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Dynamic Expression of Secreted Frizzled-related Protein 3 (sFRP3) in the developing mouse spinal cord and dorsal root ganglia

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Abstract

Wnt proteins have been implicated in regulating a variety of developmental processes in the central nervous system (CNS). Secreted Frizzled-related protein 3 (sFRP3) is a member of the sFRP family that can inhibit the Wnt signaling by binding directly to Wnts via their regions of homology to the Wnt-binding domain of Frizzleds. Recent studies suggested that sFRP3 plays an important role in cell proliferation and differentiation in various tissues. To understand the role of sFRP3 in neural development, we carried out detailed studies on the expression of *sFRP3* in the developing nervous system. Our results revealed that *sFRP3* is initially expressed in the ventricular zone of spinal cord and dorsal root ganglia (DRG), and later in the dorsal horn of spinal cord and subpopulation of DRG neurons. The spatiotemporally dynamic expression of *sFRP3* strongly suggests that sFRP3 has potential functions in the sensory neuron genesis and sensory circuitry formation.

Keywords

secreted frizzled-related protein 3; spinal cord; dorsal root ganglia; sensory circuit

INTRODUCTION

Wnts are a family of secreted proteins involved in many aspects of central nervous system (CNS) development, including neural induction, patterning, cell fate specification, cell

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proliferation, migration, axon guidance and synaptogenesis (Cadigan and Nusse, 1997; Stern, 2001; Ciani and Salinas, 2005; Bovolenta et al., 2006). Wnts transduce their signals through the Frizzled family of Wnt receptors by at least three different intracellular signaling pathways, known as the canonical Wnt/β-catenin pathway, the Wnt/Ca^{2+} pathway and the planar cell polarity pathway (Bhanot et al., 1996; Cadigan and Liu, 2006; Gordon and Nusse, 2006).

Secreted Wnt antagonists, classified as secreted Frizzled-related protein (sFRP) family, are potential negative modulators of Wnt signaling (Kawano et al., 2003). It has been shown that sFRP proteins can inhibit Wnt signaling by binding directly to Wnts via their region of homology to the Wnt-binding domain of Frizzleds (Rattner et al., 1997; Dennis et al., 1999; Ladher et al., 2000). sFRP3, also known as frizzled-related protein 1, or Frzb1 or FrzB, has been independently identified from the bovine cartilage extracts and the Spemann's organizer cells from Xenopus laevis (Hoang et al., 1996; Leyns et al., 1997; Wang et al., 1997). The sFRP3 protein shares the N-terminal cysteine-rich domain (CRD) with other members in the Frizzled family of Wnt receptors, but lacks the putative seven-transmembrane domain (Bhanot et al., 1996; Wang et al., 1996; Yang-Snyder et al.,1996).

The inhibitory activity of *sFRP3* appears to be specific, since *sFRP3* blocks WNT-1 and WNT-8 signaling in both *X laevis* embryos and a mammalian cell line (Lin et al., 1997; Wang et al., 1997; Leyns et al., 1997), but not Wnt-3A or -5A signaling (Wang et al., 1997). Murine *sFRP3* gene is expressed in the primitive streak, presomitic mesoderm, somites, and brain at early embryonic stages (Hoang et al., 1998). At later stages, it exhibits sharp boundaries of expression in the limb bud, branchial arches, facial mesenchyme, and in cartilaginous elements of the appendicular skeleton (Hu et al., 1998; Hoang et al., 1998). *sFRP3* has been implicated in osteoblast proliferation, intimal vascular disease, fibroblast proliferation and foci formation (Chung et al., 2004; Schumann et al., 2000; Mao et al., 2000; Lories et al., 2007; Scardigli et al., 2008). Although Wnts signaling pathways are known to be important for the development of various tissues, the functions of sFRP3 in neural development are not well understood.

In the present study, we describe the spatiotemporal pattern of *sFRP3* expression in the developing mouse spinal cord and dorsal root ganglia. The dynamic expression of *sFRP3* in spinal cord and dorsal root ganglia suggests that *sFRP3* has potential functions in sensory neuron genesis and sensory circuit formation.

EXPERIMENTAL PROCEDURES

Animal

C57BL/6N mice were obtained from The Jackson Laboratory. All experimental procedures conformed to National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Louisville.

In situ hybridization

In situ hybridization (ISH) was performed according to Scheer et al. (2001). Animals were deeply anesthetized and perfused with 4% paraformaldehyde (PFA), and tissues were

isolated and postfixed in 4% PFA at 4°C overnight. Fixed spinal cord tissues were embedded in OCT medium and sectioned on a cryostat. Frozen sections (16 μm thick) were subject to *in situ* hybridization with digoxigenin-labeled riboprobes according to Schaeren-Wiemers and Gerfin-Moser (1993) with minor modifications. The information of all riboprobes used for ISH is listed in Table 1.

For double in situ hybridization, tissue sections were first subjected to *sFRP3* ISH, followed by anti-Pax3, anti-Pax6, anti-Olig2, anti-Nkx6.1, anti-TrkA or IB4 immunohistochemical staining with ABC kit individually, as described previously (Zhao et al., 2007). Rabbit anti-Olig2 (a gift from Dr. Charles Stiles) was used at a final concentration of 2 μg/ml (Lu et al., 2001); Mouse anti-Pax3, Mouse anti-Pax6 and Mouse anti-Nkx6.1 (DSHB Inc.) at a final concentration of 20 μg/ml; Rabbit anti-TrkA (Epitomics) and Rabbit IB4-Biotin (Sigma) at a final concentration of 2 μg/ml. For double *in situ* hybridization experiments, tissues were hybridized simultaneously with digoxigenin-labeled *GrprDrg11*, *sFRP1*, *sFRP2*, *Wnt7b*, *FZD7*, *FZD8* or *FZD10* and fluorescin-labeled *sFRP3* riboprobes.

It is noted that DRG tissues were not included in some sections of early embryos due to slight positional differences in tissues. Therefore, expression of some genes in DRGs may be missed in some sections.

RESULTS

Selective expression of *sFRP3* in the ventricular zone of spinal cord at early embryonic stages

To examine *sFRP3* expression in the developing spinal cord, we performed RNA *in situ* hybridization in wild-type mouse spinal cord tissues at the early stages of neurogenesis. After the time of neural tube closure (E9.5), *sFRP3* was initially expressed in the ventricular zone (VZ) of the middle region of spinal cord (Fig. 1B). At E11.5, the expression of *sFRP3* was strongly detected in the ventricular neural progenitor cells of the middle spinal cord, but weak expression was also observed in more dorsal VZ of the spinal cord including the roof plate (Fig. 1C). At the same stage, two related members, *sFRP1* and *sFRP2* were also expressed in the VZ of spinal cord (Fig. 2A B) and overlapped with *sFRP3* (Fig. 2I J). In contrast, *sFRP4* and *sFRP5* did not have detectable expression in the developing spinal cord (Fig. 2C–D G–H). As development proceeded, *sFRP3* signal remained strong in the middle VZ, but its expression in the dorsal VZ was progressively reduced (Fig. 1D–E) and eventually disappeared after E14.5 (Fig. 4A B).

sFRP3 is expressed in the dp6 – p1 domains of neural progenitors at neurogenesis stage

During early neural development, the ventricular zone of the spinal cord can be divided into six distinct dorsal progenitor domains and five ventral domains. To determine the precise domains of *sFRP3* expression, we chose the E12.5 spinal cord to compare its expression with that of several neuroprogenitor genes (*Pax3*, *Pax6*, *Mash1*, *Dbx1*, *Dbx2*, *Nkx6.1* and *Olig2*) that define discrete progenitor domains along the dorsoventral axis of the neural tube (Goulding et al. 1991; Ericson et al. 1997; Pierani et al. 1999). Comparative expression analysis on consecutive sections revealed that the domain of *sFRP3* expression is nearly

identical to that of *Dbx2*, but broader than that of *Dbx1* (Fig. 3A B), suggesting that *sFRP3* is expressed in dp6, p0 and p1 domains of neural tube. Consistently, the dorsal boundary of *sFRP3* expression is positioned immediately ventral to the expression of *Mash1* (Fig. 3C), which has been shown to be expressed in dp3–p5 domains of dorsal neural tube. In further support, the ventral boundary of *sFRP3* expression is immediately dorsal to the *Nkx6.1* expression domain, but separated from the *Olig2* expression domain (Fig. 3D E). In addition, *Pax3* expression extends ventrally into the *sFRP3* expression domain (Fig. 3G), and *Pax6* expression encloses the domain of *sFRP3* expression (Fig. 3F). Together, these results demonstrate that *sFRP3* is expressed weakly in dorsal domains dp1–dp5 and strongly in dp6, p0 and p1 domains of VZ; however, its dorsal expression is down-regulated after E12.5.

sFRP3 is expressed in lamina I to II of spinal cord dorsal horn at later embryonic stages

We next examined *sFRP3* expression in the developing spinal cord at later embryonic stages. At E14, *sFRP3* expression started to be detected in dorsal horn of spinal cord (Fig. 4A), while its expression in the middle VZ rapidly diminished and completely vanished at E14.5 (Fig. 4B). At later stages, *sFRP3* expression continued to accumulate in the dorsal horn of spinal cord, and this expression pattern was maintained until P7 (Fig. 4C–H), prior to its rapid down-regulation at P15 and P30 (Fig. 4I–J), *sFRP3* was specifically expressed in the lamina of spinal cord dorsal horn as compared to the other members the sFRP family (*sFRP1*, *sFRP2*, *sFRP4* and *sFRP5*) (Fig. 2E–H).

The persistent expression of *sFRP3* in the dorsal horn of spinal cord has raised the possibility that *sFRP3* is expressed by subpopulations of sensory neurons. To examine this possibility, we performed the double ISH in E16.5 spinal cords with *sFRP3*, *Grpr* and *Drg11* riboprobes. *Grpr* is expressed in primary sensory neurons and in their projection area in the lamina I of superficial dorsal horn (Saito et al., 1995; Sun and Chen, 2007). *Drg11* is a transcription factor that is necessary for the assembly of the nociceptive circuitry in the spinal cord dorsal horn and is expressed in the lamina I–III of spinal dorsal horn. As shown in Fig. 5, double labeling experiments revealed that sFRP3+ cells occupied lamina I and II of superficial dorsal horn of the spinal cord (Fig. 5). It is also evident that a subpopulation of sFRP3+ cells in superficial dorsal horn co-expressed *Grpr* and *Drg11* (Fig. 5).

Dynamic expression of *sFRP3* in dorsal root ganglia

In the primary sensory neurons of DRG, the expression of *sFRP3* was first detected at E10.5 (Fig. 1B). At E11.5, the *sFRP3* signals were mostly distributed to the periphery of DRG neurons (Fig. 1C). Expression of other members of the sFRP family was not detected at this stage (Fig 2). As development proceeded, similar to that in the dorsal VZ of spinal cord, the number of sFRP3+ cells in DRG was gradually decreased, and no sFRP3+ cells were detected at E13.5 (Fig. 1D–E). Notably, the expression of *sFRP3* was up-regulated again at E16.5 (Fig. 4C) in a subpopulation of DRG neurons, including those expressing TrkA, a receptor for nerve growth factor (Fig. 6A), which labels peptidergic neurons in adult DRG. The expression *sFRP3* in TrkA + neurons was maintained at postnatal stages (Fig. 6B). At P8, many sFRP3+ DRG neurons co-expressed IB4 (Fig. 6C), a molecular marker for non-

peptidergic neurons. Thus, in postnatal DRG, *sFRP3* is expressed in both peptidergic and non-peptidergic subpopulations of neurons.

Expression of Wnt molecules and receptors in relation to sFPR3 expression in the spinal cord

Given that sFRP3 functions as an antagonist of Wnt signaling, it would be important to determine the potential downstream target Wnt pathway of sFRP3. Therefore we next examined the expression of various Wnt molecules and receptors in the developing spinal cord. At E11.5, of all Wnt molecules examined, Wnt4, Wnt7a and Wnt7b displayed expression in the VZ of spinal cord (Fig7), including the *sFRP3* expression domain in the middle VZ (Fig. 7I K W). This result suggested that sFPR3 may function to antagonize the Wnt4- and Wnt7-mediated signaling pathway. Consistently, among all FZD molecules examined (Fig 7 O–U), only Wnt7a receptor FZD7 is highly expressed in the VZ of the middle region (Fig. 7Q X). FZD3, the receptor for Wnt3a and Wnt4 had a weak expression in the VZ along the entire D-V axis (Fig 7O). FZD10, the receptor of Wnt7b, is highly expressed in the dorsal VZ but did not have overlapping with *sFRP3* expression domain (Fig. 7U Z). At P3, of all Wnt molecules and receptors examined, only FZD3 showed weak and specific expression in the dorsal horn interneurons overlapping the expression of *sFRP3* (Fig 7P). Examination of their expression in DRG at this stage revealed the specific expression of Wnt1, Wnt4 and FDZ8 in the peripheral DRG neurons (Fig 7B, E, T) where *sFRP3* is expressed.

DISCUSSION

Previous study has demonstrated that *sFRP3* has restricted expression in the developing forebrain and midbrain, and suggested that it plays an important role in the early development of these CNS regions (Hoang et al., 1998). However, its expression and function in the spinal cord or DRG have not been determined. Here, we report the detailed spatiotemporal pattern of *sFRP3* expression in the spinal cord and DRG, and suggest that *sFRP3* may be involved in sensory neuron genesis and the formation of sensory circuitry.

Differential expression in VZ implies distinct functions of *sFRP3*

In mouse spinal cord, six distinct dorsal interneuron (dI) populations, termed dI1–dI6 are specified by E10, on the basis of the repertoire of transcription factors that they express. The dI1–dI3 neurons migrate ventrally, whereas the dI4 and a subset of the dI5 neurons migrate laterally to populate the deep dorsal horn. Beginning at E12, three additional populations of neurons are born later (Mattise 2002). These late-born populations are derived from dp4 and dp5 neural progenitor cells, migrate to the superficial laminae of the dorsal horn, where they mediate the sensing of pain and temperature (Caspary and Anderson, 2003). The transient and weak expression of *sFRP3* in dp1–dp5 domains of VZ from E11.5 to E12.5 (Fig. 1) suggests that *sFRP3* as an antagonist of the Wnt signaling pathway may be involved in dorsal interneuron generation.

The strong and persistent expression of sFRP3 in the *Dbx1/2* expressing dp6–p1 domains of VZ from E11.5 to E14 (Fig. 3H) raises the possibility that *sFRP3* participates in V0 and V1

interneuron fate specification or consolidation (Fig 1, 3). Two related members, *sFRP1* and *2*, are similarly expressed in these domains (Fig 2I J), and the other two members sFRP4 and 5 are not expressed in spinal cord and DRG (Fig 2C D G H). Interestingly, at these stages, *Wnt7a* and its receptor *FZD7* are highly expressed in the VZ of the middle and ventral spinal cord (Fig 7I Q), including the dp6–p1 domains of *sFRP3* expression. *Wnt7a* is known to play a critical role in control of neuronal progenitor maturation (Grand et al., 2009; Horn Z et al., 2007). *Wnt7b* is strongly expressed in these regions as well, but its receptor *FDZ10* has restricted expression in the dorsal VZ (Fig. 7). Based on the strong and overlapping expression of *sFRP1–3* and *Wnt7a/Wnt7b/FZD7* in dp6–p1, it is possible that sFRP1–3 may have an important role in the fate specification of V0 and V1 interneurons by modulating the Wnt7 signaling in neural progenitor cells during early spinal cord development. The involvement of sFRPs and Wnt7/FDZ7 genes in V0 and V1 interneuron development can be tested with future genetic and experimental studies. However, since sFRP1–3 proteins are secreted molecules and their range of biological activity might well be broader, it is plausible that non-overlapping, neighboring expression domains of Wnts/Wnt-receptors may be influenced by sFRP1–3 proteins.

sFRP3 is required for spinal cutaneous sensation circuitry

sFRP3+ cells occupied lamina I and II (Fig 4) where pain and temperature are conveyed through thinner, unmyelinated axon bundles that project to this area (Altman et al., 1984; Caspary and Anderson, 2003). Double staining experiments revealed that *sFRP3* is co-expressed with *Grpr* and *Drg11* in this area (Fig 5, 6). *Drg11* is required for the projection of cutaneous sensory afferent fibers to the dorsal horn (Chen et al., 2001), and *Grpr* is essential for mediating the itch sensation at the spinal cord level (Sun et al., 2007). The persistent expression of *sFRP3* in the laminae I and II from E14 to postnatal stage P7 suggests that sFRP3-mediated inhibition of Wnt signaling may participate in the circuitry formation of cutaneous sensory neurons or itch neurons in the dorsal horn of spinal cord. In support of this concept, a recent study showed that Wnt signaling is indeed involved in the formation of spinal cutaneous sensation circuitry, as *sFRP3* mutants displayed a reduced sensory innervation in the superficial dorsal horn from TrkA+ or aquaporin1+ DRG neurons (John et al., 2012)

It is noticed that in perinatal DRG neurons, there is a significant overlapping between the expression of *sFRP3* and *TrkA* (Fig. 6A). Since *TrkA* expression in DRG is not affected in sFRP3 mutants (John et al., 2012), it is tempting to speculate at this stage that the expression of *sFRP3* in sensory neurons of dorsal horn and DRG may serve as a molecular identity for specific connections of these TrkA+ DRG neurons. It should be noted, however, that most of the sFRP3+ neurons in P8 DRG are excluded from *TrkA* expression (Fig. 6B), and instead labeled by IB4 (Fig. 6C), a molecular marker for non-peptidergic neurons as compared to those TrkA-expressing peptidergic neurons in adult DRG. This could presumably suggest a distinct role for *sFRP3* and Wnt signaling in the DRG neuron development during postnatal and adult stage. Consistently, Wnt1, Wnt4 and FZD8 signals are found in postnatal DRG neurons where *sFRP3* is expressed (Fig. 7B F T). Future studies with conditional knockouts and transgenic approaches are necessary to elucidate the functions of *sFRP3* and related Wnt/FZD signaling pathways in sensory neuron development and circuitry formation.

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Abbreviations

CRD	cysteine-rich domain
CNS	central nervous system
dI	dorsal interneuron
DRG	dorsal root ganglia
ISH	*In situ* hybridization
PFA	paraformaldehyde
FZD	Frizzled family of Wnt receptor
sFRP	Secreted Frizzled-related protein
VZ	ventricular zone
dp1–6	dorsal progenitor domains 1–6
P0–30	postnatal day 0–30
E9.5–18.5	Embryo day 9.5–18.5.

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Highlights

- We studied the expression pattern of *sFRP3* in the spinal cord and DRG in detail.
- *sFRP3* is transiently expressed in dp1-dp5 domains of VZ at early embryonic stages.
- *sFRP3* is strongly expressed in the laminae I/II of the dorsal horn of spinal cord.
- *sFRP3* may participate in the circuitry formation of sensory neurons.
- There is a distinct role of *sFRP3* in the postnatal development of DRG neurons.

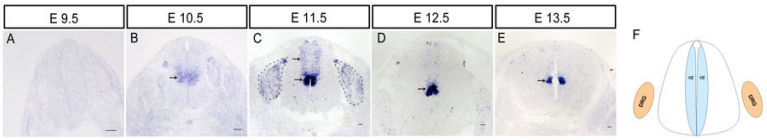

Fig. 1.

Dynamic expression of *sFRP3* in spinal cord and dorsal root ganglia at early embryo stages. **A–E:** Spinal cord sections from E9.5, E10.5, E11.5, E12.5, E13.5 were subjected to ISH with *sFRP3* riboprobe. **F:** Scheme of VZ and DRG in spinal cord. *sFRP3* expression was primarily detected in middle region of the spinal cord at E10.5. At E11.5, *sFRP3* expression in ventricular zone (VZ) was expanded to the entire dorsal half of the spinal cord and observed in most of dorsal root ganglia (DRG). By E12.5, *sFRP3* expression was down-regulated in dorsal VZ and DRG, but persisted in the middle VZ. Expression of *sFRP3* in VZ is indicated by arrows, and its expression in DRG is outlined by black dashed lines. Scale bar = 50 μm.

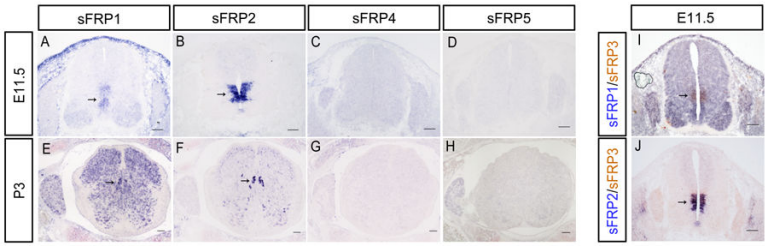

Fig. 2.

Expression of other *sFRPs* in spinal cord and dorsal root ganglia. **A–D:** E11.5 spinal cord sections were subjected to ISH with *sFRP1*, *sFRP2*, *sFRP4* and *sFRP5* riboprobes. **E–H:** P3 spinal cord sections were subjected to ISH with *sFRP1*, *sFRP2*, *sFRP4* and *sFRP5* riboprobes. **I–J:** Spinal cord sections from E11.5 embryos were subjected to ISH double labeling with digoxigenin-labeled *sFRP1* or *sFRP2* riboprobe (in blue) and fluorescein-labeled *sFRP3* riboprobe (in red). Expression of *sFRPs* and co-localization of *sFRP1/2* with *sFRP3* in the ventricular cells are indicated by arrows. DRG is outlined by black dashed lines. Scale bar = 100 μm.

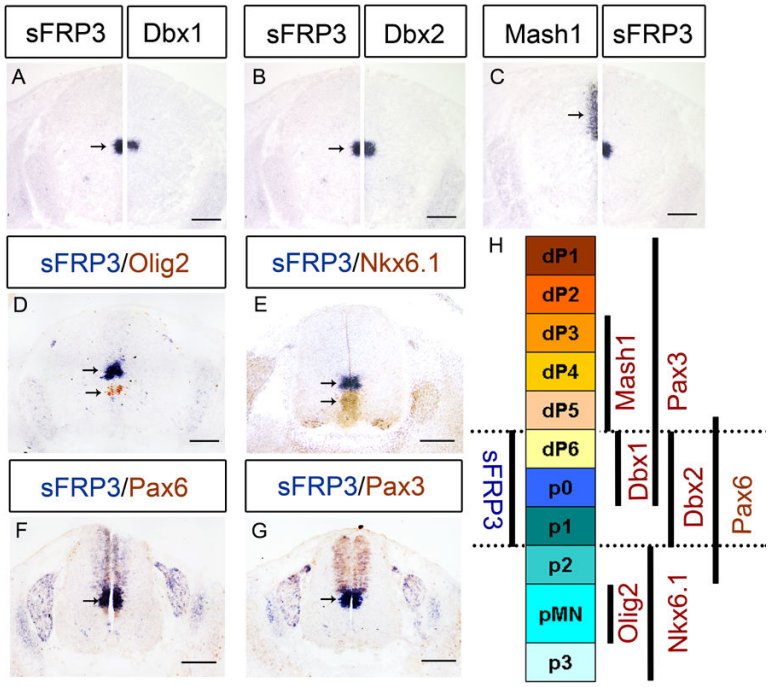

Fig. 3.

sFRP3 was expressed dp6 to p1 domains of ventricular zone at E12.5. **A–C:** Immediately adjacent sections from E12.5 spinal cord were subjected to *in situ* hybridization with *sFRP3*, *Dbx1*, *Dbx2* and *Mash1* riboprobes, and half sections were aligned at the midline. **D–G:** Spinal cords from E12.5 embryos were double-labeled with *sFRP3* (blue) and anti-Olig2 (brown in D), anti-Nkx6.1 (brown in E), anti-Pax6 (brown in F) or anti-Pax3 (brown in G). **H:** Scheme of *sFRP3* expression in progenitor domains labeled by various progenitor identity genes. Arrows indicate the ISH positive cells. Scale bar = 100 μm.

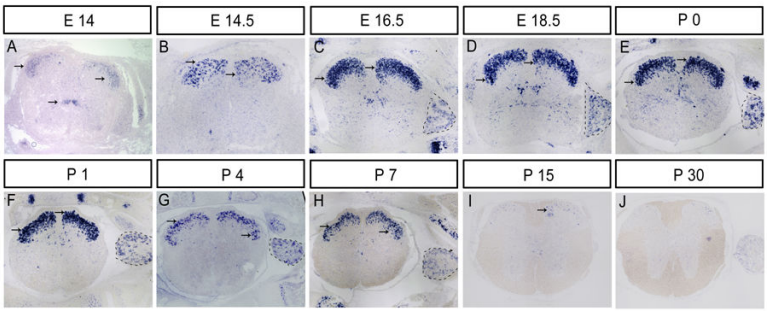

Fig. 4.

sFRP3 expression in spinal cords and dorsal root ganglia at later developmental stages. **A–J:** Spinal cord sections from E14, E14.5, E16.5, E18.5, P0, P1, P4, P7, P15 and P30 were subjected to ISH with *sFRP3* riboprobe. *sFRP3* expression was detected in spinal cord dorsal horn after E14 (indicated by arrows), and persisted till P30. *sFRP3* expression in the dorsal root ganglia (DRG) was up-regulated again at E16.5 (**C**). Arrows denote areas of *sFRP3* localization in spinal cord. DRG is outlined by black dashed lines. Scale bar = 100 μm.

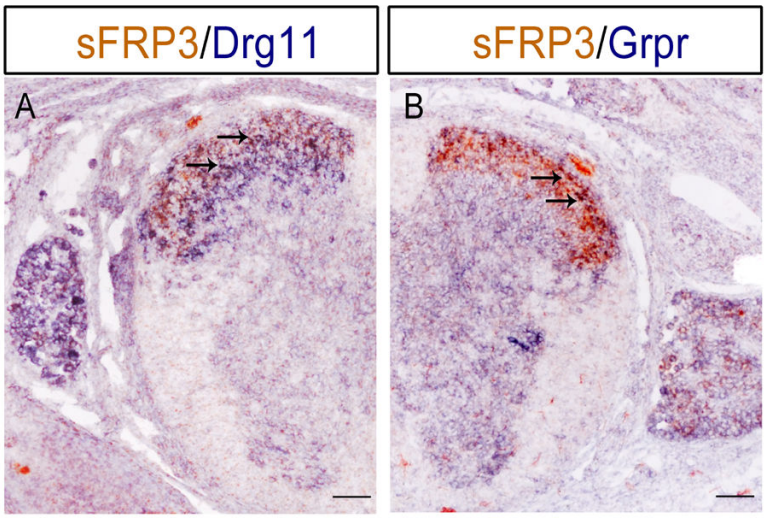

Fig. 5.

Co-expression of *sFRP3* with sensory neuronal markers in the dorsal spinal cord. Spinal cord sections from E16.5 embryos were subjected to ISH double labeling with digoxigenin-labeled Drg11 or Grpr riboprobe (in blue) and fluorescein-labeled *sFRP3* riboprobe. Representative double positive cells are indicated by arrows. Scale bar = 100 μm.

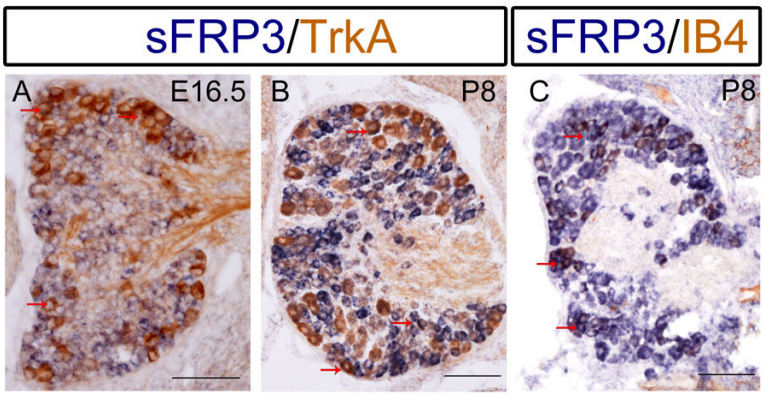

Fig. 6.

Co-expression of *sFRP3* with TrkA and IB4 in DRG neurons. DRG sections from E16.5 and P8 mice were subjected to *sFRP3* in situ hybridization (blue) followed by anti-TrkA (brown in A, B) or anti-IB4 (brown in C) immunostaining. Representative double positive cells are indicated by arrows. Scale bar = 100 μm.

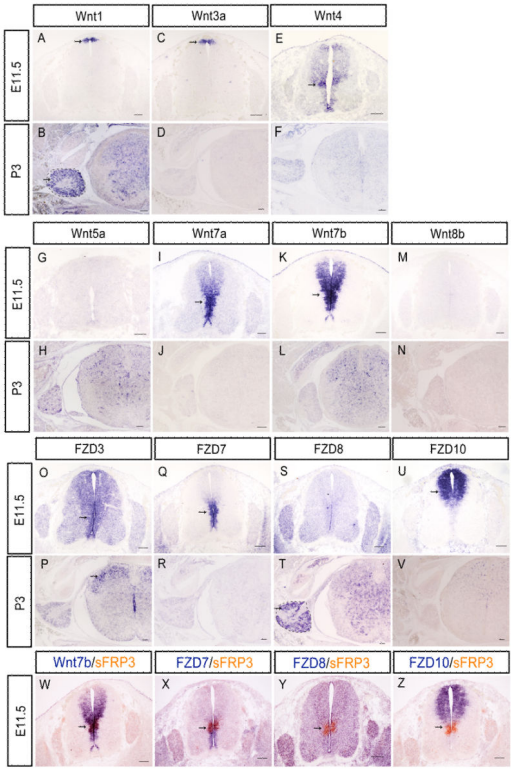

Fig. 7.

RNA in situ hybridization analysis of Wnts and their receptor gene expression in mouse embryonic (E11.5) and postanal (P3) spinal cord and DRG. **A–N:** E11.5 and P3 spinal cord sections were subjected to ISH with *Wnt1*, *Wnt3a*, *Wint4*, *Wnt5a*, *Wnt7a*, *Wnt7b* and *Wnt8b* riboprobes. **O–V:** E11.5 and P3 spinal cord sections were subjected to ISH with *FZD3*, *FZD7*, *FZD8* and *FZD10* riboprobes. **W–Z:** Spinal cord sections from E11.5 embryos were subjected to ISH double labeling with digoxigenin-labeled *Wnt7b*, *FZD7*, *FZD8* or *FZD10* riboprobe (in blue) and fluorescein-labeled *sFRP3* riboprobe (in red). The ISH positive cells are indicated by arrows, DRG is outlined by black dashed lines. Scale bar = 100 μm.

Table 1

The information of riboprobes used for ISH.

Gene	Primer (5'–3')	Length of probe (bp)
Sfrp1	CTTTAAGTGATACTGGGGCGGACT	764 bp
	AAGCAGACCCTTGCCTGGCATCC	
Sfrp2	TCGAGTACCAGAACATGCGGCTGC	1097 bp
	CAGACTGACCAAAGAGAGAAAATGCC	
Sfrp3	CGCCGTTGTGGAAGTGAAGG	1137 bp
	GAGCATCCAACAATGGGTTT	
Sfrp4	TCTATGACCGTGGAGTTTGTATCT	1109 bp
	AGTATCATTTCCCCTGCCTTTATC	
Sfrp5	CCACCTACTTGGACGACCCTAGTA	589 bp
	GGTCGTTGTCCAGGGGGAACTTGT	
Wnt1	CTTCGAGAAATCGCCCAACTTCTGC	827 bp
	AGGCATGGTTCACAGCTGTTCAATGG	
Wnt3a	GTTCCTACTTGGAGGGGTCTCTTAC	913 bp
	CTATCATACGAGGCTGTCATGCC	
Wnt4	GAGAAGTTTGACGGTGCCACGGAG	1029 bp
	TCACGGTTGCTTGCCTGGAATCTA	
Wnt5a	AGTCCCACTCCCAGGACCCACT	857 bp
	\GGCTAACACAAGATTATGG	
Wnt7a	GGACAGTCTCCAGTGCCTAGC	810 bp
	AGTGTGGTCCAGCACGTCTTAGTG	
Wnt7b	ACGCAATGGTGGTCTGGTACC	978 bp
	TGAGGAAATGGGACATTAAGCTTC	
Wnt8b	CATCTCTCAATGTTTGAGTCGCT	825 bp
	GTCTTGTTCTCCAGGCAGTAGTCTG	
Fzd3	CAGATTCCGTTACCCTGAAAGAC	894 bp
	GAGCACCTGCCGGCTCTCATTCAC	
Fzd7	CGCTTCTACCACAGACTCAGCC	745 bp
	ACCAACTTCACGCTAGGACTCT	
Fzd8	CTCTACAACCGCGTCAAGACC	776 bp
	ACGAAGCCAGCTAACAGAAACATAGTC	
Fzd10	CAAGGACATCGGCTACAACACC	790 bp
	CTCCAGTCCTTCCTGGATAACATAC	
Dbx1	GATTCTGATGAGGATGAGGAGGG	554 bp
	GCTTGCTTGATAGTGCTTTCC	
Dbx2	CACAGCTTTTTCAAGAGAAGAACACGG	529 bp
	TCAAATGGTGCTCTGGAGTCCATG	
Mash1	CTAACAGGCAGGGCTGGAAGCGCG	737 bp
	AAGGGGTGGGTGTGAGGGGGAAGGC	
Drg11	CCGTCGGCGATGTTTTATTTCCACT	1311 bp

Gene	Primer (5'–3')	Length of probe (bp)
	CCAAGAAGTTCAGTAAGCCGTAAGT	
Grpr	TCTACCTGTACCGTTCCTACCACT	1219 bp
	CAGTTAACCCTAAGCAAATACAC	