and the branch length is proportional to divergence (percentage of nucleotide changes). Numbers indicate the bootstrap confidence for each node (n=1000). Different subfamilies are coloured differently. am, *Ambystoma mexicanum* (axolotl); c, *Gallus gallus* (chick); Ce, *Caenorhabditis elegans* (nematode); ci, *Ciona intestinalis* (sea squirt); Cr, Crescent; h, *Homo sapiens* (human); m, *Mus musculus* (mouse); ol, *Oryzias latipes* (medaka fish); S, Sfrp; sp, *Strongylocentrotus purpuratus* (sea urchin); Sz, Sizzled; X, *Xenopus laevis* (African clawed frog); z, *Danio rerio* (zebrafish).

Whereas the distribution of Sizzled, Crescent and Tlc is temporally and spatially very restricted, other SFRPs in different vertebrate embryos are expressed widely throughout development. *Sfrp1* (Fig. 2A,B) as well as *Sfrp2* and *Sfrp3* are expressed in the anterior neural plate from early developmental stages. Their expression pattern changes considerably over time, because they are subsequently found – among others – in the posterior neural tube, somites, limbs and kidney, (Fig. 2C,D) (Chapman et al., 2004; Esteve et al., 2004; Esteve et al., 2000; Leimeister et al., 1998; Leyns et al., 1997; Tendeng and Houart, 2006). In some cases, individual SFRPs are expressed in a partially overlapping manner that might be complementary to the expression of certain Wnt proteins, suggesting that in some cases there is some functional redundancy in their activity as Wnt inhibitors. Indeed, there is severe shortening of the thoracic region and incomplete somite segmentation in *Sfrp1-Sfrp2* double-mutant embryos, but not in either single mutant (Satoh et al., 2006). In other cases, expression of SFRPs appears to be independent of Wnt-protein distribution: in the mouse telencephalon, *Sfrp1* and *Sfrp3* are localised in

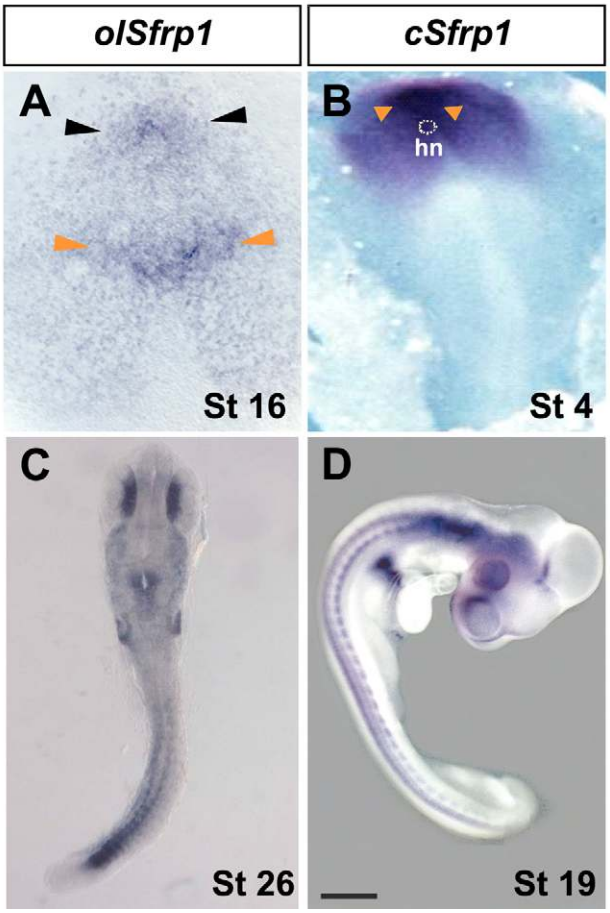

Fig. 2. Expression of *Sfrp1* in the anterior part of the embryo of medaka fish *Oryzias latipes* (olSfrp1) and chick (cSfrp1). (A-D) Panels show dorsal views of medaka fish (A,C) and chick (B,D) embryos hybridised in toto with digoxigenin-labelled species-specific probes against Sfrp. Gene transcripts accumulate in the most-anterior neural plate (black arrowheads in A) at mid-gastrula stages in both species. In both species expression is particularly abundant in the prospective eye field (orange arrowheads in A and B). In fish, expression is also observed in the future midbrain-hindbrain boundary (black arrowheads in A). During organogenesis (C,D) *Sfrp1* expression is observed in the eye, otic vesicles, neural tube, somites and limb buds. hn, Hensen's node; St, embryonic stage, numbers indicate days. Scale bar in D: 50 μm for A, 125 μm for B, 100 μm for C, 500 μm for D.

opposing gradients (Kim et al., 2001), and in the chick and mouse retina *Sfrp1* and *Sfrp2* are distributed throughout the neural retina (Esteve et al., 2003; Liu et al., 2003), whereas *Sfrp5* localises to the retinal pigmented epithelium (Chang et al., 1999).

Although the regulation of SFRP expression has not been systematically analysed, there is evidence that in embryonic fibroblasts *Sfrp1* is a direct target of the Sonic hedgehog (Shh) pathway. The Shh effector glioblastoma 1(Gli1) binds to a conserved consensus site in the *Sfrp1* promoter and activates its transcription (He et al., 2006). By contrast, Shh-mediated repression of *Sfrp1* and *Sfrp2* has been reported in mesenchymal cells (Ingram et al., 2002), whereas the transcription factor Myc seems to bind and strongly repress the *SFRP1* promoter in human mammary epithelial cells (Cowling et al., 2007). There is also good evidence that *Sfrp1* and *Sfrp2* expression in the stomach mesenchyme is directly regulated by the conserved homeobox protein BarH-like homeobox gene 1 (Barx1), which is required to promote differentiation of the abutting stomach epithelium (Kim et al., 2005). Furthermore, LIM homeobox protein 5 (Lhx5), a LIM homeodomain transcription factor, directly activates the expression of *Sfrp1a* and *Sfrp5*, which, in turn, promote development of zebrafish forebrain (Peng and Westerfield, 2006). Likewise, the expression of *C. intestinalis Sfrp1* and/or *Sfrp5* under the direct control of Ci-FoxA-a, a FoxA/HNF3 orthologue, may protect the ascidian anterior ectoderm from posteriorising signals that are known to caudalise the neurectoderm (Lamy et al., 2006). In addition, signalling through Wnt and BMP as well as the transcription factor Pax6 are thought to modify the levels of *Sfrp1* and *Sfrp2* transcripts in different tissues (Kim et al., 2001; Wawersik et al., 1999), although it remains unclear whether this regulation is direct.

Structural features of SFRPs

SFRPs are modular proteins that fold into two independent domains (Chong et al., 2002). The N-terminus contains a secretion signal peptide followed by a CRD. The CRD is characterised by the presence of ten cysteine residues at conserved positions, which form a pattern of disulphide bridges (Chong et al., 2002) identical to that reported for the extracellular CRD domains of Fz and Ror1 (Roszmusz et al., 2001). Members of the Sizzled-Crescent subgroup contain an additional cysteine residue that might form an interdomain disulphide bridge (Chong et al., 2002).

The C-terminal part of SFRP proteins is characterised by segments of positively charged residues that appear to confer heparin-binding properties (Uren et al., 2000), and by six cysteine residues that form three disulphide bridges. These features, which define the NTR module, have been identified in several other proteins, including netrin 1 (a well-characterised axon-guidance molecule), tissue inhibitors of metalloproteinases, type 1 procollagen C-proteinase enhancer protein (PCOLCE), and the complement component proteins C3, C4 and C5 (Banyai and Patthy, 1999). The NTRs of SFRP1, SFRP2 and SFRP5 share a similar pattern of cysteine spacing that is related to that of netrin 1, whereas those of the SFRP3-SFRP4 subgroup display a different cysteine-spacing pattern and, thus, a distinct pattern of disulphide bonds (Chong et al., 2002).

Post-translational modifications appear to confer additional differences that might further diversify the functions of different SFRP family members. For example, SFRP1 is N-glycosylated [shifting by approximately 2.8 kDa (Chong et al., 2002)], and sulphated at two tyrosine residues that are highly conserved in SFRP5 but absent from SFRP2, SFRP3 and SFRP4. This tyrosine sulphation is inhibited by heparin and appears to partially destabilise the protein (Zhong et al., 2007), suggesting that heparan sulphate influences protein stability in vivo.

Binding sites and specificity of Wnt-SFRP interactions: two unresolved issues

Following the discovery of SFRPs, biochemical studies established that Wnt proteins and SFRPs interact physically, with the SFRP-CRD postulated to be the binding domain because of its homology with the proposed Wnt-binding region on Fz receptors (Leyns et al., 1997; Lin et al., 1997; Wang et al., 1997). This interaction was proposed to impede Wnt binding to the Fz receptor and, therefore, prevent signal transduction (Fig. 3A). Indeed, complete removal of the SFRP3 CRD abolishes Wnt1-SFRP3 binding and ablates inhibition of Wnt1-mediated axis duplication in *Xenopus* embryos. By contrast, removal of the SFRP3 NTR only attenuates the

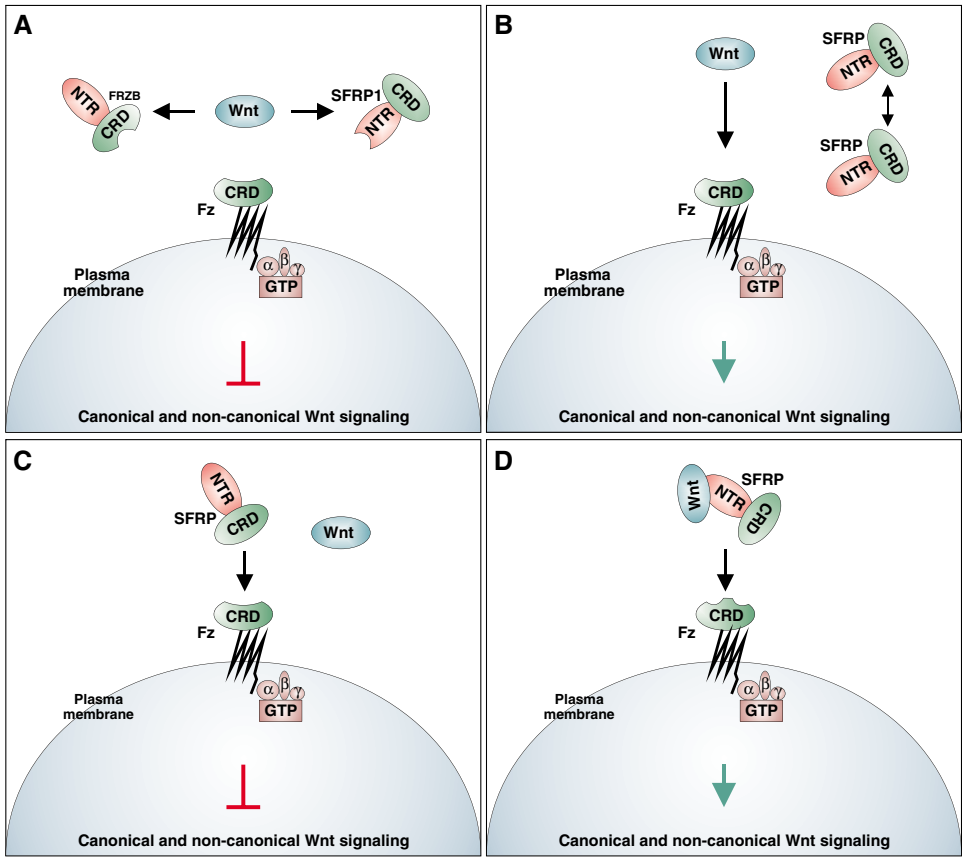

Fig. 3. Possible mechanisms by which SFRPs could modulate Wnt signalling. (A) SFRPs could sequester Wnt either through the CRD or NTR domain, thereby acting as classical antagonists. (B) They could titrate one another's activity, thereby favouring Wnt signalling. (C) SFRPs could act in a dominant-negative fashion by forming signalling-inactive complexes with Fz receptors, thereby preventing signal transduction by Wnt. (D) SFRPs might favour a Wnt-Fz interaction by simultaneously binding to both molecules and promoting signal transduction.

inhibition of axis duplication (Lin et al., 1997). Evidence for an interaction between the SFRP-CRD and Wnt was strengthened when complexes of SFRP1 and Wnt1 or Wnt2 were detected (Bafico et al., 1999). However, mutants of the human SFRP1 protein lacking the CRD retained the ability to bind to Wingless (Wg), the *Drosophila* Wnt homologue, whereas deletion of the NTR resulted in a reduction or loss of Wg binding (Uren et al., 2000). These apparently contradictory results might imply that multiple Wnt-binding sites exist on SFRP molecules, and/or that SFRP-Wnt pairs associate with differential affinities according to the different conformational and post-translational modifications of the SFRP described above. Indeed, as detailed below, there are experimental data supporting both possibilities.

A recent study of SFRP1 structure and function indicates that both domains of the protein are necessary for optimal Wnt inhibition. A conserved tyrosine residue within the CRD plays a crucial role in this process, together with the last 19 amino acid residues of the NTR, the deletion or replacement of which clearly interfere with SFRP1 function (Bhat et al., 2007). Furthermore, plasmon-resonance binding studies using SFRP1 to SFRP4, and Wnt3a and Wnt5, show that Wnt5 binds exclusively to SFRP1 and SFRP2, whereas Wnt3a binds to at least two sites in SFRP1, SFRP2 and SFRP4, and one in SFRP3. Notably, although the binding affinities for all SFRP-Wnt pairs tested were in the nanomolar range – very similar to those observed for the Wnt-Fz interaction (Wu and Nusse, 2002) – only SFRP1 and SFRP2 could inhibit Wnt3a-mediated β-catenin accumulation in cultured cells (Wawrzak et al., 2007). Several additional studies have indicated possible biochemical and/or functional specificity in the SFRP-Wnt interactions in the neural tube (Galli et al., 2006), somites (Borello et al., 1999; Lee et al., 2000), vascular endothelium (Dennis et al., 1999), and developing heart (Eisenberg and Eisenberg, 2006; Schneider and Mercola, 2001). Furthermore, Wnt-induced embryonic axis duplication and forebrain development in *Xenopus* have been used to determine the efficiency of given SFRPs to counteract specific Wnt activities, sometimes obtaining contrasting results (Bradley et al., 2000; Finch et al., 1997; Lin et al., 1997; Pera and De Robertis, 2000).

One conclusion from the studies described above is that SFRPs do not bind to Wnt proteins in an equivalent manner, neither in terms of specificity and number of binding sites nor in terms of interaction domains. However, achieving a systematic classification of SFRP-Wnt interactions may be hindered by the relatively large number of different Wnt ligands and their poor solubility, which makes their purification difficult (Logan and Nusse, 2004). Furthermore, the determination of binding affinities might not be sufficient to understand how SFRPs antagonise Wnt signalling because biochemically demonstrated Wnt-SFRP interactions are not necessarily functional in living cells (Lin et al., 1997; Wawrzak et al., 2007). This suggests that additional molecules modulate SFRP-Wnt activities in vivo. Glycosaminoglycans are certainly good candidate modulators (Uren et al., 2000) because they bind and regulate Wnt-protein diffusion (Logan and Nusse, 2004), and facilitate SFRP secretion and accumulation (Finch et al., 1997; Uren et al., 2000; Zhong et al., 2007). Alternatively, the activities of different SFRPs in vivo could reflect their ability to interfere with different Wnt signalling pathways, thereby modulating different events within the same

tissue (e.g. cell specification vs cell movement). Thus, interference with different SFRPs could generate different phenotypes.

Different SFRPs may have opposing effects on the same process

The possibility that different SFRPs have opposing effects on the same process has been suggested to explain the activities displayed by Crescent and Frzb during *Xenopus* head development. Despite the overlapping distribution of the two proteins in the prechordal plate and anterior endoderm, Crescent overexpression leads to cyclopia and reduction in size of anterior structures, whereas Frzb enlarges anterior structures without affecting proximo-distal patterning of the eye (Pera and De Robertis, 2000). These results could be reconciled if Frzb were to interfere with canonical Wnt signalling and Crescent with the non-canonical pathway, which underlies the control of morphogenetic movements (Pera and De Robertis, 2000). Indeed, this possibility is supported by the observation that Cdc42, a putative mediator of non-canonical signalling, can antagonise the effects of Crescent (Shibata et al., 2005). Differential antagonism of canonical and non-canonical signalling has also been proposed (Esteve and Bovolenta, 2006) to explain the different phenotypes observed after the knockdown of Tlc (Houart et al., 2002) and SFRP1 (Esteve et al., 2004) during telencephalic and eye development in fish.

Other, as yet unclear, mechanisms might underlie the opposing effects of SFRPs on apoptosis in breast tumours. As mentioned above, SFRPs were independently identified as secreted apoptosis-related proteins (SARPs) during a search for inhibitors of the apoptotic programme (Melkonyan et al., 1997). These studies revealed that, despite their high degree of homology, the identified proteins elicited different cellular responses in breast adenocarcinoma cells. SFRP2 (named SARP1 in Melkonyan et al., 1997) promotes β-catenin accumulation and increases cell resistance to apoptosis induced by various agents, whereas SFRP1 (named SARP2 in Melkonyan et al., 1997) favours cell death and diminishes β-catenin stability (Melkonyan et al., 1997). However, it is unclear how these observations are related to other situations in which the effects of SFRPs in apoptosis have been evaluated in cells and in vivo. For example, SFRP1 diminishes the apoptosis of dermal fibroblasts (Han and Amar, 2004), whereas SFRP2 promotes apoptosis that is associated with developmental tissue patterning in chick embryos (Ellies et al., 2000). Similarly, SFRP4 favours apoptosis that leads to the involution of the mammary gland after weaning, when the differentiated mammary epithelium is no longer needed (Lacher et al., 2003). In this case, SFRP4-mediated apoptosis involves the suppression of the phosphoinositide 3-kinase (PI3K)/Akt and the protein kinase B (PKB)/Akt survival pathways, possibly through a mechanism that is independent of canonical Wnt signalling (Lacher et al., 2003).

If apoptosis and the establishment of anterior structures are events in which different SFRPs exert opposing effects, kidney development is an example of how SFRPs can antagonise each other's activity. Both SFRP1 and SFRP2 are expressed during metanephric kidney development. In cultures of embryonic rat metanephros, SFRP1 blocks kidney-tubule formation and bud branching (Yoshino et al., 2001), processes that depend on Wnt4 activity in vivo (Kispert et al., 1998). Exposure to SFRP2 alone has no effect, but in the presence of both SFRPs, SFRP2 blocks SFRP1-mediated effects and partially restores tubule differentiation and bud branching. Binding of the Wnt effector transcription factor T cell factor (TCF) to DNA from metanephric mesenchyme is inhibited by SFRP1 but not by SFRP2, suggesting that SFRP2 does not inhibit Wnt signalling (Yoshino et al., 2001). Rather, SFRP2 could bind to and antagonise SFRP1 and/or potentiate Wnt signalling, as reported for SFRP1, which can promote Wg-mediated signalling at low concentrations but represses it at high concentrations (Uren et al., 2000). Different biochemical and crystallographic data support both possibilities, introducing the interesting idea that SFRPs can interact not only with Wnt but also with Fz receptors.

SFRPs bind to, and possibly activate, Frizzled receptors

The crystallographic resolution of the structure of the mouse SFRP3 and Fz8 CRD domains suggested that CRDs might be able to homodimerise or heterodimerise (Dann et al., 2001). This possibility has also been demonstrated in biochemical studies in which SFRPs and Fz proteins and/or their CRDs have been shown to form homo- and heteromeric complexes (Bafico et al., 1999; Carron et al., 2003; Rodriguez et al., 2005). These results provide a possible molecular basis for some of the diverse observations of SFRP activity described above, and suggest a number of different mechanisms by which SFRPs can modulate Wnt signalling. SFRPs may act in the following ways: (1) by sequestering Wnt through the CRD or NTR domains, thereby acting as classical antagonists (Fig. 3A); (2) by titrating out one another's activity and thereby favouring Wnt signalling (Fig. 3B); (3) by acting in a dominant-negative manner through the formation of inactive complexes with Fz receptors that prevent signal activation (Fig. 3C) (Bafico et al., 1999); or (4) by favouring Wnt-Fz interactions by simultaneously binding to both molecules and promoting signal activation (Fig. 3D) (Uren et al., 2000).

The final mechanism, which implies binding of the SFRP CRD to the Fz receptor, also raises the possibility of a different scenario in which, in the absence of Wnt proteins, a CRD-CRD interaction may be sufficient to activate signal transduction. This appears to be the case for Fz3 in *Xenopus* embryos, in which Fz3 receptors form homodimers through their CRD domain when overexpressed in blastula cap cells, a process sufficient to activate the β-catenin pathway. Dimer formation is unaffected by Wnt expression, supporting a ligand-independent mechanism of canonical signalling activation (Carron et al., 2003). Recent work from our laboratory provides another example of a Wnt-independent mechanism that is based on heterodimer formation. Our work also illustrates a novel function for SFRP1 as an axon guidance molecule (Fig. 4) (Rodriguez et al., 2005). In various vertebrates, SFRP1 is strongly expressed in crucial regions of the visual pathway. Consistent with this distribution, SFRP1 behaves like a Wnt-independent axon guidance cue, and modifies the behaviour of retinal ganglion cell (RGC) growth cones in several in vitro and in vivo assays (Rodriguez et al., 2005). The search for a receptor that could mediate this effect demonstrated that Fz2, which is expressed in RGCs, interacts with the CRD of SFRP1 (Rodriguez et al., 2005) (P.E., unpublished data). Furthermore, interference with Fz2 expression abolishes SFRP1-mediated activity on RGC growth cones, indicating that, in axon guidance at least, SFRP1 is an active ligand of Fz-mediated signalling (Fig. 4). Notably, the activation of the Fz receptors by a proposed ligand antagonist is not unique to SFRP1: Dickkopf2, which belongs to a different family of Wnt antagonists, can activate Wnt canonical signalling by cooperating with at least three different Fz proteins (Wu et al., 2000).

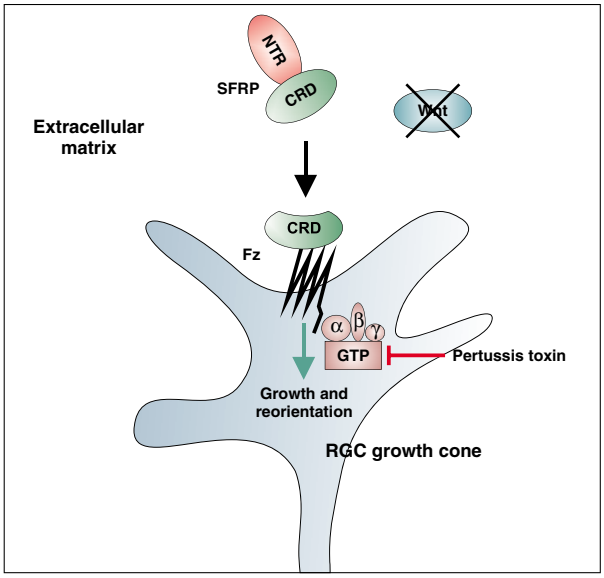

Fig. 4. Proposed mechanism of SFRP1 activity as an axon guidance cue. SFRP1 can directly modify and reorient the growth of retinal ganglion cell growth cones. This activity does not require Wnt inhibition and is mediated by Fz2. It requires pertussis-toxin-sensitive activation of Ga protein, involves protein synthesis and degradation, and is modulated by different levels of cAMP and cGMP (Rodriguez et al., 2005).

SFRPs interacts with molecules that are unrelated to Wnt signalling

Most of the functions of SFRPs that have been discussed so far relate to the effect of SFRPs on Wnt signalling in one way or another. However, SFRPs appear to be promiscuous proteins that can interact with molecules that are unrelated (to the best of our knowledge) to components of the Wnt signalling cascades and among which there is no apparent relationship. These include fibronectin, Unc5H3, receptor activator for nuclear factor kappa B (NF-κB) ligand (RANKL) and bone morphogenetic protein (BMP)/Tolloid.

SFRP2 enhances the viability of mammary tumour cells by protecting them from apoptotic stimuli (Lee, A. et al., 2004; Melkonyan et al., 1997). This effect can be explained by the accumulation of SFRP2 secreted by tumour cells in the extracellular matrix and its physical interaction with the fibronectin-integrin complex (Fig. 5A). Addition of SFRP2 to the complex favours cell adhesion and enhances viability (Lee, J. et al., 2004), possibly through the phosphorylation of the focal adhesion kinase (FAK), the activation of NF-κB – thereby confering resistance to apoptosis, and the suppression of activity of Janus kinases (JNKs) (Lee, J. et al., 2006) – stress-activated serine/threonine kinases that are involved in programmed cell death. Whether Wnt signalling participates in the regulation of apoptosis is so far unclear (Lisovsky et al., 2002); it is thus difficult to determine whether the anti-apoptotic effects that are mediated by SFRP2-fibronectin-integrin complexes represent SFRP activity that is totally independent of Wnt activity. Nevertheless, the sequence similarity of the SFRP NTR to that of other proteins present in the extracellular matrix – such as the aforementioned procollagen C-proteinase – and its interaction with heparin make this possibility attractive.

The screening of a phage display peptide library revealed that SFRP1 binds with high affinity to the peptide motif L/V-VDGRW-L/V, and showed that the DGR core is essential for binding. Surprisingly, this motif is absent from both Wnt and Fz; however, it is present in two otherwise unrelated proteins: Unc5H3 and RANKL (Chuman et al., 2004). Unc5H3 acts as a receptor in axon repulsion induced by netrin 1 in different neuronal populations (Round and Stein, 2007). Direct binding between SFRP1 and full-length Unc5H3 has not yet been tested. However, their possible interaction, particularly in the context of axon guidance, is a hypothesis worthy of investigation, especially because SFRP1 and netrin 1 expression patterns overlap in different regions of the CNS, and the properties of the two proteins as axon guidance cues have some interesting similarities (Rodriguez et al., 2005).

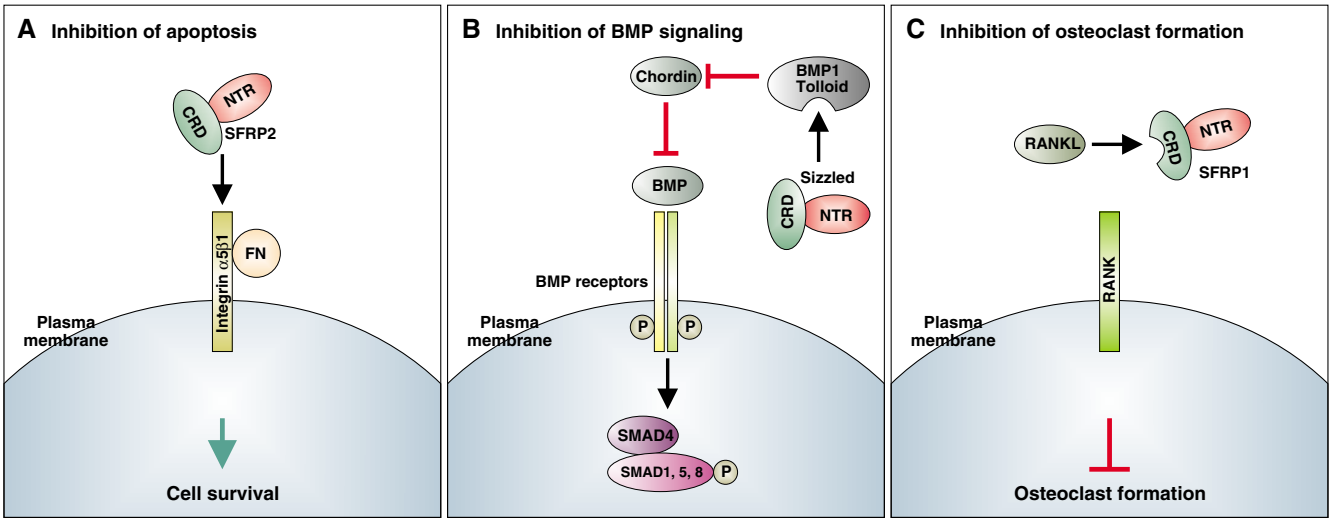

Fig. 5. SFRP interactions with molecules unrelated to Wnt signalling. (A) SFRP2 binds to the fibronectin (FN)–integrin-α5β1 complex, promoting cell adhesion and inhibiting apoptosis. (B) Sizzled binds to and inhibits the activity of BMP1/Tolloid metalloproteinases that cleave chordin, a BMP signalling antagonist. Thus, Sizzled favours chordin stabilisation, which in turn inactivates BMP signalling. (C) SFRP1 interacts with RANKL, preventing it from binding to RANK, and thus inhibits osteoclast formation.

In contrast to binding studies using Unc5H3, those that used RANKL confirmed its interaction with SFRP1 when using the entire molecule (Fig. 5C). RANKL – a member of the tumour necrosis factor family – is a major promoter of osteoclast formation, which is inhibited by the association of SFRP1 and RANKL (Hausler et al., 2004). More importantly, *Sfrp1*-null mice show decreased apoptosis of osteoblasts and osteocytes, increased osteo-progenitor differentiation, enhanced bone formation and elevated bone mineral density (Bodine and Komm, 2006; Gaur et al., 2006), indicating a possible biological role for SFRP1 beyond Wnt modulation, although an alternative interpretation for this phenotype has been proposed (Fuentealba et al., 2007).

Although the physiological activities of SFRPs activated by Unc5H3, RANKL and fibronectin require further investigation, the activity of Sizzled as an inhibitor of BMP signalling seems to be well established through a number of complementary biochemical, functional and genetic studies. Sizzled was initially identified in *Xenopus* embryos as a putative Wnt8 antagonist (Salic et al., 1997) but subsequent analyses showed that it does not block Wnt signalling in vivo, although it bind to Wnt proteins in vitro. A mutation in the *sizzled* gene is responsible for the zebrafish *ogon* phenotype (Yabe et al., 2003), which is characterised by the expansion of ventral tissue, a feature also displayed by mutants of the BMP antagonist *chordin*. In *Xenopus* and zebrafish, the formation of the dorsoventral axis depends largely on BMP signalling (Lee, H. et al., 2006; Little and Mullins, 2006). Alteration of Sizzled protein expression in both species affects this axis, which is consistent with Sizzled acting as a negative feedback regulator of BMP signalling (Collavin and Kirschner, 2003; Yabe et al., 2003). An interesting mechanistic aspect is that Sizzled binds to BMP1/Tolloid, a metalloprotease that normally degrades chordin and thereby promotes BMP signalling. The Sizzled-BMP1/Tolloid interaction prevents chordin cleavage and stabilises it, thereby indirectly inhibiting BMP signalling (Fig. 5B) (Lee, H. et al., 2006; Muraoka et al., 2006). Specifically, Sizzled acts as a competitive inhibitor of the enzymatic activity of the BMP1/Tolloid metalloprotease as shown by careful biochemical studies, which demonstrate that Chordin and Sizzled compete with similar affinities for the substrate binding site of the enzyme (Lee, H. et al., 2006).

Since the Sizzled CRD mediates inhibition of BMP signalling, it is possible that other SFRP family members have similar functions. The two pairs that were tested, SFRP2/tolloid (Lee, H. et al., 2006) and BMP1/crescent (Muraoka et al., 2006), gave positive and negative results, respectively. Such a difference, however, should not be surprising given the emerging heterogeneous behaviour of SFRPs. Moreover, other studies point to the possible cross-talk between SFRPs and BMP signalling. Thus, overexpression of SFRP2 in the embryonic chick hindbrain inhibits expression of BMP4 and prevents programmed cell death (Ellies et al., 2000), which has been reported to be mediated by BMP4 in other structures (Trousse et al., 2001). In addition, Smad-interacting protein 1 (Sip1), a transcription factor implicated in BMP signalling, binds to the *Sfrp1* promoter and probably represses its expression, because *Sfrp1* expression is strongly upregulated in Sip1 mutants in association with a progressive apoptotic degeneration in the hippocampus (Miquelajauregui et al., 2007).

The roles of SFRPs in pathological events

Wnt proteins were first identified as mammary oncoproteins (van't Veer et al., 1984). Indeed, aberrant activation of canonical Wnt signalling occurs in a large proportion of tumours, and is associated with the loss of controlled growth and the impairment of cell differentiation (Rubin et al., 2006). This constitutive activation of Wnt signalling is often associated with mutations in the downstream components of the pathway. Therefore, although one might expect that what happens at the cell surface has little influence on tumorigenesis, this does not seem to be the case – although how this influence is exerted is unclear. One possibility is that receptor activation at the cell surface results in an enhanced propagation of the signal, caused by alterations in the mutated components and, further, by the crosstalk between the tumorigenic canonical and non-canonical JNK signalling pathways. In this context, the tumour suppressor activity of SFRPs seems logical because it fits well with their different functions as Wnt-signalling modulators and underscores their importance in the aetiology of cancer (Rubin et al., 2006). However, SFRPs have been also reported to have tumour-promoting activities, in many cases linked to the aforementioned role in apoptosis (Rubin et al., 2006).

In line with a tumour suppressor function of SFRPs, loss or significant downregulation of SFRP1 or SFRP3 expression has been observed in a large proportion of invasive carcinomas, such as in breast (Turashvili et al., 2006; Zhou et al., 1998), gastric (To et al., 2001), cervical (Ko et al., 2002), hepatocellular (Huang et al., 2007) and prostate (Zi et al., 2005) tumours. Conversely, restoring SFRP expression in various cancer cells attenuates their tumorigenic behaviour (Zi et al., 2005), decreases β-catenin stabilisation and promotes cell death even when downstream components of the canonical pathway are mutated (Suzuki et al., 2004). Similarly, relief of Myc-mediated repression of SFRP1 reduces Myc-dependent transforming activity in mammary cells (Cowling et al., 2007).

Two different mechanisms participate in the loss of SFRP expression in cancer cells: allelic loss and epigenetic silencing. The *sfrp1* (8p11-12) and *sfrp3* (2q31-33) loci are commonly associated with deletions and the loss of heterozygosity in a variety of cancers (Leach et al., 1996), including those of the breast and lung, and colorectal carcinomas or neuroblastomas (Leyns et al., 1997; Ugolini et al., 1999). Promoter hypermethylation often accounts for the loss of expression of tumour suppressor genes (Herman and Baylin, 2003) and may explain low levels, or even absence, of SFRPs when no modifications of the gene copy number can be seen (Armes et al., 2004). *SFRP1*, *SFRP2*, *SFRP4* and *SFRP5*, but not *SFRP3*, have dense CpG islands that flank the first exon. These sequences have been reported to be hypermethylated in many types of carcinoma, particularly colorectal, gastric mammary and renal cell (Caldwell et al., 2004; Lee, A. et al., 2004; Stoehr et al., 2004; Suzuki et al., 2002; Takada et al., 2004; Zhou et al., 1998), possibly predisposing to pre-malignant changes (Suzuki et al., 2004).

Interestingly, the remarkably elevated SFRP4 levels in tumours associated with osteomalacia (a condition in which the bone becomes soft and flexible, often as a result of the lack of vitamin D) led to the unexpected discovery that SFRP4 plays an important role in homeostasis of phosphorus and inorganic phosphate (P_i), by inhibiting synthesis of vitamin D and, thus, intestinal absorption of P_i (Berndt and Kumar, 2007). The infusion of SFRP4 into rats results in phosphaturia that is associated with an increase in β-catenin phosphorylation, suggesting that this effect of SFRP4 involves Wnt signalling, although a decrease in abundance of Na^+-P_i co-transporters has also been reported (Berndt and Kumar, 2007). Irrespective of the mechanism of action, SFRP4 has an

important role in tissue homeostasis, because P_i is required for basic cell processes including nucleic-acid synthesis, energy metabolism, membrane function and bone mineralisation (Berndt and Kumar, 2007).

In addition to SFRP4, other SFRPs contribute to bone development and homeostasis. In particular, SFRP1 participates in this process through the aforementioned interaction with RANKL (Hausler et al., 2004) and as reflected by the phenotype of *Sfrp1*-null mice (Satoh et al., 2006). Similarly, SFRP3 has been associated with the development of osteolysis or heterotopic ossification (Gordon et al., 2007). Interestingly, a decrease in the activity of other Wnt inhibitors, such as Dickkopf-1 or sclerostin, is also associated with an increase in bone-mass formation, whereas loss-of-function mutations in LRP5 cause osteoporosis, suggesting an important general role of Wnt signalling in bone formation (Baron and Rawadi, 2007). A possible molecular basis for this function of Wnt was suggested by the recent demonstration that Wnt signalling through GSK3 activation enhances BMP signalling, which potently induces bone morphogenesis (Fuentealba et al., 2007).

Elevated levels of SFRP1 have also been reported in the retinas of patients affected by retinitis pigmentosa, an inherited disease characterised by the progressive loss of photoreceptors (Hackam, 2005; Hackam et al., 2004). Although *SFRP1* maps close to a locus associated with an uncharacterised form of retinitis pigmentosa, no mutations have been found in a cohort of screened patients (Garcia-Hoyos et al., 2004). Nevertheless, abnormal expression of SFRPs and other components of the Wnt signalling pathways have been detected in a number of mouse models of the disease (Hackam, 2005; Jones et al., 2000), supporting the possibility that alterations in the Wnt signalling pathway are involved in the progression of photoreceptor degeneration. Alternatively, elevated SFRP expression might represent an attempt by the tissue to promote the generation of photoreceptors, as seen during the development of the chick retina (Esteve et al., 2003). If this were the case, SFRPs could be considered as potentially valuable therapeutic tools, as already suggested by Mirotsou and colleagues, who found that treatment of myocardiocytes with SFRP2 promoted cell survival and repair (Mirotsou et al., 2007). Nevertheless, the elevated levels of SFRPs reported in the pathological conditions described above as well as in other less characterised examples (Imai et al., 2006; Koza et al., 2006; Mirotsou et al., 2007; Surendran et al., 2005) suggest that these molecules could also be valuable therapeutic targets.

Conclusions and perspectives

We have discussed the evidence that SFRPs are proteins with a wide range of activities beyond their role as multifunctional regulators of Wnt signalling. Extracellular antagonists exist for most cell signalling pathways as a means to provide fast and precise control of the input a cell receives. The function of SFRPs as modulators of Wnt, BMP or possibly other cell signalling pathways may deserve more attention in the future, particularly from developmental and cell biologists. Indeed, we still need to understand how embryonic cells integrate the different information provided by the multiple signals to which they are exposed. A key to this integration might be the existence of pleiotropic signal regulators. In fact, in addition to Sizzled, Shifted (the *Drosophila* homolog of WIF1) controls and facilitates the diffusion of Hedgehog (Gorfinkiel et al., 2005), whereas in vertebrates it acts as a Wnt-binding factor (Hsieh et al., 1999). Cerberus, a secreted protein that binds and antagonises Wnt, BMP and Nodal signalling (Piccolo et al., 1999) is another example.

In-depth analysis and inter-species comparisons of the phenotypes that result from the modulation of SFRP expression with different genetic and pharmacological tools might also offer new perspectives. Currently, the relatively strong phenotypes observed after knockdown of SFRP activity in lower vertebrates (Esteve et al., 2004; Houart et al., 2002) contrast with the relatively mild or absent phenotypes observed after genetic inactivation in mice (Bodine et al., 2004; Leaf et al., 2006; Satoh et al., 2006). Functional redundancy due to overlapping expression in mammals may explain this discrepancy (Satoh et al., 2006). Robust and multilayered control of key signalling pathways may be another possibility, as the abrogation of other key regulators such as Cerberus, Cerberus-like, chordin and Noggin have unexpectedly generated minor or no abnormalities in their respective mouse mutants (Borges et al., 2001). Future studies should clarify how SFRP activity is integrated into cellular signalling pathways, and might identify further Wnt-dependent and -independent roles for SFRPs.

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