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 neuroectoderm 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.

MATERIALS AND METHODS

Fish maintenance and genotyping

Zebrafish were maintained as described (Westerfield, 2000). The masterblind line (mbltm213) was provided by the Zebrafish International Resource Center (Eugene, OR). RFLP genotyping of mbltm213 embryos was carried out by PCR using the primers listed below. The mbtlm213 mutation abolishes a BpmI site in the amplified fragment. mbl-forward, 5'-GAGGTGTTTTCTCCACAGCATC-3'; and mbl-reverse, 5'-TACACCAGAAAATCCACGTC-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 (Thiss 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 (Puscel 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.
Embryo. 

We first cut and T4 polymerase-treated pCS2-lhx5 construct. The dosages for lhx5-en orf1, 200 pg of lhx5-GFP injected with a standard protocol (Oberley and Farnham, 2003). In brief, embryos were transplanted to the animal poles of late blastula stage hosts. Donor cells within host embryos were revealed in red by fluorescence labeling. 

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% bactin2. Injection of a second morpholino that blocks the splicing of lhx5 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. 10P; 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 wnt8a 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 rostral and/or elongated 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 weak 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.

**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 midblasta 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 marks 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. 10P; 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 wnt8a 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 rostral and/or elongated 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). 

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**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 coinject 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.
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 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 wnt8a 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 sfrp1a and sfrp5 mRNA together (Fig. 4I, 58%, n=12) or with sfrp5 mRNA alone (62%, n=13). Expression of six3b is also partially rescued in mbl–/– embryos injected with sfrp1a and sfrp5 together (Fig. 4M, 60%, n=10) or with sfrp5 mRNA alone (67%, n=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

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=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=42; six3b, 76%, n=59).
Lhx5 regulates secreted Wnt antagonists

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 in 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 (Fig. 5N). To test whether Lhx5 binds to this segment, we used formaldehyde-based in vivo cross-linking and chromatin immunoprecipitation (Fig. 5O). 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 lhx5-associated fragment into 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 segment, 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
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; 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).
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
wnt8b function by morpholino knockdown or the transplantation of tle-expressing cells restores emx3 expression in mbl<sup>−/−</sup> embryos, suggesting that extracellular Wnts contribute to the loss of forebrain in mbl<sup>−/−</sup> embryos (Houart et al., 2002). It is unclear whether the response to extracellular wnt8b levels in mbl<sup>−/−</sup> embryos is due to maternally deposited Axin1 (Heisenberg et al., 2001) or to a residual function of the mbl<sup>pm213</sup> 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<sup>pm213</sup> is a hypomorphic allele.

Morpholino knockdown of wnt8b function does not restore eye development in mbl<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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 (tcf711a) 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 neural plate at the onset of gastrulation, whereas lhx5 expression 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 mesendoderm at early gastrulation stages (Barnes et al., 1994; Taia et al., 1992; Toyama and Dawid, 1997). In mouse and frog embryos, Lhx1 activity is required in the mesendoderm 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 mesendoderm in mouse Lhx1 knockout embryos (see Satoh et al., 2006). In our studies, however, mesendoderm sfrp1a expression is retained in lhx5-en mRNA-injected embryos (Fig. 5C). Thus, we can conclude that Lhx1 regulation of sfrp1a expression is retained in lhx5-en mRNA-injected embryos (Fig. 5C). Therefore, we can conclude that Lhx1 regulation of sfrp1a expression is retained in lhx5-en mRNA-injected embryos (Fig. 5C). This suggests that Lhx1 regulates Wnt antagonism in the mesendoderm, allowing forebrain development to proceed in the absence of Sfrp1.

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 regulates Wnt antagonists (Zhao et al., 1999), Lhx5 may regulate Wnt signaling and antagonism of Wnt in the hippocampus (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.

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Xlim5 regulates the differential adhesion properties of early ectodermal cells. Development 130, 2695–2704.


