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Lhx5 promotes forebrain development and activates transcription of secreted Wnt antagonists

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In vertebrate embryos, induction and patterning of the forebrain require the local inhibition of caudalizing signals, such as Wnts, emanating from the mesendoderm and caudal brain. Here, we report that Lhx5, expressed in the rostral neuroectoderm, regulates the local inhibition of Wnts. Activation of Lhx5 expands forebrain structures, whereas inhibition of Lhx5 function compromises forebrain development in zebrafish embryos. Lhx5 can rescue forebrain deficiencies caused by excess Wnt activity, and inhibition of Lhx5 function results in ectopic activation of Wnt signaling. Lhx5 regulates the expression of two secreted Frizzled-related Wnt antagonists, Sfrp1a and Sfrp5. These Sfrps can reduce the ectopic activation of Wnt signaling and rescue the forebrain deficiencies caused by inhibition of Lhx5 function. Our results demonstrate that Lhx5 is a required factor that promotes forebrain development and inhibits Wnt signaling by activating the transcription of secreted Wnt antagonists.

KEY WORDS: Axin, Forebrain, Lhx5, LIM-homeobox domain factor, *masterblind*, Secreted Wnt antagonist, Sfrp, Wnt signaling, Zebrafish

INTRODUCTION

The vertebrate forebrain derives from the rostral neuroectoderm and consists of the telencephalon and diencephalon. During blastula stages, prospective rostral ectodermal cells start to develop into neural precursor cells under the influence of extrinsic signals. During gastrulation, neural precursor cells retain rostral characteristics in the presumptive forebrain region, where the activities of ventralizing and caudalizing signals such as Bmps, Wnts and Fgfs are inhibited (Wilson and Houart, 2004). Later in development, local interactions within the forebrain pattern and regulate the differentiation and growth of rostral neural precursor cells to give rise to various forebrain structures (Munoz-Sanjuan and Brivanlou, 2002; Wilson and Edlund, 2001; Wilson and Houart, 2004). Functional studies of these extrinsic signals, produced by the mesendoderm and its derivatives, and of their inhibitors have contributed significantly to our understanding of forebrain development (Harland and Gerhart, 1997).

The functional roles that the rostral neuroectoderm itself may play in formation of the forebrain are less well understood. Several studies have suggested that rostral neuroectoderm factors antagonize ventralizing and caudalizing signals. In the *Xenopus* blastula organizer, for example, cells required for brain formation express the Bmp signaling inhibitors Chordin and Noggin (Kuroda et al., 2004). In zebrafish, Tlc, a secreted frizzled-related protein expressed at the rostral margin of the neural plate, antagonizes Wnt signaling (Houart et al., 2002). In mouse, Six3 in the rostral ectoderm directly represses *Wnt1* expression (Lagutin et al., 2003). In *Xenopus*, Shisa functions in the endoplasmic reticulum to antagonize Wnt and Fgf signaling by preventing the maturation of Wnt receptors and Fgf receptors (Yamamoto et al., 2005).

How are these antagonists of caudalizing signals regulated? We discovered that Lhx5, a transcription factor expressed by rostral ectoderm, is a regulator of Wnt antagonists. Lhx5 belongs to a family of LIM-homeodomain transcription factors, and contains two LIM protein interaction domains and a homeodomain (Hobert and Westphal, 2000; Retaux and Bachy, 2002; Toyama et al., 1995). Previous studies suggested that Lhx5 regulates the differential adhesion of early ectodermal cells in *Xenopus* (Houston and Wylie, 2003), and morphogenesis and cell proliferation in the mouse hippocampus (Zhao et al., 1999). We show that Lhx5 gain of function in zebrafish inhibits Wnt signaling, whereas inhibition of Lhx5 results in ectopic activation of Wnt signaling and forebrain defects. Lhx5 regulates expression of the secreted Wnt antagonists Sfrp1a and Sfrp5, and Sfrp gene gain of function can rescue the forebrain developmental defects caused by the inhibition of Lhx5 function. We propose that Lhx5 is an intrinsic factor required for forebrain development because it inhibits Wnt signaling by regulating the local expression of secreted Wnt antagonists.

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MATERIALS AND METHODS

Fish maintenance and genotyping

Zebrafish were maintained as described (Westerfield, 2000). The *masterblind* line (*mbl*^{tm213}) was provided by the Zebrafish International Resource Center (Eugene, OR). RFLP genotyping of *mbl*^{tm213} embryos was carried out by PCR using the primers listed below. The *mbl*^{tm213} mutation abolishes a *Bpm*I site in the amplified fragment.
mbl-forward, 5′-GAGGTGTTTTCTCCACAGCATC-3′; and
mbl-reverse, 5′-TACACCAGGAAATTCATCCAGTC-3′.

Whole-mount in situ hybridization

Embryos were staged as described (Kimmel et al., 1995). The sphere-dome transition was used as the reference time point to stage blastula and early gastrula embryos.

Whole-mount in situ hybridization was performed as described (Thisse et al., 1993; Whitlock and Westerfield, 2000). The clones used in this study have been previously described: *lhx5* (Toyama et al., 1995), *ptc1* (Concordet et al., 1996), *emx3* (Morita et al., 1995), *pax6a* (Puschel et al., 1992) and *pax2a* (Krauss et al., 1991). The clone used for the synthesis of the *six3b* probe was obtained from the Zebrafish International Resource Center.

Cloning and phylogeny

Zebrafish Sfrp genes were amplified by RT-PCR based on the sequences obtained from a BLAST search of the zebrafish genome (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/d_rerio and http://www.ensembl.org/Danio_rerio/).

Multiple sequence alignment was performed with ClustalX using BLOSUM protein weight matrix (Thompson et al., 1994). The phylogenetic tree was reconstructed by a Bayesian method with MrBayes

(http://mrbayes.csit.fsu.edu/index.php), using mixed model and Gamma distributed rates. The phylogram was drawn using TreeView (Page, 1996). Gene name assignments were corroborated by synteny analyses (details available upon request) and approved by the Zebrafish Nomenclature Committee (http://zfin.org/zf_info/nomen.html).

Sequences for *sfrp1a* and *sfrp5* (Hirate et al., 2001) are essentially identical to two existing mRNA sequences in GenBank (NM_205585 and NM_131858). Zebrafish *axin2* (Shimizu et al., 2000) and *wnt8a* orf1 (Lekven et al., 2001) were amplified by RT-PCR based on the GenBank sequence NM_131561 and NM_130946, respectively.

Synthetic mRNA and morpholinos

PCR-amplified regions containing *lhx5* (–295 to 1251, using the first nucleotide of the start codon as the reference), *lhx5trunc* (–295 to 799, encodes amino acid residues 1-266, *wnt8a* orf1 (–15 to 1094), *sfrp1a* (–152 to 891) and *sfrp5* (–152 to 1605) were inserted into the pCS2 vector. The *Drosophila* Engrailed repressor domain (residues 2-297) was fused to Lhx5 at the C terminus of the homeodomain by subcloning a T4 polymerase-treated *Sph*I-*Xba*I fragment of pCS2-EnR into the *Bbs*I-*Xba*I cut and T4 polymerase-treated pCS2-lhx5 construct. The dosages for mRNA injection were 200 pg of *lhx5*, 200 pg of *lhx5trunc*, 20 pg of *wnt8a* orf1, 200 pg of *lhx5-en*, 300 pg of *sfrp1a* and 100 pg of *sfrp5* mRNA per embryo.

Morpholino (Gene Tools) *lhx5*-MO targets the translation start site and *lhx5*-e3i3 targets the third intron splice donor site. The dosages injected were 2 ng or 4 ng of *lhx5*-MO, and 5 ng of *lhx5*-e3i3 per embryo.

lhx5-MO, 5′-GTGCACCATCATTCCGCCCTGGAGG-3′; and
lhx5-e3i3, 5′-GTGCGTTGTTCTCACCTGAATCACC-3′.

Primers used in RT-PCR shown in Fig. S1 in the supplementary material are listed below:

lhx5-exon 3-forward, 5′-GATCAAATCCAGGACGACACGAAG-3′;
lhx5-exon 5-reverse, 5′-GAACCCGAGCTGAGAAGATAAGG-3′;
odc1-forward, 5′-CGAACCCTGATGTACTACGTGAATG-3′; and
odc1-reverse, 5′- CAGGCTGCACTGCTCCACAATG-3′.

Cell transplantation

Transplantations were carried out as described (Dutta et al., 2005). Cells were taken from the animal poles of midblastula stage donor embryos and transplanted to the animal poles of late blastula stage hosts. Donor embryos were injected with lysine fixable fluorescein dextran for tracing (Molecular Probes), and donor cells within host embryos were revealed in red by antifluorescein antibody labeling.

Chromatin immunoprecipitation

Chromatin immunoprecipitation experiments were carried out according to a standard protocol (Oberley and Farnham, 2003). In brief, embryos were injected with *lhx5-GFP* mRNA at the one-cell stage and fixed with 1% formaldehyde at 80% epiboly. Uninjected embryos were treated in parallel and served as controls. Cross-linked chromatin samples were fragmented to an average length of 1 kb by sonication. Rabbit anti-GFP antibody (1 μl) was added to each chromatin sample containing approximately 10^7 copies of haploid genome. DNA samples from immunoprecipitated chromatins were purified and then analyzed by PCR (primers listed below). Similar results were obtained from three independent injections and chromatin preparations.

sfrp1a promoter-forward, 5′-GTGTGGAACTCTCCAACAGGAG-3′;
sfrp1a promoter-reverse, 5′-TGGCTGTGAGTGGAAAAGTGAC-3′;
aldoaa-forward, 5′-GCAGACATTTGAGAGATGAAAGG-3′;
aldoaa-reverse, 5′-CATGCTGCTACATGCACAAACTG-3′;
bactin2-forward, 5′-TCGATTACCGATTAAACGTGGAC-3′; and
bactin2-reverse, 5′-CGCACCAATACCACTCAACAAG-3′.

RESULTS

Lhx5 promotes and is required for forebrain formation

The *lhx5* gene is broadly expressed in the early embryo but is later restricted to the nervous system (Toyama et al., 1995). We first detect *lhx5* transcripts on the presumptive dorsal side of midblastula stage embryos at 40% epiboly. By the onset of gastrulation, *lhx5* mRNA is distributed in a dorsal to ventral gradient in the rostral ectoderm (Fig. 1A,B). During gastrulation, *lhx5* expression is restricted to the presumptive forebrain (Fig. 1C,D). Later, *lhx5* expression is further restricted to subdomains in the telencephalon, diencephalon, tegmentum, hindbrain and spinal cord (Toyama et al., 1995).

We find that excess Lhx5 activity expands the size of the forebrain. Injection of *lhx5* mRNA into one-cell stage embryos results in enlarged rostral head structures by mid-somitogenesis stages when compared with uninjected control embryos (Fig. 1E,F; 40%, *n*=108; see also Fig. S2 in the supplementary material). Expansion of presumptive forebrain in the injected embryos is also indicated by an enlarged *pax6a* expression domain in the rostral neural plate by the end of the gastrulation (Fig. 1G,H; 57%, *n*=65). To examine the relative expansion of different brain regions, we labeled *lhx5* mRNA-injected embryos and uninjected controls with the presumptive telencephalon marker *emx3*, the mid-hindbrain boundary marker *pax2a*, and the hindbrain marker *egr2b* (Fig. 1I-K, 70%, *n*=30; Fig. 1L-N, 53%, *n*=32). These markers indicate that the presumptive forebrain is expanded, whereas the midbrain and hindbrain are unaffected.

Lhx5 activity is required for forebrain development. To inhibit Lhx5 function, we used two approaches: overexpression of a dominant repressor construct that produces a dominant interfering protein and injection of antisense morpholino oligonucleotides that block Lhx5 protein synthesis. We generated the dominant interfering construct, *lhx5-en*, by replacing the Lhx5 transcriptional activation domain with the *Drosophila* Engrailed repressor domain. Injection of *lhx5-en* mRNA results in embryos that lack the most rostral part of the head; posterior head structures and other parts of the embryo are unaffected (Fig. 1O,P; 36%, *n*=146). Expression of rostral neural plate markers, *emx3* (Fig. 1Q,R; 56%, *n*=32) and *six3b* (Fig. 1S,T; 62%, *n*=77), are significantly reduced or completely lost at tail bud stage in injected embryos. Expression of *wnt8b* is expanded rostrally into what remains of the forebrain by mid-somitogenesis (Fig. 1U,V; 39%, *n*=33). We obtain similar although generally less severe phenotypes with antisense morpholinos against *lhx5*. In the *lhx5* morpholino-injected embryos, the *six3b* expression domain is slightly reduced at tail bud stage (48%, *n*=67), *pax6a* in the posterior optic vesicle is significantly reduced at the 12-somite stage (Fig. 1W,X; 70%, *n*=84) and rostrolateral *pax2a* expression expands into the posterior-medial optic vesicle (Fig. 1Y,Z; 50%, *n*=96). The *lhx5* morpholino-injected embryos later develop small heads with small eyes (73%, *n*=175). Injection of a second morpholino that blocks the splicing of *lhx5* transcripts had similar effects on forebrain development (see Fig. S1A-D in the supplementary material; *pax6a*, 72%, *n*=32; *pax2a*, 78%, *n*=32).

The weaker effect of the morpholinos, when compared with the dominant interfering construct, may be due to an incomplete block of Lhx5 function. From RT-PCR analysis, we estimate that the *lhx5* splice-blocking morpholino reduces *lhx5* mRNA level to about 8% of control levels (Fig. S1H). Thus, it is possible that residual *lhx5* mRNA may have given rise to sufficient Lhx5 protein to allow a partial development of the forebrain in morpholino-injected embryos. Similarly, we cannot exclude the possibility that Lhx5-En may interfere with other LIM homeodomain factors by heterodimer formation between LIM domains (Hobert and Westphal, 2000). Nevertheless, these results together support the conclusion that Lhx5 is required for forebrain development.

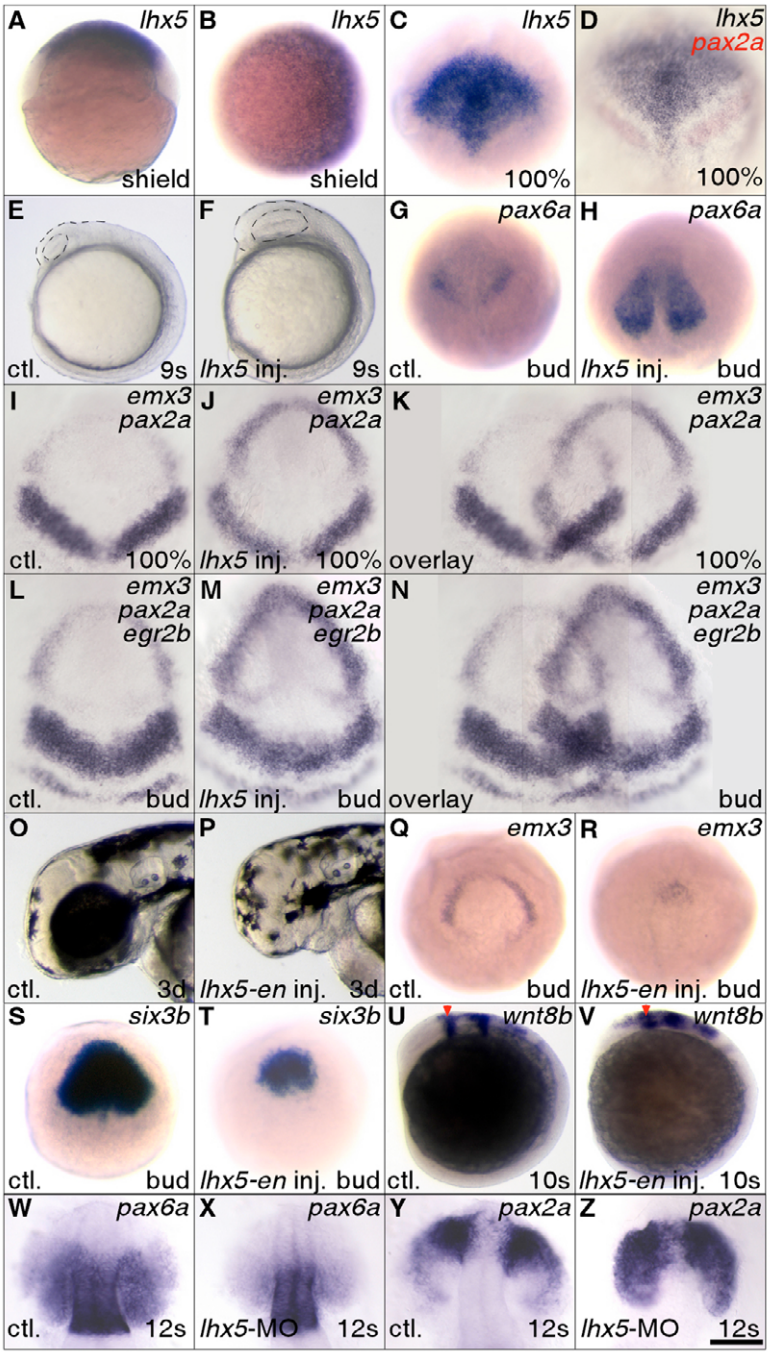

Fig. 1. Lhx5 promotes forebrain development. In this and subsequent figures, the probes used for whole-mount in situ hybridization are listed in the upper right corner of each panel. Genotypes or experimental manipulations are indicated in the lower left corners. Developmental stages are indicated in the lower right corners. Unless otherwise noted, gastrula stage embryos are orientated in animal pole view, rostral to the top; post-gastrulation stage embryos in lateral view, rostral to the top and dorsal to the right. ctl, control embryos. (**A-D**) *lhx5* is expressed in rostral regions during embryonic development. (A,B) Dorsal to the right, lateral (A) and animal pole (B) views. (D) A gap can be seen between the *lhx5* and *pax2a* expression domains. (**E-N**) *lhx5* gain of function causes expansion of the forebrain. (E,F) Forebrain boundaries are marked by broken lines. (G,H) Dorsal view, bud stage, *pax6a* expression. (I-N) Dorsal view, rostral to the top. Embryos were dissected and flat mounted in glycerol after in situ hybridization. Partial overlays of panels were made with PhotoShop (Adobe) and brightness in the overlapped regions was adjusted so that the backgrounds match. (**O-V**) Inhibition of Lhx5 function compromises forebrain development. (U,V) Red arrowheads mark the forebrain-midbrain boundary. (**W-Z**) *lhx5* morpholino knockdown alters *pax6a* and *pax2a* expression. Embryos were dissected and flat mounted in glycerol after in situ hybridization. Dorsal view, rostral to the top. Scale bar in Z: 250 μm for A-H,Q-V; 200 μm for I-K; 167 μm for L-N; 150 μm for O,P; 100 μm for W-Z.

Lhx5 activity rescues forebrain deficiencies caused by ectopic Wnt signaling

The expansion of forebrain we see in *lhx5* mRNA-injected embryos is also observed in embryos lacking *wnt8a* gene function (Erter et al., 2001; Lekven et al., 2001), and in embryos injected with Wnt inhibitors such as *dkk1* mRNA (Hashimoto et al., 2000). In addition, the expression domains of *emx3* and *six3b* are expanded in embryos injected with *wnt8b* morpholinos (Houart et al., 2002; Kim et al., 2002). Conversely, the compromised forebrain development caused by the inhibition of Lhx5 function is similar to defects in embryos with increased Wnt signaling caused by *wnt8a* mRNA injection (Kelly et al., 1995), or by mutations in the *masterblind* (*axin1*) gene (Heisenberg et al., 2001; van de Water et al., 2001). We thus examined interactions between Lhx5 activity and Wnt signaling (Fig. 2).

When we inject *wnt8a* mRNA alone into zebrafish embryos, the majority of the injected embryos (55%, *n*=102) fail to develop either one or both eyes when scored at late segmentation stages (Prim-5, 24 hours post-fertilization; Fig. 2A ; see also Table S1 in the supplementary material). The remaining affected embryos (43%) are malformed due to dorsalization during earlier development (Kelly et al., 1995). By contrast, when we co-inject *lhx5* mRNA with *wnt8a* mRNA, the effect of Wnt8a is suppressed; the majority of the injected embryos (57%, *n*=107) develop two eyes. As a control, we generated a truncated *lhx5* construct in which the transcriptional activation domain of Lhx5 is missing. When the truncated *lhx5* mRNA is co-injected with *wnt8a* mRNA, very few (14%, *n*=64) of the injected embryos form two eyes.

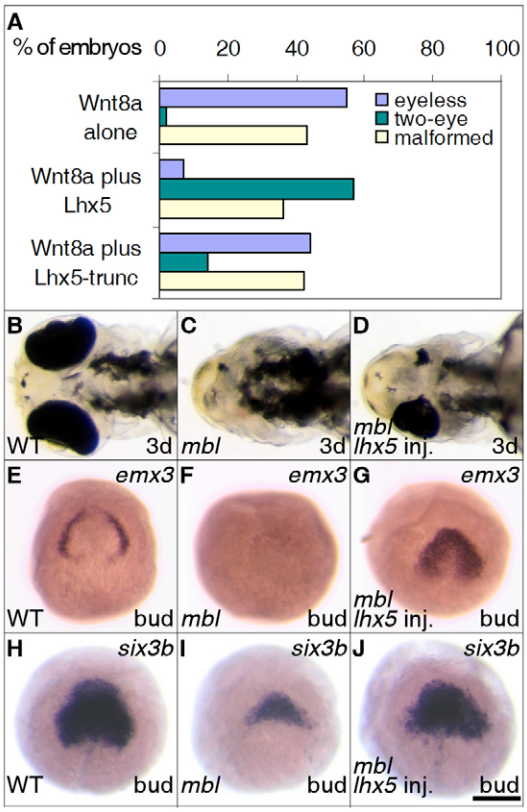

Fig. 2. Lhx5 partially rescues forebrain deficiencies induced by ectopic Wnt signaling. (**A**) *lhx5* gain of function partially rescues the eyeless phenotype of *wnt8a* mRNA-injected embryos. Injected embryos were scored after the Prim-5 stage (24 hours post-fertilization). The percentages of embryos eyeless (blue) or with two eyes (green), or malformed as a result of dorsalization or injection (yellow), were measured. (**B-D**) *lhx5* gain of function partially rescues eye development in *mbl* (*axin1*) mutants. Ventral views, rostral to the left. (**E-G**) *lhx5* gain of function partially restores *emx3* expression in *mbl* (*axin1*) mutants. Genotypes of mutant and partially rescued embryos were verified by RFLP. (**H-J**) *lhx5* gain of function partially restores *six3b* expression in *mbl* (*axin1*) mutants. WT, wild-type embryos. Scale bar in J: 150 μm for B-D; 250 μm for E-J.

We also examined the modulation of Wnt signaling by Lhx5 in *mbl* (*axin1*) mutant embryos. Previous studies suggested that the degradation of β-catenin is less efficient and Wnt signaling is overactivated in $mbl^{-/-}$ (*axin1*) embryos when compared with controls (Heisenberg et al., 2001; van de Water et al., 2001).

Due to the overactivated Wnt signaling, $mbl^{-/-}$ embryos consistently fail to form eyes (Fig. 2C). When we inject *lhx5* mRNA into embryos obtained from crosses of heterozygous $mbl^{+/-}$ parents, we observe a restoration of eye development in about a third of the $mbl^{-/-}$ homozygous mutant embryos (34%, n_{mbl}=41; affected embryos were genotyped to verify the homozygosity of the *mbl* mutation). Eye development is often better restored on one side of the rescued embryos (Fig. 2D), presumably as a result of asymmetric distribution of the injected *lhx5* mRNA. At tail bud stage, expression of *emx3* and *six3b* is significantly reduced or absent in $mbl^{-/-}$ embryos (Fig. 2F,I; *emx3*, 25%, *n*=36; *six3b*, 27%, *n*=59), whereas *emx3* and *six3b* expression is largely restored in the majority of $mbl^{-/-}$ embryos injected with *lhx5* mRNA (Fig. 2G,J; *emx3*, 58%, n_{mbl}=12; *six3b*, 76%, n_{mbl}=17).

Secreted Frizzled-related proteins, Sfrp1a and Sfrp5, antagonize Wnt signaling in the forebrain

To determine the mechanism by which Lhx5 inhibits Wnt signaling, we identified secreted frizzled-related proteins (Sfrps) (Jones and Jomary, 2002; Kawano and Kypta, 2003) as downstream targets of Lhx5. Sfrps are important Wnt regulators. Sfrps can bind directly to Wnts (Dennis et al., 1999; Lin et al., 1997; Uren et al., 2000; Xu et al., 1998) and they are dynamically expressed during development (Pera and De Robertis, 2000; Terry et al., 2000).

Based on the available genome sequence, we cloned five zebrafish Sfrp genes. All five Sfrps fall into a phylogenetic subgroup that includes Sfrp1, Sfrp2 and Sfrp5 (Fig. 3). On the basis of our mapping results (data not shown), we suggest that *sfrp1a* and *sfrp1b* have arisen from the extra genome duplication that occurred in the ray fin fish lineage (Postlethwait et al., 1998). Currently it is unknown how many Sfrp orthologs are present in the zebrafish genome.

We concentrated our studies on *sfrp1a* and *sfrp5* because their transcripts are present in the nervous system, as shown by whole-mount in situ hybridization. Transcripts for *sfrp2* and *sfrp2l* are found in cells that give rise to muscle, whereas *sfrp1b* is expressed in a region surrounding the yolk extension (data not shown) (Tendeng and Houart, 2006).

Previously, it was shown that overexpression of Sfrp1 blocks the dorsal axis duplication induced by *xwnt8* mRNA injection in *Xenopus* embryos, suggesting that Sfrp1 antagonizes Wnt signaling (Finch et al., 1997). To test whether Sfrp1a or Sfrp5 similarly antagonizes Wnt signaling in zebrafish, we injected *sfrp1a* or *sfrp5* mRNAs together with *wnt8a* mRNA. Both Sfrp1a and Sfrp5 rescue the Wnt8a-induced eyeless phenotype very effectively (Fig. 4A). In *sfrp1a* and *wnt8a* co-injected embryos, 80% of the embryos develop two eyes (*n*=64); co-injection of *sfrp5* with *wnt8a* rescues eye development in 93% of the injected embryos (*n*=70). Both Sfrps also rescue the early dorsalization of *wnt8a*-injected embryos equally well, suggesting that they have similar effects on Wnt signaling during early development (Fig. 4A).

Similar to Lhx5, overexpression of Sfrp1a or Sfrp5 promotes forebrain development. We injected *sfrp1a* or *sfrp5* mRNA into one- to two-cell stage embryos. We find that by mid-somitogenesis stages, *sfrp5*-injected embryos exhibit enlarged forebrains, whereas forebrain enlargement is less pronounced in *sfrp1a*-injected embryos (see Fig. S2 in the supplementary material). At the end of gastrulation, *sfrp1a* overexpression causes an expansion of the *emx3* and *six3b* expression domains in a small percentage of injected embryos (Fig. 4C,F; *six3b*, 10%, *n*=42; *emx3*, 22%, *n*=41), whereas *sfrp5* overexpression results in more robust expansion of the *emx3* and *six3b* domains (Fig. 4D,G; *six3b*, 60%, *n*=40; *emx3*, 53%, *n*=40, respectively).

We also examined whether the overexpression of *sfrp1a* or *sfrp5* can rescue forebrain development in $mbl^{-/-}$ embryos. *sfrp1a*, *sfrp5* or *sfrp1a* plus *sfrp5* mRNA injection fails to restore eye development to $mbl^{-/-}$ embryos when scored at 3 days of development. Nevertheless, *emx3* expression is fairly well rescued in $mbl^{-/-}$ embryos injected with *sfpr1a* and *sfrp5* mRNA together (Fig. 4J; 58%, n_{mbl}=12) or with *sfrp5* mRNA alone (62%, n_{mbl}=13). Expression of *six3b* is also partially rescued in $mbl^{-/-}$ embryos injected with *sfrp1a* and *sfrp5* together (Fig. 4M; 60%, n_{mbl}=10) or with *sfrp5* mRNA alone (67%, n_{mbl}=12). *sfrp1a* mRNA injections fail to rescue *emx3* or *six3b* expression in $mbl^{-/-}$ embryos. It is unclear what factors are responsible for the differences between *sfrp1a* and *sfrp5* in these assays. There are few functional studies of Sfrp5. Sfrp1 function is complex, involving all three branches of the

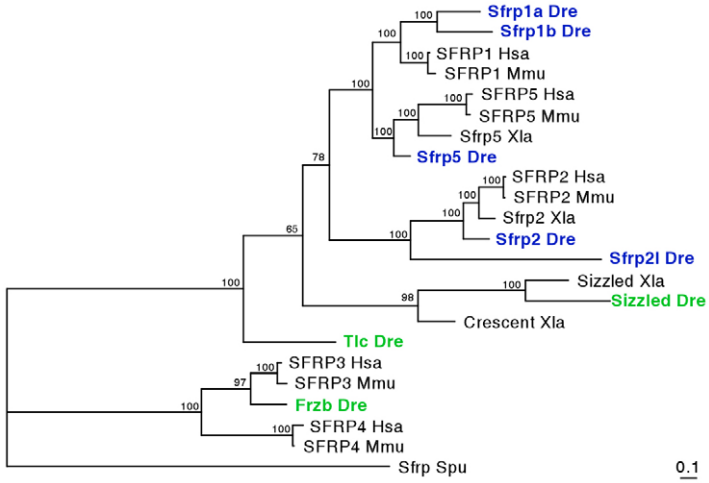

Fig. 3. Zebrafish Sfrps are closely related to Sfrps from other vertebrates. Phylogenic tree of the Sfrps from zebrafish (Dre), frog (Xla), mouse (Mmu) and human (Hsa). The Sfrp from sea urchin (Spu) is used as the outgroup to root the tree. The probability values from the MrBayes output are given at the nodes of the branches. The five Sfrps described in this study are labeled in blue and three previously described zebrafish Sfrps are labeled in green. Protein sequences used in the alignments are available upon request.

Wnt signaling pathway (Dennis et al., 1999; Esteve and Bovolenta, 2006; Esteve et al., 2004; Lin et al., 1997; Rodriguez et al., 2005; Satoh et al., 2006; Xu et al., 1998). Differences in RNA stability may also contribute to differences between the overexpression effects of *sfrp1a* and *sfrp5*.

Lhx5 regulates Sfrp1a and Sfrp5 expression

We examined whether Lhx5 regulates Sfrp1a and Sfrp5 expression. Expression of *sfrp1a* starts on the future dorsal side in mid-blastula stage embryos, in a pattern that resembles the initial *lhx5* expression. By late blastula stages, *sfrp1a* is expressed in the ectoderm in a dorsal to ventral gradient (Fig. 5A), again similar to *lhx5* (Fig. 1B). *sfrp1a* is also expressed in the marginal zone in late blastula, where *lhx5* is not expressed (Fig. 1A,B). In *lhx5* mRNA-injected embryos, expression of *sfrp1a* is dramatically elevated (Fig. 5B, 81%, *n*=67). Reducing Lhx5 function by morpholino injection decreases *sfrp1a* expression slightly in the late blastula stage (data not shown). When we inject the dominant interfering *lhx5-en* mRNA, expression of *sfrp1a* is completely lost in the rostral ectoderm, whereas its expression in the margin is retained (Fig. 5C, 68%, *n*=73). Retention of *sfrp1a* expression in the blastula margin suggests that factors other than Lhx5 are responsible for *sfrp1a* expression in this domain. Such factors may also be responsible for *sfrp1a* expression in the posterior regions of gastrula stage embryos that lack Lhx5 expression.

Lhx5 function is required for the expression of *sfrp1a* in the forebrain. In *lhx5* morpholino-injected embryos, *sfrp1a* expression in the presumptive forebrain is significantly reduced or completely lost, whereas expression in hindbrain and posterior mesoderm is relatively unaffected (Fig. 5E,F; 71%, *n*=93 and 51%, *n*=67, respectively). Later in the pharyngula period (prim-7 stage), *sfrp1a* is strongly expressed in the forebrain (Fig. 5G) and injection of the *lhx5* morpholino significantly reduces this expression in a dose-dependent manner (Fig. 5H,I; 2 ng morpholino, 80%, *n*=96; 4 ng morpholino, 77%, *n*=66). The *lhx5* splice-blocking morpholino similarly reduces *sfrp1a* expression at this stage (data not shown).

Lhx5 regulates Sfrp1a expression cell autonomously. To demonstrate this, we transplanted animal pole cells from labeled midblastula stage donor embryos to late blastula stage host embryos. The transplanted donor cells (red labeled) express *sfrp1a* (blue labeled and arrowheads) in the late gastrula, when they are distributed in the presumptive forebrain of host embryos (Fig. 5J; 100%, *n*=20). We then injected the donor embryos with the dominant interfering *lhx5-en* mRNA or the *lhx5* translation-blocking morpholino before transplanting the donor cells into the uninjected host embryos. The transplanted *lhx5-en*-expressing donor cells do not express *sfrp1a* even when they are located in the presumptive forebrain of host embryos (Fig. 5K; 65%, *n*=20), whereas morpholino-injected donor cells have a reduced *sfrp1a* expression (Fig. 5L, 33%, *n*=12). Similarly, we transplanted donor cells from *lhx5* mRNA-injected embryos into *lhx5* morpholino-injected host embryos. The transplanted *lhx5*-expressing donor cells also express *sfrp1a* in the presumptive forebrain, whereas neighboring *lhx5* morpholino-containing cells do not regain *sfrp1a* expression (Fig. 5M; 87%, *n*=30). Later during segmentation stages (18-somite stage), transplanted *lhx5* mRNA-injected donor cells continue to express *sfrp1a* in the forebrain of *lhx5* morpholino-injected host embryos (data not shown).

Lhx5 binds to the *sfrp1a* promoter. We identified *sfrp1a* promoter elements that are sufficient for *sfrp1a* expression in forebrain (Fig. 5N). We injected a series of deletion constructs of the *sfrp1a* upstream sequence fused to GFP-coding sequence into zebrafish embryos and assayed the transient GFP expression in forebrain regions (see Fig. S3 in the supplementary material). We find that a 2.7 kb *sfrp1a* upstream sequence is necessary and sufficient to drive high levels of GFP expression in forebrain. We then broke up the *sfrp1a* promoter into seven overlapping fragments and co-injected each fragment together with the *sfrp1a* (–371) basal promoter fused to the GFP-coding sequence (Muller et al., 2000). A 680 bp fragment about 1500 bp upstream of the Sfrp1a-coding sequence is most likely to be responsible for *sfrp1a* expression in forebrain (Fig. 5N). To test whether Lhx5 binds to this fragment, we used formaldehyde-based in vivo cross-linking and chromatin immunoprecipitation (Fig. 5O). We find that the *sfrp1a* promoter element is significantly enriched in Lhx5-associated chromatin, whereas upstream sequences of the housekeeping genes *aldolase a* and *bactin2* (human actin beta gene, ACTB ortholog) are not significantly enriched in Lhx5-associated chromatin. These results indicate that Lhx5 binds to the *sfrp1a* promoter element that directs Sfrp1a expression in the forebrain.

Lhx5 regulates *sfrp5* expression. *sfrp5* expression starts by the end of gastrulation in the presumptive forebrain and hindbrain (data not shown). By the 5-somite stage, *sfrp5* expression is strong in the presumptive forebrain (Fig. 5P). In embryos injected with *lhx5* mRNA, we note little change in *sfrp5* transcript levels at tail bud stage (data not shown), but by the 5-somite stage, *sfrp5* expression is expanded in the forebrain (Fig. 5Q; 52%, *n*=61). The expansion of *sfrp5* expression correlates with the increase in size of the forebrain from this stage onwards. In *lhx5* morpholino-injected

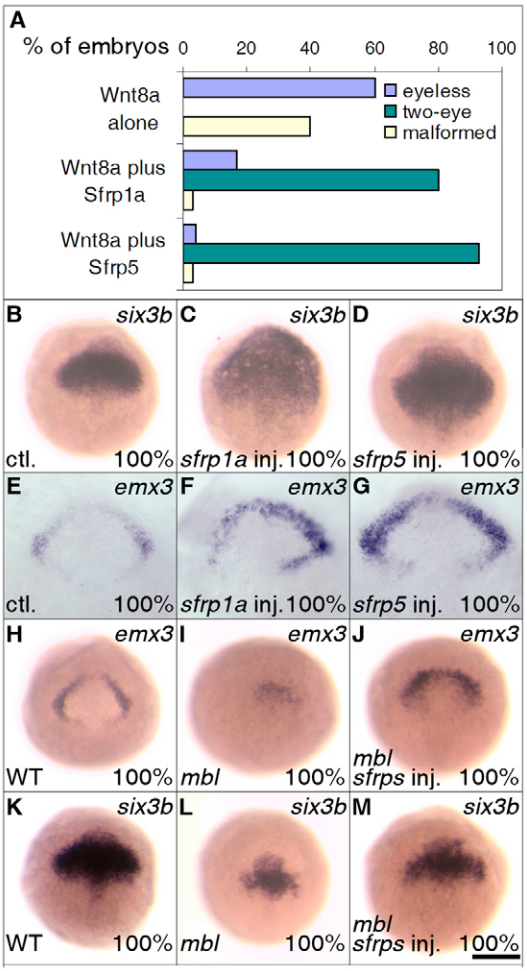

Fig. 4. Sfrps antagonize Wnt signaling and partially restore forebrain marker expression in *mbl* mutant embryos. (**A**) Sfrp1a and Sfrp5 rescue eye development in *wnt8a* mRNA-injected embryos. Injected embryos were scored after 24 hours post-fertilization. (**B-D**) *sfrp1a* or *sfrp5* gain of function expands the *six3b* expression domain. (**E-G**) *sfrp1a* or *sfrp5* gain of function expands the *emx3* expression domain. Embryos were dissected and flat mounted in glycerol after in situ hybridization. Animal pole views, rostral to the top. (**H-M**) Overexpression of Sfrps partially restores *emx3* (J) and *six3b* (M) expression in *mbl* embryos. Dosage injected was 150 pg of *sfrp1a* mRNA and 100 pg of *sfrp5* mRNA per embryo. Genotypes of mutant and partially rescued embryos were verified by RFLP. WT, wild-type embryos. Scale bar in M: 250 μM for B-M.

embryos, *sfrp5* expression is reduced at the 5-somite stage (Fig. 5R; 70%, *n*=82). Thus, Lhx5 is apparently required for normal *sfrp5* expression, although it is not sufficient to activate *sfrp5* expression. Later in development, *sfrp5* transcripts are distributed in the forebrain in a pattern similar to, but distinct from, the expression pattern of *sfrp1a*. Expression of *sfrp5* is still reduced in *lhx5* morpholino-injected embryos at these later stages (Fig. 5T,U; 65%, *n*=62 and 57%, *n*=67, respectively).

Sfrp1a or Sfrp5 activity can rescue forebrain deficiencies caused by inhibition of Lhx5 function

Our observation that Lhx5 regulates the expression of *sfrp1a* and *sfrp5*, and binds to the *sfrp1a* promoter, suggests that these Sfrp genes may be genetically downstream targets of Lhx5. We tested this hypothesis by examining whether exogenous Sfrp1a or Sfrp5 can compensate for loss of Lhx5 function (Fig. 6A). We took advantage of our observation that the injection of dominant-interfering *lhx5-en* mRNA blocks the expression of *sfrp1a* very effectively before tail bud stage (Fig. 5C) and severely compromises subsequent forebrain development (Fig. 1P). When *lhx5-en* mRNA alone is injected, 44% of the injected embryos fail to develop either one or both eyes (*n*=207). By contrast, when either *sfrp1a* or *sfrp5* is co-injected with *lhx5-en* mRNA, the majority of the co-injected embryos develop two eyes (*sfrp1a*, 64%, *n*=256; *sfrp5*, 55%, *n*=161), and the fraction of eyeless embryos is reduced to 7% and 10%, respectively. This result indicates that Sfrp1a and Sfrp5 act genetically downstream of Lhx5.

Lhx5 regulates Wnt signaling

We used expression of the endogenous *axin2* gene as a Wnt pathway reporter gene. The mouse *Axin2* gene contains multiple TCF-binding sites in its promoter and introns, and is a direct target of the canonical Wnt signaling pathway (Jho et al., 2002). We identified multiple TCF-binding sites in the zebrafish *axin2* genomic sequence (data not shown) and examined the expression of *axin2* in response to changes in Wnt signaling. We find that the zygotic *axin2* gene expression pattern closely matches Wnt signaling activity. At blastula stages, *axin2* is expressed in the marginal zone (Fig. 6B), where *wnt8a* and other Wnt genes are known to be expressed (Kelly et al., 1995). When Wnt signaling is ectopically activated by *wnt8a* mRNA injection, the entire blastula expresses *axin2* (Fig. 6C; 93%, *n*=96), and this ubiquitous activation of *axin2* expression persists during gastrula stages (data not shown) (Weidinger et al., 2005). By the end of the gastrulation, *axin2* is normally restricted to posterior tissues; presumptive forebrain exhibits little *axin2* expression (Fig. 6D). In *mbl*$^{-/-}$ embryos that have expanded Wnt signaling (Heisenberg et al., 2001; Houart et al., 2002), *axin2* expression expands rostrally into the presumptive forebrain (Fig. 6E; 21%, *n*=63). These results demonstrate that the zebrafish *axin2* gene can be used as a Wnt pathway reporter.

Lhx5 regulates *axin2* expression. In *lhx5* morpholino-injected embryos, *axin2* expression is not significantly affected during gastrulation (data not shown). However, we observe elevated *axin2* expression in the forebrain at the 16-somite stage in *lhx5* morpholino-injected embryos (Fig. 6G,I; 53%, *n*=68), consistent with the view that Lhx5 negatively regulates Wnt signaling. When the dominant interfering *lhx5-en* construct is injected, *axin2* expression is ectopically activated in rostral ectoderm during gastrulation (Fig. 6K; 80%, *n*=79), indicating ectopic activation of Wnt signaling. By contrast, when *sfrp1a* or *sfrp5* mRNA is co-injected together with *lhx5-en* mRNA, the ectopic expression of *axin2* is blocked such that rostral ectoderm is largely free of *axin2* expression (Fig. 6L,M; 59%, *n*=64 and 67%, *n*=63, respectively).

DISCUSSION

Lhx5 acts upstream of Sfrps to modulate Wnt signaling

Our *lhx5* gain- and loss-of-function studies suggest that Lhx5 plays a crucial role in forebrain formation. Activation of Lhx5 expands forebrain structures, whereas blocking the early function of Lhx5 compromises forebrain development (Fig. 1). Both *lhx5* gain- and loss-of-function experiments indicate that Lhx5 is upstream of Sfrp1a and Sfrp5 (Fig. 5). Sfrp1a and Sfrp5, in turn, block Wnt signaling (Fig. 4). We propose that Lhx5 promotes forebrain development through its regulation of secreted Wnt antagonists (Fig. 7).

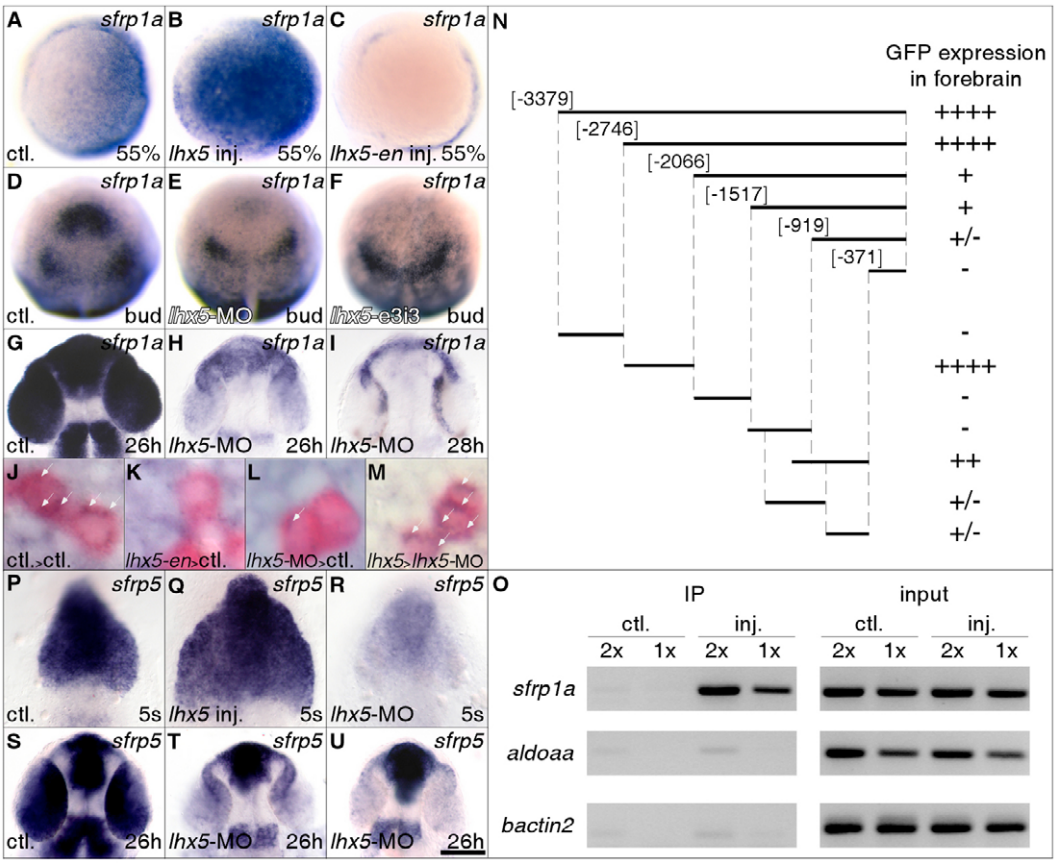

Fig. 5. Lhx5 regulates Sfrp1a and Sfrp5 expression. (**A-I**) Lhx5 regulates Sfrp1a expression. (A-C) Animal pole views, dorsal to the right. (D-F) Dorsal views, bud stage, *sfrp1a* expression. (G-I) Flat mounts in glycerol; dorsal views, rostral to the top. 2 ng (H) or 4 ng (I) of *lhx5* morpholino was injected. (**J-M**) Lhx5 cell autonomously regulates Sfrp1a expression. Transplanted cells are labeled in red. *sfrp1a* transcripts are highlighted by white arrowheads and are apparent as blue dots in the narrow cytoplasm around the nuclei of transplanted cells in controls (J, ctl) and *lhx5* mRNA-injected cells transplanted into *lhx5* morpholino injected hosts (M). *sfrp1a* transcripts are not detected in *lhx5-en* injected cells transplanted into control hosts (K). In transplanted *lhx5* morpholino-injected cells, *sfrp1a* expression is reduced (L). (J-L) 90% epiboly stage; (M) one-somite stage. (**N**) Mapping of *sfrp1a* upstream enhancers by transient GFP reporter expression in the forebrain. Fragments are numbered using the first nucleotide of the Sfrp1a start codon as the reference. GFP expression in the forebrain was scored at the 3 somite stage and the Prim-5 stage. The number of + signs indicate relative GFP expression levels. (**O**) Lhx5 binds to the *sfrp1a* promoter. Input represents 0.2% (2 μl, 2x) or 0.1% (1 μl, 1x) of starting material for each immunoprecipitation reaction. 2 μl (2x) or 1 μl (1x) of purified DNA product from each sample was used in the PCR. ctl., uninjected embryos treated in parallel; inj., *lhx5-GFP* mRNA-injected embryos. Mock immunoprecipitation with no antibody fails to amplify from either uninjected or injected groups (data not shown). (**P-U**) Lhx5 regulates Sfrp5 expression. Flat mounts in glycerol, dorsal views, rostral to the top. (R,T,U) 2 ng (R,T) or 4 ng (U) of *lhx5* morpholinos was injected. Scale bar in U: 250 μm for A-F; 100 μm for G-I; 8 μm for J-M; 100 μm for P-U.

Wnt proteins play crucial roles during nervous system development (Ciani and Salinas, 2005; Wilson and Edlund, 2001; Wilson and Houart, 2004). During gastrula stages, Wnts expressed by the posterior paraxial mesoderm (Kelly et al., 1995) are thought to act as graded signals that caudalize the neural plate (McGrew et al., 1995; Nordstrom et al., 2002). In zebrafish, mutations in the *headless* (*tcf7l1a*) or *masterblind* (*axin1*) genes, which both encode negative regulators of Wnt signaling, truncate the prosencephalon (Heisenberg et al., 2001; Kim et al., 2000; van de Water et al., 2001). We used *axin2* expression as a marker of Wnt signaling; rostral ectoderm normally exhibits little *axin2* expression, and overactivation of Wnt signaling by injection of *wnt8a* mRNA (Fig. 6C) or *masterblind* (*axin1*) mutation results in the ectopic activation of *axin2* (Fig. 6E). Dominant interference with Lhx5 function also results in the ectopic activation of *axin2* in the rostral ectoderm (Fig. 6K), whereas Sfrp mRNA injection reduces ectopic *axin2* expression and the forebrain defects that result from loss of Lhx5 function (Fig. 6L,M). Therefore, it is likely that Lhx5-regulated Sfrp expression is an important part of the mechanism that normally suppresses Wnt signaling in the rostral ectoderm during early development.

Extracellular Wnts contribute to forebrain loss in *mbl* (*axin1*) mutants

Overexpression of Lhx5 or Sfrps partially rescues forebrain development in *mbl*$^{-/-}$ embryos. *axin1* encodes the intracellular scaffold protein of the β-catenin degradation complex. The current view of Wnt signaling suggests that the docking of Axin1 to Lrp (the LDL receptor related protein) and the concomitant removal of Axin1 from the β-catenin degradation complex is the crucial step in the activation of the Wnt signaling pathway (Logan and Nusse, 2004; Tolwinski and Wieschaus, 2004). Therefore, it is somewhat surprising that changes in extracellular Wnt signaling affect *mbl*$^{-/-}$ embryos. Consistent with our results, however, the reduction of

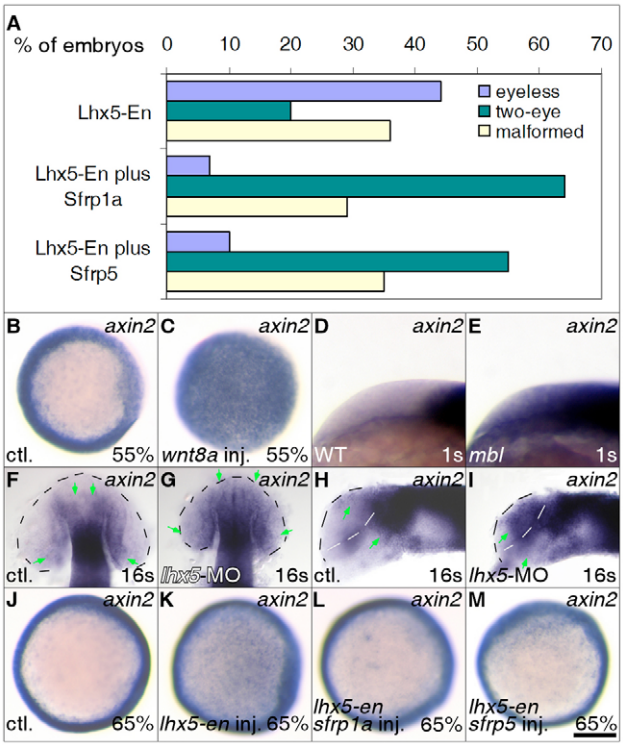

Fig. 6. Sfrp compensates for inhibition of Lhx5 function. (**A**) Sfrp1a and Sfrp5 rescue eye development in *lhx5-en* mRNA-injected embryos. The percentages of embryos malformed, as a result of dorsalization or injection (yellow), eyeless (blue) or two eyed (green) were measured. (**B-E**) Ectopic Wnt signaling results in ectopic *axin2* expression. (B,C) Animal pole views; (D,E) lateral views, rostral to the left, dorsal to the top. Genotypes were determined by RFLP. (**F-I**) Ectopic *axin2* expression in *lhx5* morpholino-injected embryos. Flat mounts in glycerol. Brain boundaries are marked by broken lines. Green arrows indicate the borders of *axin2* labeling. (F,G) Dorsal views, rostral to the top; (H,I) ateral views, rostral to the left, dorsal to the top. Eyes were removed to expose the brain. (**J-M**) *sfrp1a* or *sfrp5* gain of function reduces ectopic *axin2* expression in *lhx5-en*-injected embryos. Animal pole views, dorsal to the left. Scale bar in M: 250 μm for B,C; 125 μm for D,E; 100 μm for F-I; 250 μm for J-M.

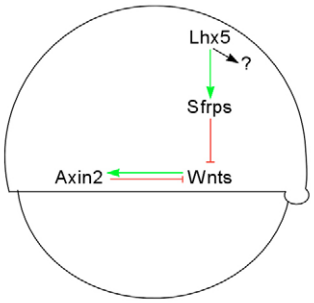

Fig. 7. Lhx5 regulates Sfrps that antagonize Wnt signaling in zebrafish forebrain. At gastrula stages, Wnt genes are expressed in the posterior paraxial mesoderm and Wnt signaling activates *axin2* expression. Rostral ectodermal cells express Lhx5, which activates the expression of Sfrps. Sfrps in turn antagonize Wnt activity in the rostral ectoderm and *axin2* expression is prevented. Similar events are likely to also occur in the presumptive forebrain at segmentation stage. Question mark denotes that other unknown Lhx5 downstream factors may also contribute to the antagonism of Wnt signaling.

wnt8b function by morpholino knockdown or the transplantation of *tlc*-expressing cells restores *emx3* expression in *mbl*$^{-/-}$ embryos, suggesting that extracellular Wnts contribute to the loss of forebrain in *mbl*$^{-/-}$ embryos (Houart et al., 2002). It is unclear whether the response to extracellular *wnt8b* levels in *mbl*$^{-/-}$ embryos is due to maternally deposited Axin1 (Heisenberg et al., 2001) or to a residual function of the *mbl*^{tm213} allele. Axin1 is present only at very low levels in *Xenopus* egg extracts (Lee et al., 2003) and the degradation of Axin1 is regulated by Wnt signaling (Tolwinski and Wieschaus, 2004). These features, if conserved in the zebrafish Wnt signaling pathway, raise the possibility that the missense mutation of *mbl*^{tm213} is a hypomorphic allele.

Morpholino knockdown of *wnt8b* function does not restore eye development in *mbl*$^{-/-}$ embryos (Houart et al., 2002). The presumptive eye field is specified as part of the forebrain anlage and, subsequently, eye vesicles evaginate from the forebrain to form eyes. Wnt signaling is implicated in eye formation (Esteve and Bovolenta, 2006; Wilson and Houart, 2004) and there is, apparently, a cell-autonomous requirement for Axin1 function for eye development (Heisenberg et al., 1996; Houart et al., 2002). Our studies show that the overexpression of Sfrps does not restore eye development in *mbl*$^{-/-}$ embryos and results in limited rescue of *six3b* expression. By contrast, overexpression of Lhx5 largely restores *six3b* expression and partially rescues eye development in *mbl*$^{-/-}$ embryos. Although we cannot exclude the possibility that factors such as mRNA or protein stability may contribute to the different outcomes, a parsimonious model might include other Lhx5 downstream factors that contribute to the restoration of eye development in *mbl*$^{-/-}$ embryos (Fig. 7).

Multiple mechanisms restrict Wnt signaling in the rostral neural plate

Previously it was shown that Six3 is required for forebrain development and that Six3 directly suppresses *Wnt1* expression in the rostral neural plate (Lagutin et al., 2003). Overexpression of Six3 in zebrafish or medaka leads to the enlargement of forebrain structures (Kobayashi et al., 2001; Loosli et al., 1999), and inhibition of Six3 function in mouse or medaka results in truncation of the prosencephalon (Carl et al., 2002; Lagutin et al., 2003). Remarkably, overexpression of mouse Six3 in zebrafish rescues the forebrain deficiency of *headless* (*tcf7l1a*) mutant embryos (Lagutin et al., 2003). Thus, Six3 and Lhx5 appear to have similar functions, although there are differences. In zebrafish, *six3* expression is initiated in the rostral ectoderm at late gastrulation stages, whereas *lhx5* is already expressed by the onset of gastrulation. We find that *six3* expression in *masterblind* (*axin1*) mutant embryos is significantly reduced at the end of gastrulation and that overexpression of Lhx5 can largely restore Six3 expression and rescue the forebrain deficiency. Thus, although both Lhx5 and Six3 suppress Wnt signaling and promote forebrain development, their molecular targets and the timing of their activities probably differ. A recent study showed that Six3 also functions to control cell proliferation by sequestering Geminin from Cdt1, a key component in the assembly of the pre-replication complex (Del Bene et al., 2004). Lhx2 may function downstream of Six3 to regulate cell proliferation in the developing forebrain (Ando et al., 2005). Currently it is unknown whether Lhx5 plays a similar role in cell proliferation.

Function of Lhx5 in vertebrate development

Lhx5 belongs to the Lin-11 group of LIM-homeodomain factors (Hobert and Westphal, 2000). Vertebrate *Lhx5* orthologs share conserved expression patterns and are implicated in the

establishment of the prosomeric organization of the forebrain (Bachy et al., 2001). In frogs or fish, *lhx5* is broadly expressed by early gastrulation stages. Inhibition of Lhx5 function in frogs causes the dissociation of rostral ectodermal cells during gastrulation (Houston and Wylie, 2003), and our studies in zebrafish suggest that Lhx5 regulates Wnt antagonism in the gastrula. In mice, however, no gastrulation defects have been reported in *Lhx5* knockout animals (Zhao et al., 1999). Published data show that the earliest mouse *Lhx5* expression is detected in the rostral neural plate at E8.0 when gastrulation is ending and the late head fold is forming (Sheng et al., 1997). It is unclear whether the absence of gastrulation defects in *Lhx5* knockout mice is due to a late onset of *Lhx5* expression in rostral ectoderm or to unknown mechanisms that compensate for loss of *Lhx5* function. Mouse *Sfrp2* is broadly expressed in the ectoderm (Mukhopadhyay et al., 2003) and *Sfrp5* is expressed in the rostral visceral endoderm during gastrulation (Finley et al., 2003). Thus, either Sfrp2 or Sfrp5 may antagonize Wnt signaling and allow forebrain development in the absence of Sfrp1.

Lhx5 shares high sequence identity with Lhx1 (Hobert and Westphal, 2000). Vertebrate *Lhx1* orthologs share conserved expression in the mesoendoderm at early gastrulation stages (Barnes et al., 1994; Taira et al., 1992; Toyama and Dawid, 1997). In mouse and frog embryos, Lhx1 activity is required in the mesoendoderm for the formation of anterior head structures (Hukriede et al., 2003; Shawlot and Behringer, 1995; Tam et al., 2004). If zebrafish Lhx1 plays a similar role in anterior head development, the severe phenotype caused by Lhx5-En may be due partly to its interference with Lhx1 function. Interestingly, *Sfrp1* expression is reduced in the anterior mesoendoderm in mouse *Lhx1* knockout embryos (see Satoh et al., 2006). In our studies, however, mesoendoderm *sfrp1a* expression is retained in *lhx5-en* mRNA-injected embryos (Fig. 5C). Thus, we can conclude that Lhx5-En does not significantly interfere with the Lhx1 regulation of *sfrp1a* expression. Nevertheless, we cannot exclude the possibility that Lhx5-En may interfere with other functions of Lhx1 in the mesoendoderm. Further studies are needed to determine the functional relationship between Lhx5 and Lhx1 in zebrafish forebrain development.

Lhx5 regulation of Sfrps has not been previously reported. In mouse, *Lhx5* knockout results in excess neural-precursor cell proliferation and migration defects during hippocampus formation (Zhao et al., 1999). Because Wnt signaling induces mitogenic activity in the nervous system (Megason and McMahon, 2002) and Wnt3a positively regulates mouse hippocampal development (Lee et al., 2000), it is possible that Wnt signaling is elevated in the hippocampus of *Lhx5* knockout mice. *Sfrp1* is also expressed in the mouse hippocampus. Thus, Lhx5 regulation of Wnt antagonists may be a conserved mechanism that also functions during development of the hippocampus.

Previous studies have shown that members of the LIM homeodomain protein family play crucial roles in the terminal differentiation of neuronal cells (Hobert and Westphal, 2000; Jessell, 2000; Shirasaki and Pfaff, 2002). In addition to its early expression in the presumptive forebrain, *lhx5* is also expressed later in subsets of interneurons (Toyama et al., 1995). A recent study showed that mouse Lhx5 marks neurons in the rostral part of the medial amygdala that project primarily to the hypothalamic nuclei associated with defensive behavior (Choi et al., 2005). Further studies are needed to determine whether Lhx5 acts in the context of Sfrps and Wnt signaling to regulate the terminal differentiation of these interneuron cells.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/16/3191/DC1

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Table S1. Exogenous Lhx5 partially rescues Wnt8a-induced forebrain deficiency				
		% Eyeless (*n*)	% Two-eye (*n*)	% Malformed (*n*)
10 pg *wnt8a*	Alone	52% (22)	5% (2)	43% (18)
	Plus *lhx5*	7% (3)	59% (26)	34% (15)
20 pg *wnt8a*	Alone	56% (49)	6% (5)	38% (34)
	Plus *lhx5*	4% (3)	54% (37)	42% (29)
	Alone	55% (56)	2% (2)	43% (44)
	Plus *lhx5*	7% (7)	57% (61)	36% (39)
	Plus *lhx5-trunc*	44% (28)	14% (9)	42% (27)
40 pg *wnt8a*	Alone	53% (20)	0% (0)	47% (18)
	Plus *lhx5*	30% (12)	15% (6)	55% (22)