Pax-6 Regulates Expression of *SFRP-2* and *Wnt-7b* in the Developing CNS

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Wnt signaling regulates a wide range of developmental processes such as proliferation, cell migration, axon guidance, and cell fate determination. In this report, we studied the expression of *secreted frizzled related protein-2* (*SFRP-2*), which codes for a putative Wnt inhibitor, in the developing nervous system. *SFRP-2* is expressed in several discrete neuroepithelial domains, including the diencephalon, the insertion of the eminentia thalami into the caudal telencephalon, and the pallial–subpallial boundary (PSB). We also noted that *Wnt-7b* expression was similar to *SFRP-2* expression. Because many of these structures are disrupted in *Pax-6* mutant mice, we examined *SFRP-2* and *Wnt-7b* expression in the forebrains of *Pax-6 Sey/Sey* mice. We found that *Pax-6* mutants lack *SFRP-2* expression in the PSB and diencephalon. Interestingly, *Pax-6* mutants also lack *Wnt-7b* expression in the PSB, but *Wnt-7b* expression in the diencephalon is preserved. Furthermore, in the spinal cord of *Pax-6* mutants, *SFRP-2* and *Wnt-7b* expression was greatly reduced. Our results suggest that by virtue of its apposition to *Wnt-7b* expression, *SFRP-2* may modulate its function, particularly at boundaries such as the PSB, and that changes in Wnt signaling contribute to the phenotype of *Pax-6* mutants.

Key words: pallial–subpallial boundary; zona limitans intrathalamica; SFRP-2*;* Wnt-7b*;* Pax-6*; small eyes (Sey); prosomere; lateral ganglionic eminence; caudal ganglionic eminence; eminentia thalami*

The restricted expression of transcription factors and secreted molecules suggests that the developing forebrain is organized into a series of specific transverse and longitudinal domains (Bulfone et al., 1993). The boundaries between these regions correlate with sites in which there are discontinuities in cell fate, cell migration, and the trajectories of axons (Neyt et al., 1997; O. Marin and J. L. Rubenstein, unpublished observations). The analysis of mutants with defects in forebrain patterning has shed light on the formation and function of these boundary zones. In particular, *Pax-6* mutants have a defect in forming the pallial–subpallial boundary (PSB) (Stoykova et al., 1997; Chapouton et al., 1999; Toresson et al., 2000; Yun et al., 2001).

Wnts are a family of secreted proteins involved in several processes, including cell proliferation (Ikeya et al., 1997; S. M. Lee et al., 2000), dorsal–ventral patterning (Saint-Jeannet et al., 1997), and axonal remodeling and synaptogenesis (for review, see Wodarz and Nusse, 1998; Hall et al., 2000). Wnts transduce their signals through the Frizzled family of Wnt receptors (Bhanot et al., 1996). The secreted frizzled related protein (SFRP) family 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).

Here we studied the expression of *SFRP-2* in the embryonic mouse forebrain and found that its expression matches a subset the expression of *Wnt-7b*. We show that *SFRP-2* is expressed in progenitor cells that flank the PSB and abut the boundary between the eminentia thalami and the caudal basal telencephalon. *SFRP-2* is also expressed in a discrete diencephalic longitudinal domain. We also show that *Pax-6 Sey/Sey* mutant mice, which are known to have defects at the PSB, lack *SFRP-2* and *Wnt-7b* expression at the PSB and lose *SFRP-2* expression in the diencephalon. *SFRP-2* and *Wnt-7b*, which are normally expressed in overlapping regions in the ventral and intermediate zones of the spinal cord, are also missing in this region in *Pax-6* mutants. Our analysis shows that *Pax-6* is required for *SFRP-2* and *Wnt-7b* expression at the PSB and in the spinal cord and raises the possibility that Wnt signaling is involved in the formation and/or function of the PSB.

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MATERIALS AND METHODS

Animals, genotyping, and tissue preparation. All animals were handled according to protocols approved by the Committee on Animal Research at the University of California, San Francisco. Mouse embryos were obtained from timed pregnancies and genotyped according to previously described protocols [*Pax-6 Sey-1neu* allele (Hill et al., 1991); *Dlx-1*/*Dlx-2* mutants (Anderson et al., 1997a)]. We examined two pairs of *Dlx-1*/*Dlx-2* mutants and wild types at embryonic day 13.5 (E13.5), one pair at E14.5, two pairs at E16.5, and two pairs at postnatal day 0 (P0). We examined three pairs of *Pax-6 Sey*/*Sey* and *Pax-6* +/*Sey* mutants at E10.5 and E13.5. In addition, for the wild-type observations, we examined at least three to five CD1 mice at each time point. Postnatal animals were anesthetized by cooling or pentobarbital and perfused with and then post-fixed in 4% paraformaldehyde (PFA) in diethypyrocarbonate (DEPC)-treated PBS. Embryos were immersion-fixed in 4% PFA in DEPC–PBS for 24 hr. After fixation, brains or embryo heads were either cryoprotected in 30% sucrose, frozen in embedding medium (optimal cutting temperature medium) and sectioned using a cryostat, or used for whole-mount *in situ* hybridization.

Riboprobes. Transcription of plasmids containing cDNAs of interest was performed with RNA polymerase (Roche, Basel, Switzerland) in the presence of digoxigenin-labeled UTPs (Roche). Probes included *SFRP-2* (J. Nathans, Johns Hopkins University, Baltimore, MD), *Wnt-7b* (A. McMahon, Harvard University, Cambridge, MA), *Pax-6* (P. Gruss, Max Planck Institute, Gottingen, Germany), and *Dlx-2* (Bulfone et al., 1993). Riboprobes were hydrolyzed to 250 bp before use.

In situ *hybridization.* Nonradioactive *in situ* hybridization of tissue sections was performed using a protocol obtained from D. Anderson (California Institute of Technology, Pasadena, CA), which was modified from published protocols (Schaeren-Wiemers and Gerfin-Moser, 1993) as described previously (Pleasure et al., 2000). Whole-mount *in situ* hybridization of E10.5 mouse embryos was performed using protocols obtained from D. Anderson; after staining, the embryos were sectioned at 100 μm using a vibrating microtome.

RESULTS

Expression of *SFRP-2* is concentrated at the PSB, diencephalon, and spinal cord during embryonic CNS development

Other reports have focused on expression of Wnts in the developing CNS, but there have been few studies describing the expression of SFRPs (Parr et al., 1993; Leimeister et al., 1998; Wawersik et al., 1999; Baranski et al., 2000; Ladher et al., 2000; C. S. Lee et al., 2000). None of these reports have given a detailed description of *SFRP* expression during later development of the forebrain and none have compared the expression patterns of Wnts and SFRPs.

We examined *SFRP-2* expression in coronal sections of the developing mouse forebrain. *SFRP-2* was detectable weakly throughout the developing neuroepithelium at E10.5, with a sharply demarcated region of higher expression in a restricted domain in the diencephalon. Its posterior boundary approximated the zona limitans intrathalamica (ZLI). [The ZLI is the boundary between the dorsal and ventral thalamus and corresponds to the prosomere 2 (p2)/p3 boundary of the prosomeric model (Puelles and Rubenstein, 1993).] Its rostral boundary approximated the p4/p5 limit. The dorsal part of this domain, the eminentia thalami (EMT), entered the caudal telencephalon (Fig. 1*A*). This pattern of expression was maintained at E14.5, although expression in p5 was more prominent (Fig. 1*C*). Expression in the EMT is topographically complex (Fig. 1*B*); its limit in the caudal telencephalon is a small domain flanking the caudal ganglionic eminence (CGE) (Fig. 1*C*).

By E12.5–E13.5, *SFRP-2* expression is found on the pallial side of the PSB, in the ventricular zone of a progenitor zone known as the ventral pallium (VP) (Puelles et al., 2000) (Fig. 1*B*) (K. Yun and J. L. Rubenstein, unpublished observation). Its expression overlaps with strong *Pax-6* expression (Fig. 2*A,B*) and abuts the *Dlx-2* expression in the lateral ganglionic eminence (LGE) (Fig. 2*C,D*). *SFRP-2* expression in the VP domain widens in more caudal regions of the telencephalon and includes the lateral (dorsal) half of the CGE (Fig. 1*C,D*). The medial (ventral) side of the CGE has *SFRP-2* expression at the insertion of the EMT (Fig. 1*D*). The *SFRP-2*-negative domain within the CGE in Figure 1*D* may represent an oblique section including a portion of the subventricular zone, although the possibility of a *SFRP-2*-negative domain is this region cannot be ruled out (Puelles et al., 2000).

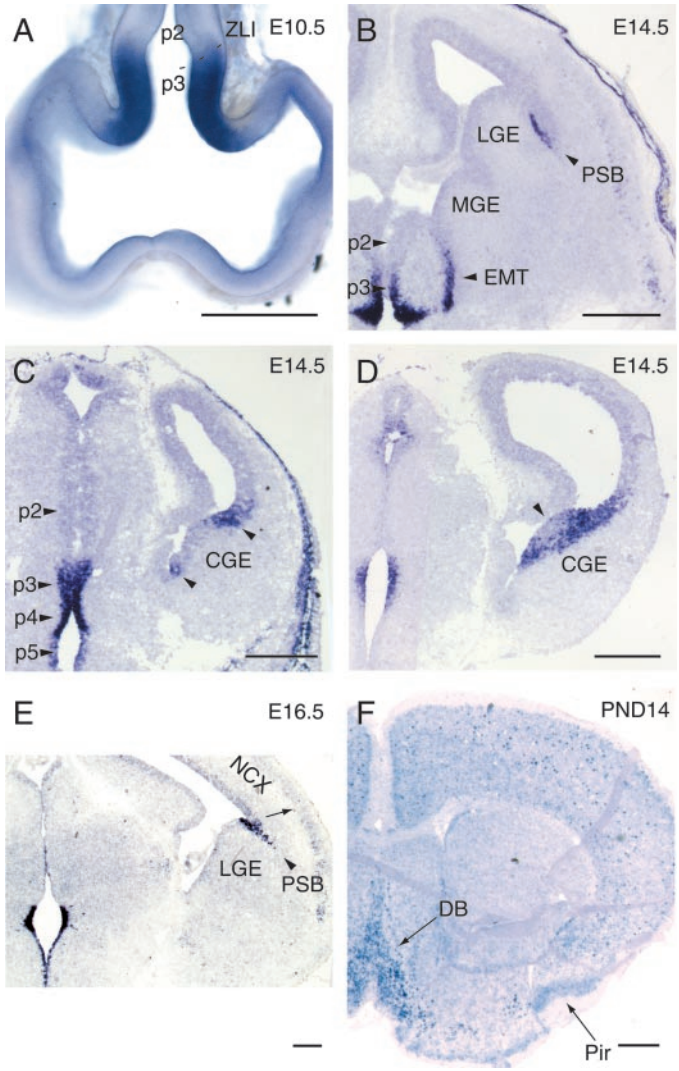

Figure 1. Expression of *SFRP-2* in the mouse embryonic and early postnatal forebrain. *A* is a 100 μm coronal section of an embryo after whole-mount *in situ* hybridization. The ZLI at the p2/p3 border is shown as a *dashed line*. *SFRP-2* expression in the ventricular zone begins to consolidate into the developing PSB and diencephalon at E14.5 (*B, C, D* show three different coronal levels from anterior to posterior to indicate the topographic relationship of the diencephalic expression domain and the expression in the CGE) and continues through E16.5 (*E*). *Arrowheads* in *C* point to *SFRP-2* expression at the PSB and at the insertion of the EMT. The *arrowhead* in *D* indicates an area of decreased *SFRP-2* expression in the CGE between the previously distinct PSB expression and EMT expression domains seen anteriorly in *C*. *SFRP-2* also begins to be expressed in scattered cells in the neocortex (*NCX*) at E16.5 (*arrow* in *E*). By postnatal day 14 (*PND14*), *SFRP-2* is expressed in scattered large cells in the cortical plate, amygdala, piriform cortex (*Pir*), and in the vertical limb of the diagonal band (*DB*) (*F*). Scale bars, 0.5 mm.

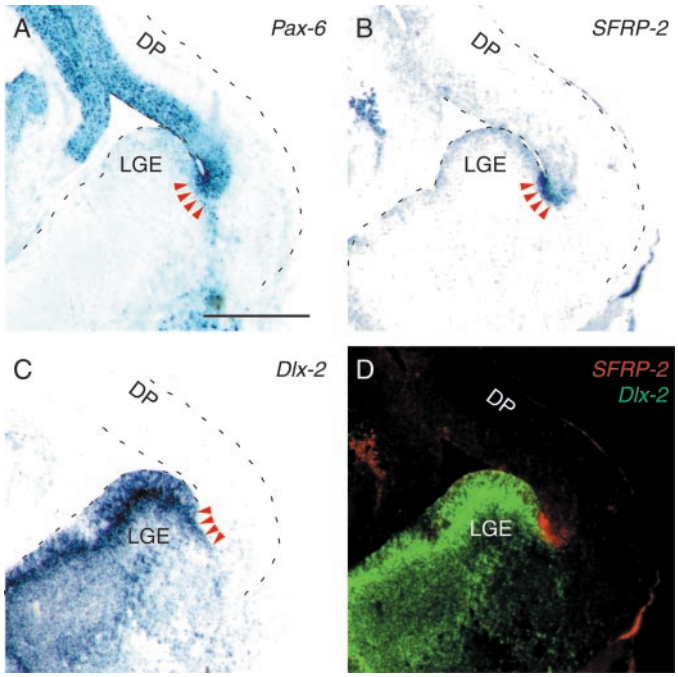

Figure 2. *SFRP-2*, *Dlx-2*, and *Pax-6* expression at the PSB in serial sections of E13.5 mouse forebrain. The *SFRP-2* and *Pax-6* expression domains are overlapping at the PSB (*A, B, D*). In contrast, the *SFRP-2* expression domain ends in a short border (*red arrowheads* in *B*) that corresponds to the edge of *Dlx-2* expression in the LGE (*red arrowheads* in *C*). *D* is a false color overlay of *B* and *C* (*SFRP-2*, *red*; *Dlx-2*, *green*) demonstrating the complementary expression of *SFRP-2* and *Dlx-2* at the PSB. *DP*, Dorsal pallium. Scale bars, 0.5 mm.

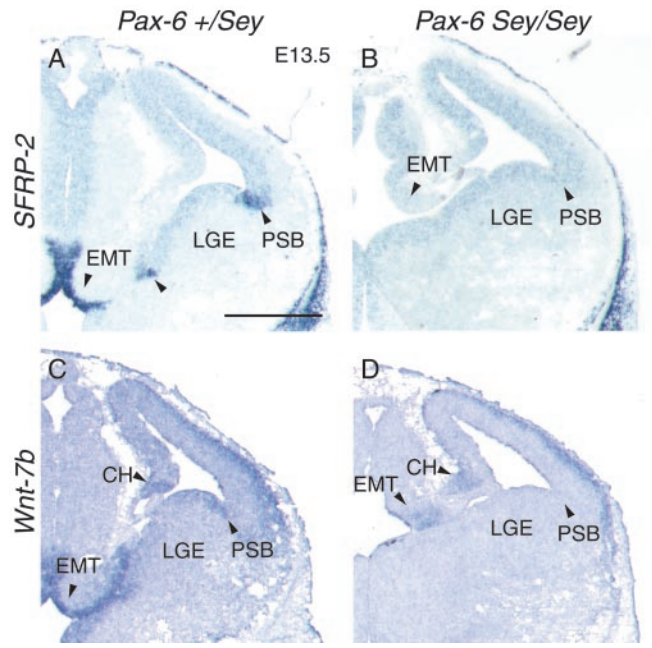

Figure 3. *SFRP-2* and *Wnt-7b* expression in the forebrain of E13.5 *Pax-6 +/Sey* and E13.5 *Pax-6* mutant animals. *SFRP-2* expression at the PSB and the diencephalon in the wild-type mice (*A*) is undetectable in the *Pax-6* mutant mice (*B*). *Wnt-7b* is undetectable near the PSB in *Pax-6* mutants (*D*) but remains in the diencephalon, cortical hem (*CH*), and cortical plate in wild-type mice (*C*). Note that *Wnt-7b* is expressed in the LGE near the PSB. Scale bars, 0.5 mm.

Later in embryonic development (E16.5), a new *SFRP-2* expression domain is detected in a laminar pattern in the lateral cerebral cortex (Fig. 1*E*), and there are scattered *SFRP-2*-positive cells in the amygdala, which is derived from the CGE. By P2, the expression in the diencephalon and the PSB was not detectable (data not shown). By P14, there are numerous *SFRP-2*-expressing cells scattered in the neocortex, amygdala, piriform cortex, and vertical limb of the diagonal band (Fig. 1*F*).

SFRP-2 expression is not affected by mutations in *Dlx-1*/*Dlx-2*

Mutation of the homeobox genes *Dlx-1* and *Dlx-2* causes developmental abnormalities in the subpallium and a reduction in the migration of immature interneurons across the PSB (Anderson et al., 1997a,b; Marin et al., 2000). Because these transcription factors are necessary for the development of the domain directly adjacent to the *SFRP-2* expressing neuroepithelium (the VP), and because *Dlx-1* and *Dlx-2* are also expressed in some of the regions of the diencephalon that express *SFRP-2*, we tested the effect of *Dlx-1*/*Dlx-2* mutations on the normally sharp border of the *SFRP-2* expression domain. However, we found that *SFRP-2* expression in both the VP and the diencephalon was unaffected in the forebrains of E13.5, E14.5, and E16.5 *Dlx-1*/*Dlx-2* mutant mice (data not shown).

Pax-6 regulates *SFRP-2* and *Wnt-7b* expression at the PSB, diencephalon, and spinal cord

Mice with mutations in the *Pax-6* paired homeobox gene have defects in many regions of the developing CNS, including in the PSB, EMT, and the ventral spinal cord. All of these regions express *SFRP-2*. Defects in the PSB include altered expression of transcription factors and cell adhesion molecules and disruption of radial glial fascicles (Stoykova et al., 1997; Götz et al., 1998; Chapouton et al., 1999; Toresson et al., 2000; Yun et al., 2001). It is postulated that changes in the PSB in *Pax-6* mutants may account for the increase in subpallial-to-pallial tangential migration observed in these embryos (Chapouton et al., 1999).

The *Pax-6* mutation also leads to a disruption of the ZLI at the p2/p3 boundary, to an abnormality in axon guidance of corticothalamic fibers, and to an altered expression of a variety of markers, including a broadening of the *Wnt-7b* expression domain (Stoykova et al., 1997; Grindley et al., 1997; Warren and Price, 1997). Because of these phenotypic changes, we decided to examine *SFRP-2* and *Wnt-7b* expression in *Pax-6* mutant mice with particular attention to the PSB and diencephalon.

In the *Pax-6* mutant, *SFRP-2* continued to be expressed at low levels throughout the ventricular zone of the forebrain, but strong *SFRP-2* expression was undetectable in the VP and diencephalon (Fig. 3*C,D*). Surprisingly, *Wnt-7b* expression was not detectable in the LGE and abutting the PSB in *Pax-6* mutants as well, but the expression of *Wnt-7b* was intact in the diencephalon, fimbria, and cortical plate (Fig. 3*E,F*).

In the spinal cord, *Pax-6* regulates dorsoventral patterning; *Pax-6* mutants have a dorsal expansion of ventral regulatory gene expression (Ericson et al., 1997). A previous report, which was focused on the regulation of lens development by *BMP-7*, showed that *Pax-6* mutants lack the *SFRP-2* expression in the ventral spinal cord (Wawersik et al., 1999). Another report indicated that *Wnt-7b* expression in the hindbrain and spinal cord was altered but not absent in *Pax-6* mutants (Osumi et al., 1997). To further investigate the relationship between the regulation of *SFRP-2* and *Wnt-7b*, we compared their spinal cord expression in normal mice and in *Pax-6* mutants. We found that *SFRP-2* and *Wnt-7b* are

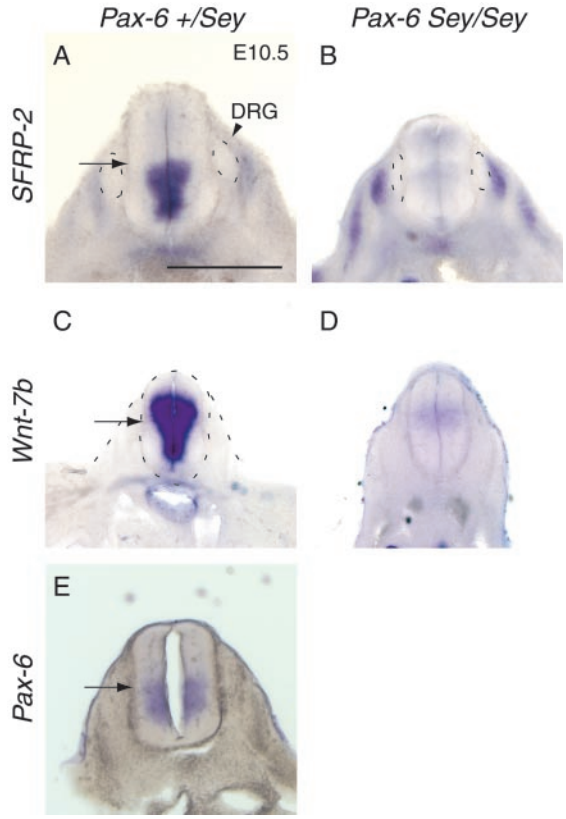

Figure 4. *SFRP-2*, *Wnt-7b*, and *Pax-6* expression in the E10.5 spinal cord as detected by whole-mount RNA *in situ* hybridization. The expression of *Wnt-7b* extends more dorsally than *SFRP-2* expression (*A, C*). Note the lack of *SFRP-2* expression in the dorsal root ganglion (*DRG*). *Pax-6* mutants lack both *SFRP-2* and *Wnt-7b* expression in the spinal cord (*B, D*). *Pax-6* expression is shown for comparison (*E*). *Arrows* show the dorsoventral midline of the spinal cord in *A* and *C*. The dorsal root ganglia, which do not express *SFRP-2* or *Wnt-7b*, are outlined with *dashed lines* in *A* and *B*. Scale bars, 0.5 mm.

expressed in overlapping domains in the intermediate and ventral zones of the spinal cord in wild-type E10.5 mice, with *Wnt-7b* expression extending more dorsally (Fig. 4*A,C*). The expression of both *SFRP-2* and *Wnt-7b* in the spinal cord was greatly reduced in *Pax-6* mutant mice (Fig. 4*B,D*). The previously described expression pattern of *Pax-6* in the spinal cord is shown in Figure 4*E* for comparison (Walther and Gruss, 1991; Goulding et al., 1993). The *Pax-6* expression in the spinal cord closely matches the *SFRP-2* expression domain but does not extend as far dorsally as the *Wnt-7b* expression domain.

DISCUSSION

We have shown that *SFRP-2* and *Wnt-7b* are expressed in spatially restricted patterns in the telencephalon, diencephalon, and spinal cord, and that their expression is reduced in *Pax-6* mutants but not in *Dlx-1*/*Dlx-2* mutants. These results suggest that some of the phenotypes in the *Pax-6* mutant may occur because of alterations in Wnt signaling.

SFRP-2 is expressed adjacent to the pallial–subpallial boundary and in the CGE

Previous studies have implicated Wnt signaling in regulation of the formation of boundaries in the developing CNS (Wodarz and Nusse, 1998). For example, *Wnt-1* is involved in the formation of the hindbrain–midbrain boundary (Bally-Cuif et al., 1995). Interestingly, there is evidence that the inhibition of cell migration through the proliferative zone at the PSB is regulated by an unidentified short-range diffusible signal (Neyt et al., 1997). Our data on *SFRP-2* expression are consistent with a potential role for *SFRP-2* in PSB function. Further studies will be required to determine whether *SFRP-2* directly regulates cell migration, proliferation, or differentiation at the PSB. Also, *SFRP-2* expression in much of the CGE (but not in the LGE and medial ganglionic eminence) and the amygdala, which is derived largely from the CGE, may imply an important role for Wnt signaling in the development of the CGE and its derivatives.

Pax-6 regulates *SFRP-2* and *Wnt-7b* expression

We have examined the expression of *SFRP-2* and *Wnt-7b* in mutants for three regionally expressed homeodomain transcription factors: *Pax-6* and *Dlx-1*/*Dlx-2*. *SFRP-2* expression appears to be unaffected in the forebrains of *Dlx-1*/*Dlx-2* mutant mice. However, *SFRP-2* expression is altered in the *Pax-6* mutants, and our findings suggest that the *Wnt* and *Pax-6* pathways appear to interact in a complex manner. Although *SFRP-2* expression is eliminated from the entire forebrain and spinal cord of *Pax-6* mutants, *Wnt-7b* expression appears to be eliminated only on the subpallial side of the PSB and in the spinal cord; *Wnt-7b* is still expressed in the diencephalon, cortical plate, and cortical hem, and *SFRP-2* continues to be expressed in the mesonephric duct and hindbrain (Wawersik et al., 1999; this study). The coordinate regulation of *Wnt-7b* and *SFRP-2* at the PSB may imply a functional association between these two molecules. In the diencephalon, where *Wnt-7b* expression persists, perhaps *SFRP-2* has a similar association with other Wnts expressed in the diencephalon (Puelles and Rubenstein, 1993). *Wnt-7b* expression extends into the PSB and the dorsal spinal cord beyond its region of overlap with *Pax-6* and *SFRP-2*. However, the entire *Wnt-7b* domain is disrupted in these regions in *Pax-6* mutants, which may imply a similarity in these two regions as postulated previously by others (Toresson et al., 2000; Yun et al., 2001).

Wnt and *Wnt* inhibitor interactions

The overlapping and adjacent expression patterns of *SFRP-2* and *Wnt-7b* at the PSB, the diencephalon, the hindbrain, and the spinal cord invite questions about the complex functional interactions of Wnts and Wnt inhibitors (Christian, 2000). For instance, at the PSB, *SFRP-2* and *Wnt-7b* expression appear to be complementary, whereas in the diencephalon, *SFRP-2* and *Wnt-7b* expression is largely overlapping. Similarly, the ventral half of the spinal cord expresses both *Wnt-7b* and *SFRP-2*, whereas the dorsal spinal cord expresses only *Wnt-7b*. Two other Wnts, *Wnt-3* and *Wnt-3a*, are also expressed in the diencephalon, but they have stronger expression in the dorsal thalamus, whereas *Wnt-7b* and *SFRP-2* are expressed in the ventral thalamus (Roelink and Nusse, 1991; Bulfone et al., 1993).

Why might Wnts and Wnt inhibitors be expressed in overlapping domains? One possibility is that SFRPs function to modulate the effects of Wnt by selectively inhibiting certain Wnts in a localized region. SFRP-2 has been shown to inhibit XWNT-8 activity (Ladher et al., 2000), and SFRP-1 has been shown to selectively inhibit WNT-1 signaling but not WNT-5a signaling (Dennis et al., 1999). In this model, Wnt signaling in a region may be determined by the combination of graded differences in Wnt inhibition by SFRPs and graded expression of Wnts.

Another possibility is that SFRPs bind and concentrate Wnts within a localized region. This could serve to potentiate Wnt

activity by increasing its local concentration in a given area or it could work to delimit the area of Wnt activity by binding to and limiting the diffusion of Wnts. As an example, SFRP-2 at the PSB might prevent WNT-7b from diffusing across the PSB to the neocortex, thereby limiting WNT-7b activity to the subpallial side of the PSB. Further studies examining the precise interactions between specific Wnts and SFRPs will be required to evaluate these possibilities.

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