The Wnt co-receptors Lrp5 and Lrp6 are essential for gastrulation in mice

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Summary

Recent work has identified LDL receptor-related family members, Lrp5 and Lrp6, as co-receptors for the transduction of Wnt signals. Our analysis of mice carrying mutations in both Lrp5 and Lrp6 demonstrates that the functions of these genes are redundant and are essential for gastrulation. Lrp5;Lrp6 double homozygous mutants fail to establish a primitive streak, although the anterior visceral endoderm and anterior epiblast fates are specified. Thus, Lrp5 and Lrp6 are required for posterior patterning of the epiblast, consistent with a role in transducing Wnt signals in the early embryo. Interestingly, Lrp5+/−;Lrp6−/− embryos die shortly after gastrulation and exhibit an accumulation of cells at the primitive streak and a selective loss of paraxial mesoderm. A similar phenotype is observed in Fgf8 and Fgfr1 mutant embryos and provides genetic evidence in support of a molecular link between the Fgf and Wnt signaling pathways in patterning nascent mesoderm. Lrp5−/−;Lrp6−/− embryos also display an expansion of anterior primitive streak derivatives and anterior neurectoderm that correlates with increased Nodal expression in these embryos. The effect of reducing, but not eliminating, Wnt signaling in Lrp5+/−;Lrp6−/− mutant embryos provides important insight into the interplay between Wnt, Fgf and Nodal signals in patterning the early mouse embryo.

Key words: Wnt, Lrp5, Lrp6, Gastrulation, Nodal, Fgf, Mouse

Introduction

Intercellular signaling by the Wnt family of secreted proteins governs many inductive and patterning processes throughout vertebrate embryonic development (reviewed by Woodard and Nusse, 1998; Uusitalo et al., 1999). In mammals, 19 Wnt genes have been identified and mutational analyses of some of these genes in mice have demonstrated diverse roles for Wnt genes in axis formation, brain development, limb patterning and organogenesis (Liu et al., 1999; McMahon and Bradley, 1990; Parr and McMahon, 1995; Stark et al., 1994). In the canonical Wnt pathway, binding to the frizzled-type receptor initiates a cascade of cytoplasmic events that results in stabilization and translocation of β-catenin to the nucleus, where it complexes with TCF/LEF proteins to activate transcription of Wnt target genes (reviewed by Woodard and Nusse, 1998).

Analyses of Wnt gene mutations in mice have pointed to important roles for Wnt signals during gastrulation (reviewed by Yamaguchi, 2001). The first morphological sign of gastrulation is the formation of the primitive streak on the posterior side of the embryo. At the primitive streak, ectodermal epiblast cells delaminate and either ingress between the ectoderm and the visceral endoderm to become mesoderm, or displace the overlying visceral endoderm to form the embryonic definitive endoderm (reviewed by Tam and Behringer, 1997). Fate-mapping studies have shown that cells emerging from the posterior and middle regions of the streak give rise to extra-embryonic mesoderm and trunk mesoderm, respectively. Cells exiting the anterior primitive streak and later the node contribute to the axial mesoderm and definitive endoderm. Opposite the streak, a region known as the anterior visceral endoderm (AVE) imparts anterior fates on the epiblast, principally by restricting posterior signals (reviewed by Lu et al., 2001; Perea-Gomez et al., 2001). Subsequently, the anterior primitive streak and its derivatives are important for inducing/maintaining anterior epiblast fates initiated by the AVE (reviewed by Martinez-Barbera and Beddington, 2001). The loss of either β-catenin or Wnt3 function has severe effects on gastrulation (Huelsken, 2000; Liu et al., 1999). Both mutants lack a primitive streak and mesoderm, although the AVE is specified. Interestingly, the AVE fails to move anteriorly in β-catenin mutants, suggesting that proper positioning of the AVE either involves additional Wnt signals or is independent of Wnt genes. Although defects in Wnt3a mutants are less severe, these mutants also display perturbations of posterior development. Wnt3a−/− embryos lose posterior somites, and ectopic neural tissue forms at the expense of paraxial mesoderm (Takada et al., 1994; Yoshikawa et al., 1997). Thus, Wnt signals are required for posterior patterning of the embryo during gastrulation.

Recent genetic and biochemical studies have identified
members of the LDL receptor-related (Lrp) family, specifically Lrp5 and Lrp6 in vertebrates and Arrow in Drosophila, as important components of the canonical Wnt signaling pathway (Wehrli et al., 2000; Pinson et al., 2000; Tamai et al., 2000) (reviewed by He et al., 2004). Lrp5/Lrp6 are single-pass transmembrane proteins that have been proposed to act as Wnt co-receptors by binding to Wnt and frizzled in a ternary complex (Tamai et al., 2000; Semenov et al., 2001; Schweizer and Varmus, 2003), although a direct interaction between Drosophila Wnt/Wingless and Arrow has not yet been demonstrated (Wu and Nusse, 2002). The mechanism by which Wnt signals are transduced through Lrp5/6 appears to involve binding of Lrp5/6 to axin, a downstream negative regulator of the pathway that controls degradation of β-catenin (Mao et al., 2001b; Liu et al., 2003; Tolwinski et al., 2003; Tamai et al., 2004). Wnt signals stimulate phosphorylation of reiterated PPPSP motifs in the intracellular domain of Lrp5/6, thereby recruiting binding of axin (Tamai et al., 2004) and resulting in stabilization of β-catenin. Lrp5/6 function can be inhibited by binding to the secreted protein dickkopf (Mao et al., 2001a; Baico et al., 2001; Semenov et al., 2001), which is believed to disrupt Wnt-induced interactions between frizzled and Lrp5/6 (Semenov et al., 2001). A newly identified class of transmembrane proteins called kremens form a complex with Lrp5/6 and dickkopf, resulting in rapid internalization and hence, sequestering Lrp5/6 away from the frizzled receptor (Mao et al., 2002). Thus, these biochemical studies demonstrate a key role for Lrp5/6 in modulating cellular responses to Wnts.

To investigate the function of Lrp5 and Lrp6, mutational analyses have been performed in mice. Mice homozygous for a targeted mutation in Lrp5 are viable and exhibit subtle defects in bone ossification and eye vasculature that recapitulate aspects of the human disease, osteoporosis-pseudoglioma syndrome (Kato et al., 2002; Gong et al., 2001). Although there is ample evidence to support the role of Wnts in osteoblast proliferation and bone formation, the specific Wnt proteins involved have not been identified by genetic analysis (reviewed by Patel and Karsenty, 2002). In addition, Lrp5 mutant mice have impaired glucose tolerance (Fujino et al., 2003). In mice, disruption of Lrp6 causes severe developmental defects (Pinson et al., 2000). These abnormalities include a deletion of caudal midbrain, axis truncation and limb patterning defects (Wu and Nusse, 2002). The mechanism by which Wnt signals are transduced through Lrp5/6 appears to involve phosphorylation of reiterated PPPSP motifs in the intracellular domain of Lrp5/6, thereby recruiting binding of axin (Tamai et al., 2004) and resulting in stabilization of β-catenin (Mao et al., 2001b; Liu et al., 2003; Tolwinski et al., 2003; Tamai et al., 2004). Lrp5/6 and dickkopf, resulting in rapid internalization and hence, sequestering Lrp5/6 away from the frizzled receptor (Mao et al., 2002). Thus, these biochemical studies demonstrate a key role for Lrp5/6 in modulating cellular responses to Wnts.

Materials and methods

Generation of Lrp5 and Lrp6 mutant mice

The Lrp5 targeting vector was constructed using the Lambda KOS system (Wattler et al., 1999) in which a genomic fragment from the Lrp5 locus was identified by PCR using exon 1-specific primers (5′-GGCGGCCGACAGAT and 5′-CCGGGCGGGGACCAAG). A yeast selection cassette containing the URA3 marker was generated by PCR using gene-specific sense and antisense primers (5′-AGCCGGTGAGCGGGCGCCTCCGGCGCGGACGC and 5′-CGTTCCAGGGAGGGGCCTCGGGGAGAGAAGG-GC, respectively) and introduced into the genomic clone by recombination in yeast. The URA3 cassette was replaced with an IRES-β-galactosidase reporter and an MC1-neo selection cassette, resulting in the deletion of 197 bp containing the entire coding portion of exon 1. Norl-digested vector was electroporated into 129 Sv/Eybnd (LEX1) ES cells and Q418/FIAU resistant ES cell clones were isolated. Positive clones were identified by Southern blot analysis of EcoRI digested genomic DNA using a 265 bp 5′ external probe generated by PCR (5′-GATCCACGACCTGAATGATG; 5′-GA-GTCCAAAGGCCAGTTGATC) that detected a 9 kb wild-type and a 7.4 kb mutant fragment. Positive clones were verified using a 313 bp 3′ external probe generated by PCR (5′-ATGCCCTCTCTGTCG-TGAATCC; 5′-GGTTCTTGAAGACACTTCTCC) that detected a 13.5 kb wild-type and a 6.4 kb mutant fragment on Southern blots of EcoRV-digested genomic DNA. Several correctly targeted ES cell clones were identified, one of which was injected into C57BL/6 (albino) blastocysts. The resulting chimeras were mated to C57BL/6 (albino) females to generate heterozygous animals (official designation Lrp5tm1Hes), Lrp6 mutant mice (official designation Lrp6tm5ggt1t8tm187tmg) carrying a gene-trap insertion in intron 5 have been described previously (Pinson et al., 2000).

PCR genotyping of Lrp5-/- and Lrp6-/- deficient mice and embryos

Weanlings were genotyped at 3 weeks of age by PCR of DNA prepared from tail samples. Tail tissue (1 mm) was boiled at 100°C for 20-30 minutes in 50-150 μl of 1 N NaOH/1mM EDTA under mineral oil. After neutralization with an equal volume of 40 mM Tris-HCl pH 7.5-8.0, 1-5 μl of the DNA extract was used for PCR amplification. For both Lrp5 and Lrp6 PCR genotyping, an annealing temperature of 60°C and 30-35 cycles of amplification were used. The Lrp5 mutant allele was detected by the amplification of a 350 bp product using a forward primer in neo (5′-GGCGGCCGACAGAT and a reverse primer in the Lrp5 genomic sequence (Lrp5-D1: 5′-CTTCTCTCCAGACTCCTCCAAAG). The Lrp5 wild-type allele was detected by the amplification of a 520 bp product using the Lrp5-D1 primer and a forward primer from sequence within the genomic region deleted during homologous recombination (Lrp5-U1: 5′-GAGCTCC-CAAAGCTCAGCCCGC). Using genomic DNA from wild-type and Lrp6-/- embryos, PCR was performed with overlapping primer sets within intron 5 of the Lrp5 gene and the gene-trap insertion site was mapped to within the first 650 bp of the intron. Two primers from this region (Lrp6-U1: 5′-GAGGTGTAGCGCCCTTGGAG and Lrp6-D1: 5′-ACTACAAGCCGCTCAGCTGCC) were used to PCR amplify a 285 bp DNA product corresponding to the wild-type allele. A vector-specific primer (en2: 5′-GTGAGATTCCATTGGAGACCCGCGCGCCGCGCGCGCGC) complemented to the En2 intron sequence of the gene trap vector (Skarnes et al., 1995) in combination with primer Lrp6-U1 amplified a fragment of approximately 385 bp from the mutant allele.

Timed matings were used to collect embryos at desired stages with noon on the day of the copulation plug designated as day 0.5 dpc. Unfixed embryos and embryos following whole-mount RNA in situ hybridization were genotyped by PCR after removal of maternal tissues. Normal littersmates from several genotypic classes were used as controls for comparisons with the Lrp5+/-, Lrp6+/-, Lrp5+/-, Lrp6-/- mutant embryos.
Northern blot analysis
Total RNA from adult kidney and brain of wild-type and Lrp5 homozygous animals was prepared using Trizol reagent (Gibco-BRL). Northern blot analysis was performed using the following probes: Lrp5, a 400 bp EcoRI/NorI cDNA fragment from the 3’ untranslated region (mouse expressed sequence tag, GenBank #AI119858); and actin, a 610 bp PstI fragment of the mouse β-actin cDNA fragment.

Expression analysis of whole-mount embryos
For detection of β-galactosidase activity, embryos were stained with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) as previously described (Skarnes, 2000). RNA in situ hybridization was performed on embryos according to the protocol developed by Henrique and Ish-Horowicz (see http://iprotocol.mit.edu/protocol/59.htm), partly described by Henrique et al. (Henrique et al., 1995). With the following modifications: (1) the hybridization mix contained SSC at a pH of 7.0; (2) hybridization and subsequent high temperature washes were carried out at 67°C; (3) the anti-DIG-AP antibody (Roche #1093274) was used at a dilution of 1:4000; (4) post-antibody washes consisted of four one hour washes at room temperature and one overnight wash at 4°C; and (5) embryos were stained in 2.25 μl/ml NBT (75 mg/ml) and 1.75 μl/ml BCIP (50 mg/ml).

Antisense riboprobes were synthesized using a DIG RNA labeling kit (Roche, #171 025) for the following genes: Bmp4 (Winnier et al., 1995), Cer1 (Belo et al., 1997), Fgf8 (Mahmoood et al., 1995), Foxa2 (Sasaki and Hogan, 1993); Gbx2 (Wassarman et al., 1997), Hex (Bedford et al., 1993), Hexl (Thomas et al., 1995), Ebf (previously Lefty2) (Meno et al., 1999), Lhx1 (Shawlowt and Behringer, 1995), Lrp5 (Gong et al., 2001), Nodal (Meno et al., 1998), Pou5f1 (A. Smith), sprouty 2 (Minowada et al., 1999), Sfx3 (Oliver et al., 1995), Shh (Echelard et al., 1993), T (Wilkinson et al., 1990), Tbx6 (Image clone #599230) and Wnt5 (Roelink et al., 1990).

Skeletal preparations and histology
Skeletal preparations were made using standard methods (Parr and McMahon, 1995). Histological sections of embryos were processed as previously described (Pinson et al., 2000). Following in situ hybridization, selected embryos were fixed in 60% ethanol/30% formaldehyde/10% acetic acid, dehydrated in ethanol, embedded in paraffin wax, sectioned at 8 μm and counterstained with nuclear fast red (Vector, #H-3403) for 1 minute.

Results
Generation of Lrp5/6-deficient mice
To create Lrp5/6-deficient mice, we crossed animals carrying a targeted mutation in the Lrp5 gene (Fig. 1A, official designation Lrp5<sup>SmI1Hes</sup>) to animals carrying an insertional mutation in the Lrp6 gene (Fig. 1B; official designation Lrp6<sup>GntpG7118Tm6I157Wc2</sup>) (Pinson et al., 2000). The Lrp5 mutation was generated by homologous recombination in which a portion of exon 1 of the Lrp5 gene was replaced with the IRES-β-galactosidase reporter and the MC1-neo cassette (Fig. 1A). The genotypes of animals were determined by gel electrophoresis of PCR products amplified from genomic DNA (Fig. 1C) using primer pairs specific to each mutant locus (Fig. 1A,B). Homozygous Lrp5 mice are viable and fertile as was reported for a second targeted mutation of Lrp5, Lrp5<sup>SmI1Kry</sup> (Kato et al., 2002). Northern blot analysis of adult kidney (Fig. 1D) and brain (data not shown) confirms the absence of Lrp5 transcripts in Lrp5 homozygous mice. Thus, the Lrp5 allele used in our experiments is likely to be a null mutation. Crosses between Lrp5 and Lrp6 heterozygous mice yield double heterozygous animals at the expected Mendelian frequency. Intercrosses of these Lrp5<sup>+/–</sup>:Lrp6<sup>+/–</sup> animals were performed and a summary of the phenotypes associated with each genotypic class is shown in Table 1.

Lrp5 homozygotes and Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup> double heterozygotes are viable and fertile, whereas Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup> animals are found at a lower-than-expected Mendelian frequency at weaning (2.4% of 166 animals compared with 12.5% expected). To determine whether Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup> animals die before or after birth, timed matings between Lrp5<sup>–/–</sup> and Lrp5<sup>+/–</sup>:Lrp6<sup>–/–</sup> animals were performed, and litters were collected at 18.5 dpc for genotyping. Of 85 pups delivered by Cesarean section, 21 (25%) were of the Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup> genotype, demonstrating that the loss of these animals occurs sometime after birth. Although the precise nature and timing of lethality in the Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup> animals was not investigated further, we did not observe respiratory distress in the pups delivered by Cesarean section. The Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup>-pups were 20% smaller than their littermates at 18.5 dpc, and those that survived remained smaller into adulthood (data not shown). Despite their reduced size, we successfully mated Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup>-males to Lrp5<sup>+/–</sup>:Lrp6<sup>–/–</sup>-females to maximize the number of double mutant embryos for analysis in subsequent crosses. In contrast to other genotypic classes, no Lrp5<sup>–/–</sup>:Lrp6<sup>6/–</sup> or double homozygous mutants were found at weaning. Furthermore, matings between double heterozygotes and Lrp6 heterozygotes failed to produce any Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup>-progeny at 18.5 dpc (n=72). From timed matings, we determined that Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup>-and Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup>-embryos arrest prior to 10.5 dpc.

Lrp6 is expressed ubiquitously during embryonic development (Pinson et al., 2000) and Lrp5 is expressed in many adult tissues (Kato et al., 2002). The early lethality of Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup>-and Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup>-embryos prompted us to examine Lrp5 and Lrp6 expression during gastrulation (Fig. 1E-N). Insertion of the gene-trap vector into the 5th intron of Lrp6 creates a fusion protein with β-galactosidase (Mitchell et al., 2001), which was used as a reporter of Lrp6 expression. Lrp6 is broadly expressed in the embryo, although expression is stronger in the embryonic region (Fig. 1F,G). Lrp6 is expressed throughout the embryonic ectoderm and in nascent mesoderm and endoderm emerging from the primitive streak (Fig. 1H,I). Although the targeted allele of Lrp5 is tagged with IRES-β-galactosidase, the reporter is not functional, as we failed to detect β-galactosidase activity in Lrp5<sup>–/–</sup> or Lrp5<sup>–/–</sup>-embryos. Instead, we used whole-mount RNA in situ hybridization to examine Lrp5 expression in wild-type embryos. Expression is strongest in visceral endoderm overlying the extra-embryonic ectoderm, but is excluded from the visceral endoderm surrounding the epiblast (Fig. 1K,L, data not shown). Lrp5 is also expressed throughout the embryonic and extra-embryonic ectoderm. Unlike Lrp6, Lrp5 expression is not detected in mesoderm or definitive endoderm exiting the primitive streak (Fig. 1M,N). Thus, the phenotypes associated with Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup>-and Lrp5<sup>–/–</sup>:Lrp6<sup>–/–</sup>-mutants are consistent with the overlapping expression of both genes in the early embryo.

Limb defects in Lrp5<sup>–/–</sup>:Lrp6<sup>6/–</sup> and Lrp 5<sup>–/–</sup>:Lrp6<sup>–/–</sup>-mutants
Mice homozygous for the published Lrp5<sup>SmI1Kry</sup> mutation have defects in phalanx ossification at P4 (Kato et al., 2002).
Neonatal limbs of pups from intercrosses of double heterozygous mutant animals were examined for similar defects (Fig. 2). Lrp5–/– mutants also demonstrate a loss of middle phalanx ossification at 18.5 dpc (Fig. 2B). Interestingly, Lrp5/6 double heterozygotes are more severely affected than Lrp5 homozygotes, exhibiting the absence of multiple ossification centers (Fig. 2C). This result clearly shows that Lrp5 and Lrp6 have redundant functions in the limb. In addition to osteogenic defects, aberrant limb patterning is apparent in these animals. Approximately 39% of Lrp5+/–;Lrp6+/– animals display postaxial digit loss. Typically one digit on the right forelimb is missing (Fig. 2E). By contrast, Lrp5–/–;Lrp6+/– animals show more severe limb defects, which exhibit a loss of one to two postaxial digits in the forelimb, missing carpals, shorter metacarpals (Fig. 2G,J) and in more severe cases a missing zeugopod element (Fig. 2G,I). Hindlimbs are generally more affected than forelimbs, displaying only one to two digits and loss of the fibula (Fig. 2G,L,M). The defects observed in Lrp5–/–;Lrp6+/– limbs are similar to those seen in Lrp6–/– mice (Pinson et al., 2000), providing further evidence that Lrp5 and Lrp6 share overlapping functions in the limb. As previously noted for Lrp6–/– mutants (Pinson et al., 2000), the defects in limb patterning in Lrp5–/–;Lrp6+/– mutants resemble Wnt7a mutants.
Wnt co-receptors in gastrulation (Parr and McMahon, 1995). More recently it has been shown that conditional removal of Wnt3 function in the limb ectoderm and of β-catenin function in the ventral limb ectoderm result in similar limb phenotypes (Barrow et al., 2003; Soshnikova et al., 2003).

Mesodermal migration and patterning defects in Lrp5+/–;Lrp6+/– embryos

At the onset of gastrulation, Lrp5+/–;Lrp6+/– embryos appear morphologically indistinguishable from normal littermates (data not shown). However, by 7.5 dpc these embryos display an accumulation of cells protruding into the amniotic cavity on the posterior side of the epiblast. To characterize this phenotype more extensively, 7.5 to 8.0 dpc Lrp5+/–;Lrp6+/– embryos were assayed for the expression of various markers of the primitive streak and nascent mesoderm (Fig. 3). Bmp4, a marker of the posterior primitive streak and of extra-embryonic mesoderm lining the exocoelom (Winnier et al., 1995), is expressed in Lrp5+/–;Lrp6+/– embryos (data not shown), and posterior mesodermal derivatives such as the allantois and amnion are formed (arrow, Fig. 3F). The primitive streak marker T/Brachyury (Fig. 3A,C) (Wilkinson et al., 1990) is expressed in epiblast cells and in some mesenchymal cells within the bulge (Fig. 3B,D). Although fewer mesodermal cells migrate laterally in mutant embryos (Fig. 3D) compared with normal embryos (Fig. 3C), T is appropriately downregulated in those mesodermal cells that do migrate away from the primitive streak.

Fig. 2. Functional redundancy between Lrp5 and Lrp6 in the limb. (A–C) Dorsal views of skeletal preparations of 18.5 dpc forelimbs stained with Alizarin Red showing regions of ossification in Lrp5+/– (A), Lrp5–/– (B) and Lrp5+/–;Lrp6+/– (C) littermates. Arrows show ossification centers in the phalanx that are lost in Lrp5–/– and Lrp5+/–;Lrp6+/– forelimbs. (D,E) Ventral views of adult forelimbs showing loss of a post-axial digit (arrow) in Lrp5+/–;Lrp6+/– mice (E) compared with a wild-type littermate (D). (F,G) Side views of newborn pups showing normal limbs (F) compared with defects in a Lrp5+/–;Lrp6+/– mutant littermate (G). (H–M) Skeletal preparations of newborn limbs stained with Alizarin Red and Alcian Blue to indicate bone and cartilage formation, respectively. Lrp5+/–;Lrp6+/– forelimbs (I,J) and hindlimbs (L,M) show severe defects compared with a wild-type forelimb (H) and hindlimb (K). The mutant forelimb in I is from the newborn pictured in G.

Table 1. Phenotypes associated with different genotypic classes of Lrp5/6 mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Viability</th>
<th>Phenotype</th>
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<tr>
<td>Lrp5+/–</td>
<td>Viable</td>
<td>Normal</td>
</tr>
<tr>
<td>Lrp6+/–</td>
<td>Viable</td>
<td>Tail kinks (10%)*</td>
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<tr>
<td>Lrp5–/–</td>
<td>Viable</td>
<td>Osteogenic, eye vasculature† and glucose tolerance defects‡</td>
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<tr>
<td>Lrp5+/–;Lrp6+/–</td>
<td>Sub-viable</td>
<td>Osteogenic defects, post-axial loss of one digit</td>
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<tr>
<td>Lrp5+/–;Lrp6–/–</td>
<td>Perinatal lethal</td>
<td>Osteogenic defects, post-axial loss of multiple digits</td>
</tr>
<tr>
<td>Lrp5–/–;Lrp6+/–</td>
<td>Embryonic lethal</td>
<td>Axis truncation, limb patterning and CNS defects*</td>
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<tr>
<td>Lrp5–/–;Lrp6–/–</td>
<td>Embryonic lethal</td>
<td>Cell accumulation at the PS, expansion of ADE and ANE, and loss of PAM</td>
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ADE, anterior definitive endoderm; ANE, anterior neuroectoderm; CNS, central nervous system; PAM, paraxial mesoderm; PS, primitive streak

*Pinson et al., 2001
†Kato et al., 2002
‡Fujino et al., 2003
Tbx6 and Fgf8 is absent in 1999; Deng et al., 1994; Yamaguchi et al., 1994). In addition, Fgf8 characteristic of their failure to properly migrate away from the streak is not form visible somites at later stages (data not shown).

In examining T expression, we noted perturbations in its expression, which are indicative of defects in mesodermal patterning in Lrp5+/–;Lrp6+/– embryos. T expression is diminished in the distal epiblast of mutant embryos (Fig. 3B). The primitive streak marker Ebaf (previously Lefty2) is expressed within trunk mesoderm that exits the mid-region of the primitive streak, but is specifically excluded from the axial mesendoderm emerging from the anterior primitive streak (Fig. 3I) (Meno et al., 1999). In Lrp5+/–;Lrp6+/– mutants, Ebaf is absent from the distal and lateral regions of the embryo (Fig. 3J). Moreover, the primitive streak marker Tbx6 (Fig. 3K) (Chapman et al., 1996) is absent in mutant embryos (Fig. 3L). In Tbx6-deficient embryos, no posterior somites form (Chapman and Papoiaannou, 1998), and in various mutants that lack somites, Tbx6 expression is extinguished (Sun et al., 1999; Deng et al., 1994; Yamaguchi et al., 1994; Ciruna and Rossant, 2001). Thus, the absence of Tbx6 expression combined with the aberrant expression of T and Ebaf in Lrp5+/–;Lrp6+/– embryos is consistent with a failure to specify paraxial mesoderm, and not surprisingly, Lrp5+/–;Lrp6+/– embryos do not form visible somites at later stages (data not shown).

The accumulation of cells at the primitive streak and their failure to properly migrate away from the streak is characteristic of Fgfr1+/– and Fgfr1 mutant embryos (Sun et al., 1999; Deng et al., 1994; Yamaguchi et al., 1994). In addition, the expression of Tbx6 is absent in Fgfr1+/– (Sun et al., 1999) embryos and significantly reduced in Fgfr1+/– mutants (Ciruna and Rossant, 2001). This similarity in phenotypes could be explained if Lrp5/6 is required to maintain the expression of Fgfr1 and/or its receptor in the primitive streak. To address this issue, we examined the expression of Fgfr1 and sprouty 2 (Spry2), a downstream target of Fgf signaling (Crossley and Martin, 1995; Mahmood et al., 1995; Maruoka et al., 1998; Minowada et al., 1999). Both Spry2 and Fgfr1 and are readily detected at the primitive streak of wild-type and Lrp5+/–;Lrp6+/– embryos (Fig. 3M,N and data not shown), suggesting that Fgf signaling is intact in these embryos.

Anterior primitive streak derivatives are expanded in Lrp5+/–;Lrp6+/– embryos

The expression of Lhx1 in the anterior region of Lrp5+/–;Lrp6+/– embryos (Fig. 3F) suggests that unlike the paraxial mesoderm, the axial mesendoderm, a derivative of the anterior primitive streak, is specified. The prolonged expression of Lhx1 at the primitive streak (Fig. 3F,H) and the reduction in the domain of Ebaf expression (Fig. 3J), however, suggest that the anterior primitive streak may be expanded. Thus, we next examined the expression of various molecular markers that delineate different populations of axial mesendoderm cells that emerge from the anterior primitive streak/node in Lrp5+/–;Lrp6+/– embryos (Fig. 4). Indeed, there is a dramatic expansion of Foxa2 expression at 7.5 dpc, a
marker of the anterior primitive streak and axial mesendoderm (Fig. 4A) (Ang et al., 1993; Monaghan et al., 1993; Sasaki and Hogan, 1993), in mutant embryos (Fig. 4B). Moreover, Hex expression is broader in 7.5 dpc Lrp5+/–;Lrp6–/– embryos (Fig. 4D), indicative of an increase in anterior definitive endoderm (ADE) (Fig. 4C) (Thomas et al., 1998). Examination of Shh and T expression at 8.5 dpc, however, shows that although axial mesoderm is specified in the Lrp5+/–;Lrp6–/– embryo, it is not expanded (Fig. 4E-J) (Echelard et al., 1993; Wilkinson et al., 1990). In fact, in more severely affected embryos, axial mesoderm appears reduced (Fig. 4F). Together, these results demonstrate that derivatives of the anterior primitive streak, in particular the ADE, are expanded in Lrp5+/–;Lrp6–/– embryos.

An expansion of anterior primitive streak derivatives is observed in some classes of Cer1+/–;Lefty1+/– mutants and in Drap1 mutants (Perea-Gomez et al., 2002; Iratni et al., 2002). Moreover, both these mutants fail to specify paraxial mesoderm and Drap1 mutants produce an excess of mesodermal cells at the primitive streak. As Cer1 and Leftb (previously Lefty1) are Nodal antagonists and Drap1 is probably a transcriptional co-repressor for Nodal, these defects in patterning of the primitive streak appear to be a consequence of increased levels of Nodal signaling. Therefore, we were prompted to examine Nodal expression in Lrp5+/–;Lrp6–/– embryos. Nodal is normally expressed within the primitive streak (Fig. 4K), but by 7.5-8.0 dpc, its expression in the primitive streak is downregulated (Fig. 4M) and eventually becomes restricted to the node (Fig. 4O) (Zhou et al., 1993; Conlon et al., 1994; Varlet et al., 1997). In Lrp5+/–;Lrp6–/– embryos, Nodal expression is not properly down-regulated in the primitive streak compared to normal littermates (Fig. 4K-P).

Anterior neurectoderm is expanded in Lrp5+/–;Lrp6–/– embryos

As axial mesendoderm is required for maintenance of anterior neural fates in the epiblast (reviewed by Martinez-Barbera and Beddington, 2001), we next assayed the expression of neurectoderm markers in Lrp5+/–;Lrp6–/– embryos (Fig. 5). Expression of Six3, a marker of anterior neurectoderm (Fig. 5A,C) (Oliver et al., 1995), is broader both posteriorly and laterally in mutant embryos at 8.0 dpc (Fig. 5B). The consequence of this broader domain is clearly evident by 8.5 dpc by the formation of a greatly enlarged forebrain in Lrp5+/–;Lrp6–/– embryos (Fig. 5D). To determine if more posterior neurectoderm forms, we examined Gbx2 expression, a marker of anterior hindbrain at 8.5 dpc (Fig. 5E) (Bouillet et al., 1995; Wassarman et al., 1997). In Lrp5+/–;Lrp6–/– embryos there is a stripe of Gbx2 expression in the head region, indicating the formation, but lack of expansion, of anterior hindbrain (Fig. 5F). Thus, in Lrp5+/–;Lrp6–/– embryos, there is a specific expansion of anterior neurectoderm.

Whereas the expanded headfold region of Lrp5+/–;Lrp6–/– embryos is fairly consistent, the development of the posterior is more variable at 8.5 dpc. In some cases, a shortened embryo forms that is devoid of somites (Fig. 4H,J; Fig. 5D,F, data not shown). In more severely affected embryos, the posterior region is a highly abnormal tube structure (Fig. 4F, Fig. 5H) that continues to express high levels of Pou5f1, a marker of undifferentiated epiblast not normally observed at this stage (Fig. 5G) (Rosner et al., 1990). Thus, in some Lrp5+/–;Lrp6–/– embryos, the posterior portion of the embryo remains largely undifferentiated.

Fig. 4. Expanded anterior primitive streak derivatives and increased Nodal expression in Lrp 5+/–;Lrp6–/– embryos. Whole-mount RNA in situ analysis of marker gene expression in normal and Lrp5+/–;Lrp6–/– mutant embryos. Anterior is towards the left in all panels except B, which is an anterolateral view. Embryos in A-D and K-P are between 7.5 dpc and 8.0 dpc stages, whereas embryos in E-J are at 8.5 dpc. (A,B) Analysis of Foxa2 expression. A control embryo (A) shows expression in the axial mesendoderm, which is greatly expanded in the mutant embryo (B). (C,D) Analysis of Hex expression. A control embryo (C) shows expression in the ADE, which is broader in the mutant embryo (D). (E-H) Analysis of Shh expression. Control embryos (E,G) show expression in the axial mesendoderm that is greatly (F) or mildly (H) reduced in mutant embryos. (L,J) Analysis of T expression. Control (I) and mutant (J) embryos show expression in the axial mesendoderm. (K-P) Analysis of Nodal expression. Control embryos show expression in the primitive streak (K) that is downregulated by the late streak stage (M) and restricted to the node by the headfold stage (O). Mutant embryos (L,N,P) fail to properly downregulate expression in the primitive streak as development proceeds. Photos taken at the same magnification are grouped as follows: A-D; E,F; G-I; K-P.
Posterior patterning is absent in Lrp5/6-deficient embryos

At the onset of gastrulation, double homozygous mutant embryos appear normal (data not shown), but by 7.5 dpc, they are visibly smaller and abnormal compared with their littermates. In normal gastrulating embryos, mesoderm exits the primitive streak on the posterior side of the embryo and migrates between the ectoderm and endoderm layers, and the definitive endoderm displaces the overlying visceral endoderm (Fig. 6A). Sagittal sections of double homozygous embryos show a complete absence of mesoderm and definitive endoderm, while the ectoderm appears slightly thickened and collapsed (Fig. 6B). In addition, development of extra-embryonic tissue is severely compromised and structures such as the amnion and allantois are not visible.

To confirm the absence of mesoderm formation in Lrp5+/–;Lrp6+/– mutants, we examined 7.0-7.5 dpc embryos with a panel of molecular markers expressed in the primitive streak and nascent mesoderm (Fig. 6). Bmp4 is expressed in the extra-embryonic ectoderm adjacent to the proximal epiblast prior to gastrulation and later in posterior primitive streak and extra-embryonic mesoderm (Fig. 6C) (Winnier et al., 1995). In double homozygous mutant embryos, Bmp4 transcripts are limited to a faint proximal ring at the junction of the embryonic and extra-embryonic ectoderm, confirming a lack of posterior mesoderm (Fig. 6D). Other markers of the primitive streak, such as Fgf8 and T (Fig. 6E,G) (Crossley and Martin, 1995; Mahmood et al., 1995; Maruoka et al., 1998; Wilkinson et al., 1999), are absent in double homozygous mutants (Fig. 6F,H). Similarly, markers of both the primitive streak and nascent mesoderm, including Tbx6, Wnt3 and Lhx1 (Fig. 6I,K,M) (Chapman et al., 1996; Shawlot and Behringer, 1995; Belo et al., 1997; Liu et al., 1999) are not detectable in double homozygous mutant embryos (Fig. 6J,L,N). Thus, histological and molecular marker analyses demonstrate that Lrp5+/–;Lrp6+/– embryos fail to form a primitive streak and nascent mesoderm.

We noted a patch of Lhx1 expression on the presumptive anterior side of double homozygous mutant embryos (Fig. 6N, arrow), suggesting that AVE is formed. This was not surprising as the AVE is specified prior to gastrulation and forms independently of the primitive streak (reviewed by Lu et al., 2001). Other markers of the AVE, Hex and Cerl (Fig. 6Q) (Thomas et al., 1998; Belo et al., 1997; Biben et al., 1998; Pearce et al., 1999; Shawlot et al., 1998), are also expressed in double homozygous mutant embryos, but in fewer cells than normal (Fig. 6P,R). Although both Hex and Cerl are also normally expressed in the primitive streak (Fig. 6O,Q) (Thomas et al., 1998; Belo et al., 1997; Biben et al., 1998; Pearce et al., 1999; Shawlot et al., 1998), the remaining Hex and Cerl-expressing cells in double homozygous mutants most probably correspond to the AVE as the definitive endoderm is lost subsequent to the failure to establish a primitive streak (Fig. 6B,P,R). Together, these results show that while the AVE is specified in double homozygous mutant embryos, the primitive streak and its mesodermal and endodermal derivatives do not form.

It has been suggested that the AVE is not sufficient to specify anterior fates in the epiblast, instead requiring additional signals from the anterior primitive streak (Tam and Steiner, 1999) (reviewed by Martinez-Barbera and Beddington, 2001; Lu et al., 2001). Surprisingly, however, an expanded domain of Hesx1 expression, a marker of anterior axial mesendoderm and overlying anterior neuroectoderm (Fig. 6S) (Thomas et al., 1995; Hermesz et al., 1996), is observed in double homozygous mutant embryos at 7.5 dpc (Fig. 6T) and at 8.5 dpc (data not shown). Because these embryos fail to form primitive streak derivatives, the domain of Hesx1 expression most probably corresponds to anterior neuroectoderm. We therefore hypothesize that AVE cells are sufficient to specify anterior neuroectodermal fate in the absence of a primitive streak. The expanded domain of Hesx1 expression may reflect the absence of posterior signals that normally counteract anterior signals (reviewed by Lu et al., 2001).

Discussion

Lrp5 and Lrp6 have redundant functions during embryonic development

The generation of mice with null mutations in both Lrp5 and Lrp6 demonstrate that these two Wnt co-receptors share overlapping functions during embryonic development. A comparison of the limbs of Lrp5+/– and Lrp5+/–;Lrp6+/– mutants reveals a similar defect in ossification of the digits, although this phenotype is more pronounced in Lrp5/6 double heterozygous limbs. Furthermore, Lrp6+/– limbs and
Lrp5<sup>−/−</sup>;Lrp6<sup>−/−</sup> limbs both show loss of multiple post-axial digits. The removal of one copy of the paralogous gene in a Lrp5<sup>−/−</sup> or Lrp6<sup>−/−</sup> background also has a dramatic effect on the severity of the phenotype. For example, Lrp5<sup>−/+</sup>;Lrp6<sup>−/−</sup> animals exhibit a severe malformation of the limbs, in contrast to the normal appearance of Lrp5<sup>−/−</sup> mutant limbs. Conversely, the loss of Lrp5 function dramatically enhances the severity of the Lrp6 homozygous mutant phenotype. Whereas Lrp6<sup>−/−</sup> embryos die late in gestation, Lrp5<sup>−/+</sup> and Lrp5<sup>−/−</sup>;Lrp6<sup>−/−</sup> embryos arrest prior to mid-gestation (Table 1). These results provide formal genetic proof for functional redundancy between Lrp5 and Lrp6.

Lrp5 and Lrp6 are very similar in their overall structure (Brown et al., 1998), and the intracellular portion of each receptor contains five PPPSP motifs important for recruiting axin (Tamai et al., 2004). Previous work, however, has suggested that Lrp5 and Lrp6 are not equivalent in their ability to transduce Wnt signals. For example, in Xenopus assays Lrp6 is sufficient to induce axis duplication on its own, whereas Lrp5 is not. However, both Lrp5 and Lrp6 potentiate axis duplication in the presence of sub-threshold levels of Wnt (Tamai et al., 2000). In addition, Lrp6 is much more potent in activating a Wnt-responsive promoter in cultured mammalian cells in response to a Wnt signal (Holmen et al., 2002). In keeping with these studies, it is interesting that the loss of Lrp6 alleles consistently produces more severe phenotypes compared with the loss of Lrp5 alleles (Table 1). Thus, an allelic series that presumably reflects the progressive loss of Wnt signaling can be constructed as follows: Lrp5<sup>−/+</sup> < Lrp6<sup>−/+</sup> < Lrp5<sup>−/−</sup>;Lrp6<sup>−/−</sup> < Lrp5<sup>−/−</sup>;Lrp6<sup>−/+</sup> < Lrp6<sup>−/−</sup>;Lrp6<sup>−/+</sup> < Lrp5<sup>−/−</sup>;Lrp6<sup>−/−</sup>;Lrp6<sup>−/+</sup> < Lrp5<sup>−/−</sup>;Lrp6<sup>−/−</sup>;Lrp6<sup>−/+</sup>. We conclude that Lrp5 and Lrp6 perform the same function during embryonic development, albeit with different efficiencies. We note, however, that while Lrp5 and Lrp6 are both widely expressed during embryonic development (Fig. 1), they are not co-expressed in all cells. Thus, it is formally possible that Lrp5 and Lrp6 may have separate functions that we were not able to detect in our assays. We found no evidence that would suggest qualitative differences in the specificity of Lrp5 and Lrp6 for different subsets of Wnts, although further genetic and biochemical studies will be required to settle this point.

**Lrp5 and Lrp6 are required for primitive streak formation**

Targeted disruption of Wnt3 has firmly established a role for Wnts in posterior patterning of the early embryo. Wnt3 is
initially expressed in the proximal epiblast and overlying visceral endoderm of the pre-gastrula embryo. Wnt3 mutants fail to form a primitive streak and lack mesoderm (Liu et al., 1999). Thus, Wnt3 is the earliest-acting of the mammalian Wnts identified to date. Based on morphology and marker gene expression, Lrp5/6 double homozygous mutant embryos are very similar to Wnt3 mutant embryos. Like Wnt3 mutants, Lrp5/6-deficient embryos fail to express markers of the primitive streak (T, Fgf8 and Wnt3 itself), as well as markers of nascent mesoderm (Lhx1, Tbx6, Wnt3) and definitive endoderm (Cer1, Hex, Lhx1). By contrast, anterior patterning is correctly established in both Wnt3 (Liu et al., 1999) and Lrp5/6 mutant embryos as indicated by the expression and localization of AVE markers (Hex, Cer1 and Lhx1). Thus, we conclude that Lrp5/6 function is probably required for the reception of Wnt3 signals to establish posterior pattern in the epiblast. The absence of Wnt3 expression in the Lrp5/6 double mutant gastrula embryo may reflect a failure to induce and/or maintain the precursors of the primitive streak in the proximal epiblast of the pre-gastrula embryo because of loss of signaling through Lrp5/6. Alternatively, it is formally possible that Wnt3 is the target of another unidentified Wnt with an earlier role in development that signals through Lrp5/6, a hypothesis supported by slight differences in phenotypes between Wnt3 and Lrp5/6–/– mutants, as discussed below.

Interestingly, the phenotype of the classical mutation mesoderm-deficient (mesd) is very similar to Wnt3 and Lrp5/6-deficient mutants (Holdener et al., 1994; Liu et al., 1999; Hsieh et al., 2003). The mesd gene and its Drosophila homolog boc has been recently identified as chaperones required for the proper expression of Lrp5/6 on the surface of cells (Hsieh et al., 2003; Culi and Mann, 2003). The Lrp5/6 and mesd mutant embryos arrest at a slightly earlier stage than Wnt3-deficient embryos. Signaling by other Wnt genes expressed at gastrulation, such as Wnt3a (Gavin et al., 1990; McMahon et al., 1992) may account for the continued growth of the epiblast in Wnt3 mutants. However, considering the well-known role of LDL receptor family members in the binding and uptake of macromolecules (reviewed by Willnow, 1999), it seems reasonable that Lrp5/6 may have additional functions in the growth of the embryo that are independent of Wnt signaling.

The expression of Hexsl in the epiblast represents another important difference between Lrp5/6- and mesd-deficient embryos compared with Wnt3 mutants (Fig. 6T). Hexsl expression is reportedly absent in Wnt3 mutants, and this observation has led to the hypothesis that the AVE is not sufficient to induce anterior neurectoderm (Liu et al., 1999). Based on transplant and explant studies, AVE signals are instead believed to repress posteriorizing signals from the primitive streak, thereby priming the epiblast to respond to additional anterior signals emanating from the anterior primitive streak (Tam and Steiner, 1999; Kimura et al., 2000) (reviewed by Lu et al., 2001; Perea-Gomez et al., 2001).

The expression of Hexsl in the epiblast of Lrp5/6 double mutants and mesd mutants suggests that the AVE is, in fact, sufficient to induce anterior neurectodermal cell fates in the absence of a primitive streak. Presumably, the absence of posterior signals from the primitive streak allows the AVE to induce anterior fates in a broader region of the Lrp5/6 and mesd mutant epiblast. It is unclear why Wnt3 mutants fail to express Hexsl; however, one possibility is that another unidentified Wnt normally represses anterior fates in the epiblast and that this inhibition is relieved in Lrp5/6 and mesd, but not Wnt3, mutant embryos. It will be important to assay other markers of anterior neurectoderm to determine whether Hexsl expression in Lrp5/6 double mutants reflects a 'pre-neuralized' state or a complete induction of anterior neural tissue.

**Mesoderm defects in Lrp5+/–;Lrp6+/– mutants resemble mutants with defects in Fgf signaling**

The elimination of all but one copy of Lrp5 in Lrp5+/–;Lrp6–/– embryos has interesting and dramatic effects on mesoderm formation and anterior development. The characterization of this genotypic class allows us to examine the effects of severely reducing, but not completely eliminating, Wnt signaling in the early embryo. The morphology of these embryos, as well as changes in marker gene expression, appear remarkably similar to the phenotypes of Fgf8 and Fgfr1 mutant embryos (Sun et al., 1999; Deng et al., 1994; Yamaguchi et al., 1994; Ciruna and Rossant, 2001). For example, shortly after gastrulation Lrp5+/–;Lrp6–/– mutants exhibit an excess of cells accumulating at the primitive streak, forming a distinctive bulge that protrudes into the amniotic cavity. This defect is characteristic of Fgf8 and Fgfr1 mutant embryos in which mesoderm forms but fails to properly migrate away from the primitive streak. The migration defect is more severe in Fgf8 mutants, which fail to form any identifiable tissues by 8.5 dpc. Thus, in this regard, Lrp5+/–;Lrp6–/– mutants more closely resemble Fgfr1 mutants. In both cases, expression of Tbx6 is lost or significantly reduced and mutant embryos lack somites. Since the expression of Spry2, a downstream target of Fgf signaling, is maintained in Lrp5+/–;Lrp6–/– embryos (Fig. 3N), the striking similarity between Lrp5+/–;Lrp6–/– embryos and Fgf8 and Fgfr1 mutants cannot be explained by a downregulation of Fgf signaling. Thus, the gastrulation defects in Lrp5+/–;Lrp6–/– embryos are probably due to a reduction in Wnt signaling, rather than to an effect on Fgf signaling.

A recent study has shown that Wnt signaling is attenuated in Fgfr1 mutant embryos, establishing a molecular link between the Fgf and Wnt signaling pathways (Ciruna and Rossant, 2001). In Fgfr1–/– embryos, cells of the primitive streak express abnormally high levels E-cadherin, and as a consequence, β-catenin is sequestered at the membrane. Thus, one of the normal responses to signaling through Fgfr1 is to downregulate E-cadherin in cells undergoing the epithelial-to-mesenchymal transition, thereby releasing β-catenin from the membrane and priming these cells to respond to Wnt signals. In this light, Wnt and Fgf signaling appear to define parallel signaling pathways that converge on β-catenin to regulate the specification and migration of trunk mesoderm. In addition, it has recently been shown that Wnts can downregulate E-cadherin expression in other tissues (Jamora et al., 2003). These observations readily explain how a reduction in Wnt signaling in Lrp5+/–;Lrp6–/– embryos can mimic the loss of Fgf8/Fgfr1 function. The generation of hypomorphic alleles of Wnt3 will be very useful in testing this hypothesis.

**Expansion of anterior primitive streak derivatives and anterior neurectoderm in Lrp5+/–;Lrp6–/– embryos**

Lineage studies have established that cells emerging from the anterior region of the primitive streak and later the node,
migrate anteriorly and give rise to definitive endoderm and axial mesoderm (Tam and Behringer, 1997). Lrp5\(^{+/+}\);Lrp6\(^{-/-}\) embryos have an excess of anterior primitive streak derivatives, in particular the ADE, as judged by an increase in the expression of Hex and Foxa2. Interestingly, an abundance of anterior primitive streak derivatives is also observed in Fgfr1-deficient embryos. In this case, an excess of axial mesoderm rather than definitive endoderm is formed as judged by an increase in expression of Shh and T (Deng et al., 1994; Yamaguchi et al., 1994). Signals from the ADE and prechordal mesoderm are thought to be required to induce and maintain anterior neur ectoderm fates in the epiblast (reviewed by Martinez-Barbera and Beddington, 2001). Thus, the dramatic expansion of anterior neur ectoderm expressing the most rostral neural marker Six3 can be explained by the overproduction of ADE in Lrp5\(^{+/+}\);Lrp6\(^{-/-}\) embryos. Inhibition of Wnt signaling is known to be an important aspect of anterior development (reviewed by Niehrs et al., 2001). Overexpression of the Wnt antagonist dickkopf1 leads to enlarged heads in Cer1 and Leftb are Nodal antagonists, and Drap1 is probably a transcriptional co-repressor for Nodal, these defects appear to be a consequence of increased levels of Nodal signaling. In support of this idea, mutant embryos with the loss-of-function of two Nodal signaling modulators, Foxh1 and arka dia (Rnf111 – Mouse Genome Informatics), fail to establish an anterior primitive streak/node and its derivatives (Hoodless et al., 2001; Yamamoto et al., 2001; Episkopou et al., 2001). Finally, either conditional loss-of-function of Smad2, a Nodal effector, in the epiblast or decreased Nodal signals in the primitive streak results in a specific loss of the ADE and prechordal mesoderm (Vincent et al., 2003). Consistent with these data and the defects observed in Lrp5\(^{+/+}\);Lrp6\(^{-/-}\) embryos, Nodal expression is aberrantly maintained at elevated levels in the primitive streak of Lrp5\(^{+/+}\);Lrp6\(^{-/-}\) mutants (Fig. 4K-P). Interestingly, Nodal expression is also upregulated in Fgfr mutant embryos (Sun et al., 1999). Thus, one important role for Fgf and Wnt signaling may be to antagonize Nodal signaling in the primitive streak.

The mechanism by which Fgfs and Wnts antagonize anterior primitive streak fates is unclear. The expression of Eba f, a Nodal antagonist expressed in nascent mesoderm, is decreased in Lrp5\(^{+/+}\);Lrp6\(^{-/-}\) embryos (Fig. 3J). As Ebf normally acts to suppress the activity of Nodal in nascent mesoderm (Men et al., 1999), decreased Ebf expression in Lrp5\(^{+/+}\);Lrp6\(^{-/-}\) embryos may further amplify Nodal activity in the primitive streak (Fig. 4K-P) and could account for the dramatic expansion of axial mesendoderm at the expense of paraxial mesoderm. Whether Ebf is a direct target of Wnt signaling will require further study.

In summary, the generation of Lrp5\(^{+/+}\);Lrp6\(^{-/-}\) mutants provides a unique opportunity to examine the effect of reducing, but not completely eliminating, Wnt signaling in the embryo. The defects observed in these embryos reveal interesting links between the Wnt, Fgf and Nodal signaling pathways. Future experiments will be directed towards understanding the complex interplay of these pathways in patterning and specification of cells fates in the mammalian embryo.

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