Vertebrates are characterised by an external bilateral symmetry and an internal left-right (LR) asymmetry. This asymmetry is genetically determined and is crucial for the placement and orientation of internal organs. Failure in the development of LR-asymmetry can lead to diseases in human, including, for example, complex congenital heart disease (CCHD) and randomisation of organ position (situs ambiguous) (Schneider and Brueckner, 2000). Recently, substantial progress has been made in identifying mechanisms and specific molecules underlying the establishment of ‘left’ and ‘right’ in the embryo (Blum et al., 1999; Essner et al., 2002; Hamada et al., 2002; Mercola and Levin, 2001; Nonaka et al., 2002).

Several genes playing key roles in the LR pathway are conserved in vertebrates. Among these genes are the TGF-β superfamily members Nodal and Leftb (Lefty2), which are asymmetrically expressed during a narrow window of development in the left lateral plate mesoderm (LPM) before morphological differences between the left and right body halves are evident (Bisgrove et al., 1999). In addition, both Nodal and Leftb loss-of-function mutants die early in development and interfere with gastrulation (Conlon et al., 1994; Meno et al., 1999). Embryos that are compound heterozygotes for a Nodal hypomorphic and a Nodal null allele display LR abnormalities, including randomised LR cardiac asymmetry (Lowe et al., 2001). Also asymmetrically expressed in the LPM is the Drosophila bicoid-related homeobox gene Pitx2, which is expressed predominantly in left halves of developing organs, such as the heart and gut (Ryan et al., 1998). Pitx2 null mutants show multiple abnormalities including right isomerism of the lung and atria (Gage et al., 1999; Lin et al., 1999; Lu et al., 1999). The first visible morphological indication for a LR sidedness in vertebrates is bending of the bilaterally organised, linear heart tube to the right body side (Beddington and Robertson, 1999). In mammals, a twisting of the embryo along its rostrocaudal axis follows this process (Faisst et al., 2002). Subsequently, LR asymmetric morphogenesis of the visceral organs such as the stomach and the spleen occurs.

Manipulating experiments in gastrulating embryos, for example by extirpation of organiser cells in Xenopus or the ablation of the node in mouse, identified the organiser or the node, respectively, as an early inducer of laterality (Danos and Yost, 1995; Davidson et al., 1999). Experiments in Xenopus embryos and studies on zebrafish mutants revealed that besides the organiser, intact axial midline tissues such as the notochord and floorplate are required for correct LR development (Bisgrove et al., 2000; Danos and Yost, 1995; Danos and Yost, 1996; Lohr et al., 1998). The existence of mouse mutants with abnormal midline tissue and laterality defects strongly suggests that these processes may be a common feature of vertebrates.

**INTRODUCTION**

Vertebrates are characterised by an external bilateral symmetry and an internal left-right (LR) asymmetry. This asymmetry is genetically determined and is crucial for the placement and orientation of internal organs. Failure in the development of LR-asymmetry can lead to diseases in human, including, for example, complex congenital heart disease (CCHD) and randomisation of organ position (situs ambiguous) (Schneider and Brueckner, 2000). Recently, substantial progress has been made in identifying mechanisms and specific molecules underlying the establishment of ‘left’ and ‘right’ in the embryo (Blum et al., 1999; Essner et al., 2002; Hamada et al., 2002; Mercola and Levin, 2001; Nonaka et al., 2002).

Several genes playing key roles in the LR pathway are conserved in vertebrates. Among these genes are the TGF-β superfamily members Nodal and Leftb (Lefty2), which are asymmetrically expressed during a narrow window of development in the left lateral plate mesoderm (LPM) before morphological differences between the left and right body halves are evident (Bisgrove et al., 1999). In addition, both Nodal and Leftb loss-of-function mutants die early in development and interfere with gastrulation (Conlon et al., 1994; Meno et al., 1999). Embryos that are compound heterozygotes for a Nodal hypomorphic and a Nodal null allele display LR abnormalities, including randomised LR cardiac asymmetry (Lowe et al., 2001). Also asymmetrically expressed in the LPM is the Drosophila bicoid-related homeobox gene Pitx2, which is expressed predominantly in left halves of developing organs, such as the heart and gut (Ryan et al., 1998). Pitx2 null mutants show multiple abnormalities including right isomerism of the lung and atria (Gage et al., 1999; Lin et al., 1999; Lu et al., 1999). The first visible morphological indication for a LR sidedness in vertebrates is bending of the bilaterally organised, linear heart tube to the right body side (Beddington and Robertson, 1999). In mammals, a twisting of the embryo along its rostrocaudal axis follows this process (Faisst et al., 2002). Subsequently, LR asymmetric morphogenesis of the visceral organs such as the stomach and the spleen occurs.

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**SUMMARY**

Axes formation is a fundamental process of early embryonic development. In addition to the anteroposterior and dorsoventral axes, the determination of the left-right axis is crucial for the proper morphogenesis of internal organs and is evolutionarily conserved in vertebrates. Genes known to be required for the normal establishment and/or maintenance of left-right asymmetry in vertebrates include, for example, components of the TGF-β family of intercellular signalling molecules and genes required for node and midline function. We report that Notch signalling, which previously had not been implicated in this morphogenetic process, is required for normal left-right determination in mice. We show, that the loss-of-function of the delta 1 (Dll1) gene causes a situs ambiguous phenotype, including randomisation of the direction of heart looping and embryonic turning. The most probable cause for this left-right defect in Dll1 mutant embryos is a failure in the development of proper midline structures. These originate from the node, which is disrupted and deformed in Dll1 mutant embryos. Based on expression analysis in wild-type and mutant embryos, we suggest a model, in which Notch signalling is required for the proper differentiation of node cells and node morphology.

**Key words:** Notch signalling, Gastrulation, Node formation, Notochord, Floorplate, Midline, Left-right asymmetry, Mouse
The breaking of the initial bilateral symmetry of vertebrates seems to be caused by an asymmetrical signal associated with the organiser (Fujinaga, 1997). In mammals, for example, it has been suggested that the directional rotation of cilia in the ventral node may generate a laminar leftward flow transporting a morphogen (Nonaka et al., 1998; Okada et al., 1999). Interestingly, mouse mutants with immotile or absent cilia display laterality defects reminiscent to human primary ciliary dyskinesia (Ibanez-Tallon et al., 2002). Recently, it was demonstrated that an artificially generated rightward flow is sufficient to reverse the situs of mouse embryos (Nonaka et al., 2002). In Xenopus, chicken and zebrafish, such a mechanism has not yet been identified, but the existence of cilia in the organiser suggests that they may be required for LR asymmetry in all vertebrates (Essner et al., 2002). However, it is not clear which kind of essential molecules are transported by the nodal flow and which molecular factor triggers the expression of Nodal, the earliest gene expressed asymmetrically in all vertebrates. Sonic hedgehog (Shh) and Fgf8 are asymmetrically expressed in and around the chicken organiser (Hensen’s node), whereas the same genes in mouse embryos have a symmetrical expression pattern (Boettger et al., 1999; Levin et al., 1995; Meyers and Martin, 1999). However, both Shh and Fgf8 mouse mutants display numerous LR-asymmetry abnormalities (Meyers and Martin, 1999; Tsukui et al., 1999).

The identification of novel, so far unknown mutants with impaired laterality is essential for the further understanding of LR-axis formation in vertebrates.

Predominantly bilateral symmetric, LR differences are also present in invertebrates. In C. elegans, for example, stereotyped cleavages of early AB blastomere descendants lead to an invariant handedness of the intestine (Hutter and Schnabel, 1995), which is dependent on the LIN-12/Notch-like signalling pathway (Hermann et al., 2000).

In vertebrates, the evolutionarily conserved Notch-signalling pathway had not been implicated in LR development to date. Notch signalling is thought to act predominantly in a ligand/receptor-like manner and mediates various cell-fate decisions, which are important for the morphogenesis and development of numerous organs and tissues in many vertebrates and invertebrates (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Lewis, 1998). During neurogenesis, Notch signalling regulates the proliferation of various neural stem cells, either keeping them in an undifferentiated state or promoting glial differentiation (De Bellard et al., 2002; de la Pompa et al., 1997; Fristen and Lendahl, 2001). Other important processes with a crucial involvement of Notch signalling are, for example, somitogenesis, pancreas development and inner ear sensory development (Apelqvist et al., 1999; Beckers et al., 1999; Hrabé de Angelis et al., 1997; Jiang et al., 2000; Kiernan et al., 2001; Kusumi et al., 1998; Morrison et al., 1999).

We describe an as yet unknown requirement of Notch signalling for the normal development of LR asymmetry in vertebrate embryos. We observe randomisation of the direction of heart looping and embryonic turning in embryos homozygous for a loss-of-function allele of the Dll1 gene (Hrabé de Angelis et al., 1997). In addition, expression studies and scanning electron microscopy analysis of late gastrulating embryos show that Dll1 function is also required for the development of proper embryonic midline structures and normal node morphology. The requirement of Dll1 function for node development represents the earliest function of Notch signalling during mammalian development described until now.

MATERIALS AND METHODS

Mice and embryo collection

Mice carrying the Dll1lacZ knock-in allele have been described previously and were maintained on a mixed 129Sv/C57BL/6J background (Hrabé de Angelis et al., 1997). Mice carrying the Htu mutation have been described recently and were maintained on a C3HeB/FeJ background (Kiernan et al., 2001). Embryos were obtained from timed pregnancies, dissected in phosphate buffered saline (PBS, pH 7.3) and fixed at 4°C overnight in 4% paraformaldehyde (PFA) in PBS. Genotypes were controlled by yolk-sac PCR as described (Hrabé de Angelis et al., 1997; Kiernan et al., 2001).

Whole mount lacZ staining and RNA in situ hybridisation

lacZ staining was carried out as described by Wurst and Gossler (Wurst and Gossler, 2002). Antisense riboprobes were generated using the DIG-RNA labelling system (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions. In situ hybridisation was performed using the InsituPro robot from ABIMED (Langenfeld, Germany) following a protocol previously described (Spörle and Schughart, 1998). Embryos were stained with BM Purple AP substrate (Roche Molecular Biochemicals, Mannheim, Germany) and postfixed with 4% PFA in PBS. The following probes were used: Hand1 (eHAND) (Srivastava et al., 1995); Nodal (Zhou et al., 1993); Lefty1 (EbaF) and Lefty2 (Leftb) (Meno et al., 1997); Pitx2 (Campione et al., 1999); T (brachyury) (Herrmann, 1991); Hnf3b (Ang et al., 1993); Dll1 (Bettenhausen et al., 1995); Dll3 (Dunwoodie et al., 1997); Jag1 (Mitsiadis et al., 1997); Notch1 (Del Amo et al., 1992); Notch2 (Larsson et al., 1994); and Lfng (Cohen et al., 1997).

Histological analysis

Stained embryos were dehydrated through an ethanol series, embedded in Spurr’s resin (Spurr, 1969), sectioned at 7.5 µm and counterstained with safranin. Alternatively, stained embryos were cryoprotected in 30% sucrose/PBS at 4°C overnight, subsequently embedded in Cryoblock (Medite Medizintechnik GmbH, Burgdorf, Germany) and cryosectioned (35 µm) at –25°C. Processed sections were mounted under coverslips in KAISER’S glycerol gelatine (Merck, Darmstadt, Germany). For quantitative analysis of cell numbers, embryos were fixed in 3% glutaraldehyde, 4% sucrose, 0.1 M Na-cacodylate/HCl pH 7.6 and 2 mM CaCl2; washed for 2 hours with 2% OsO4; dehydrated in ethanol; and embedded in Epon®. Embryos were cut at 1 µm and counterstained with Toluidine Blue. Cell numbers were collected from five sections (every 20 µm of the first segment posterior to the forelimb bud) of five individuals each per age and genotype. Statistical significance was proven by the repeated measures analysis of variance using the SAS 6.12 software.

Scanning electron microscopy

Dehydration of embryo samples (developmental stage E7.5 to E10.5) was performed in a graded series of ethanol. The embryos were critical-point dried from CO2 by a routine procedure and sputter-coated (K375 EMITECH LTD, Ashford, UK) with 1-3 nm platinum. Coated specimens were examined in a field emission scanning microscope (Jeol JSM-6300F, Tokyo, Japan) with accelerating voltage of 2-10 kV in secondary electron mode.
Table 1. Heart looping and embryonic turning in offspring from Dll1lacZ heterozygous crosses

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Phenotypic trait</th>
<th>+/lacZ or +/+</th>
<th>lacZ/lacZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>E8.5</td>
<td>Heart looping</td>
<td>n=67</td>
<td>n=20</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>100%</td>
<td>60.0%</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>0%</td>
<td>40.0%</td>
</tr>
<tr>
<td>Embryonic turning</td>
<td>Anti-clockwise</td>
<td>n=59</td>
<td>n=16</td>
</tr>
<tr>
<td></td>
<td>Clockwise</td>
<td>96.6%</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.4%</td>
<td>50%</td>
</tr>
<tr>
<td>E9.5</td>
<td>Heart looping</td>
<td>n=84</td>
<td>n=33</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>100%</td>
<td>39.4%</td>
</tr>
<tr>
<td></td>
<td>Incomplete right</td>
<td>0%</td>
<td>9.1%</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>0%</td>
<td>42.4%</td>
</tr>
<tr>
<td></td>
<td>Incomplete left</td>
<td>0%</td>
<td>9.1%</td>
</tr>
<tr>
<td>Tail placement</td>
<td>Right</td>
<td>97.6%</td>
<td>51.5%</td>
</tr>
<tr>
<td></td>
<td>Left or abnormal*</td>
<td>2.4%</td>
<td>48.5%</td>
</tr>
</tbody>
</table>

Embryos were obtained from timed pregnancies and examined for heart looping and embryonic turning. Based on their phenotype (abnormal somites and undulated neural tube), ~25% of all embryos examined were phenotyped as homozygous for the Dll1lacZ allele. Genotypes were controlled by yolk-sac PCR as described previously (Hrabé de Angelis et al., 1997).

*In three out of 33 homozygous Dll1lacZ mutant embryos at E9.5, determination of the tail position was not possible because of the severity of the phenotype.

RESULTS

Randomisation of heart looping and embryonic turning in homozygous Dll1lacZ mutants

One of the first morphological events leading to asymmetry between the left and right body halves in the developing embryo is looping of the tubular heart to the right. In rodents, this process is accompanied by an anti-clockwise rotation of the lordotic embryo along its anteroposterior (AP) axis (Beddington and Robertson, 1999). To analyse the requirement forDll1 function in these processes, we examined heart morphology and direction of turning in Dll1 mutant embryos. To analyse the requirement for Dll1 function in these processes, we examined heart morphology and direction of turning in Dll1 mutant embryos from day 8.5 (E8.5) to day 10.5 (E10.5) of embryonic development. Owing to their severe haemorrhagic phenotype, beginning at E10.0, Dll1-deficient embryos are rapidly resorbed and die around E12.0 (Hrabé de Angelis et al., 1997).

Whereas 100% of the wild-type embryos showed looping of the tubular heart to the right, close to 50% of homozygous Dll1lacZ mutant embryos at E8.5 and E9.5 had an abnormal heart looping, either completely or incompletely to the left (Table 1). As a consequence of the anti-clockwise rotation of wild-type embryos, the developing tail curves to the right side in the vast majority (97.6%) of E9.5 embryos (Fig. 1A, left). By contrast, axial rotation at E8.5 was clockwise in 50% and the positioning of the tail at E9.5 was either to the left in 48.5% or abnormal in Dll1-deficient embryos (Table 1; Fig. 1A, right). Apparently, the direction of heart looping and embryonic turning are not linked in mutant embryos, as all combinations between normal and abnormal tail placement and heart orientation were observed in homozygous Dll1lacZ mutant embryos at E9.5. These data show that, in Dll1-deficient embryos, the asymmetric development of the heart and the direction of embryonic turning are randomised.

To investigate the morphology of Dll1 mutant hearts in more detail, we used scanning electron microscopy. Heart morphology at E10.5 was either as in wild-type embryos, with complete looping of the heart to the right in 39.4% of the mutants (Fig. 1C), or abnormal with incomplete looping to the right or left (in 18.2% of the mutants, Fig. 1D) or with complete looping to the left (in 42.2% of the mutants, Fig. 1E), resulting in mirror-imaged morphology when compared with wild-type hearts. Despite the reversed orientation all morphologically distinct subunits, such as the bulbous cordis; L, left body side; pv, future pulmonary ventricle; R, right body side; sv, future systemic ventricle.

Fig. 1. Tail placement in E9.5 and heart morphology in E10.5 embryos. (A) As a result of an anti-clockwise rotation, the tail lies at the right body side of the wild-type embryo (+/+). In half of the homozygous Dll1lacZ mutants (−/−), the tail was left-sided or abnormal (three out of 33), due to the randomisation of the direction of axial rotation. (B) Expression of Hand1 in the wild-type heart (+/+), Hand1 expression was detected in the appropriate position, although in reversed locations. (C-E) Scanning electron micrographs of a normal, fully looped wild-type heart (C), an incomplete looped mutant heart (D) and of a mirror-imaged heart (E), where the future pulmonary ventricle is on the left and the future systemic ventricle on the right. The arrow in E indicates the beginning bifurcation into branchial arteries. a, atrium; bc, bulbus cordis; L, left body side; pv, future pulmonary ventricle; R, right body side; sv, future systemic ventricle.
Fig. 2. Analysis of LR-asymmetric gene expression at E8.5 (A-N). (A-E) Expression of Nodal in the left lateral plate mesoderm (LPM) and lateral to the node (arrow). (B) Cross-section of the embryo in A: arrows indicate Nodal expression in the left LPM and lateral to the node. In homozygous Dll1lacZ mutant embryos Nodal expression in the LPM was either left-sided (4/21), right-sided (4/21; E), bilateral (3/21; arrows in D) or not detectable (10/21). Nodal expression in the node region was either normal (4/21), changed [3/21 with a right bias (arrow in E) or equal to both sides] or absent (14/21). (C) Cross-section of the embryo shown in D; arrows indicate the bilateral expression domains of Nodal in the LPM. (F-H) Leftb expression at the four to seven somite stage. Leftb expression in wild-type embryos (F) was exclusively in the left LPM, whereas in homozygous Dll1lacZ mutant embryos Leftb expression was either left-sided (5/16, H), right-sided (3/16), bilateral (4/16; arrows in G) or not detectable (4/16). (I-L) Pitx2 expression at the six to 10 somite stage. Expression in wild-type embryos (I) was found in the head mesenchyme and the left LPM. In homozygous Dll1lacZ mutant embryos, Pitx2 expression was found in the head mesenchyme and either in the left LPM (5/25), right LPM (1/25; arrow in K), bilateral in the left and right LPM (15/25; arrows in L) or absent from the LPM (4/25). (M,N) Floorplate-specific expression of Ebf1 at the two to six somite stage. In wild-type embryos (M), expression of Ebf1 was found in the left half of the floorplate, whereas in homozygous Dll1lacZ embryos (N), Ebf1 expression was not detectable (0/11). (O,P) Ventral view of brachyury (T) expression at the six to 10 somite stage; pictures were taken in a transmitted-light mode. A continuous expression of T was found in the notochord of wild-type embryos (arrowheads in O), whereas notochordal expression of T in homozygous Dll1lacZ embryos (P) was strongly reduced or absent.

that during embryonic development, Dll1 function may be required for directional looping of the heart primordial, but not for the differentiation of particular heart subunits. The loss-of-function mutants display a randomisation characteristic of a situs ambiguous phenotype.

To investigate whether this phenotype is specific for the Dll1 gene, we analysed LR sidedness in a second mutant allele of a putative Notch ligand, the Jag1 gene (Kiernan et al., 2001). Among 33 homozygous headturner (Htu) mutants at E10.5, only one showed a complete reversed looping of the heart together with a right-sided tail, whereas all others remained unchanged compared with the wild type (data not shown). These data show that the situs ambiguous phenotype is more pronounced in Dll1 mutant embryos.

Randomised expression of LR-specific genes

To investigate in more detail the determination of LR asymmetry in Dll1 mutant embryos, we analysed the expression pattern of specific molecular markers. The TGFβ family members Nodal, Ebf1 (also called Lefty1) and Leftb (also called Lefty2), together with the homeobox gene Pitx2, are either required for proper LR development or are specific markers of LR determination (Hamada et al., 2002). These genes have side-specific expression patterns prior to heart looping and embryonic turning (Capdevila et al., 2000; Hamada et al., 2002). To test whether Dll1 is required for the asymmetric expression of these genes (Nodal, Ebf1, Leftb and Pitx2) we performed whole-mount RNA in situ-hybridisation in Dll1 mutant embryos.

In wild-type (Dll1+/+) and heterozygous mutant embryos (Dll1+/-) at the early somite stage (E8.5, 0 to 6 somite pairs) Nodal expression is confined to the left lateral plate mesoderm (LPM) and to small domains to the left and right of the node, which is stronger to the left and weaker to the right with an increasing number of somites (Fig. 2A,B). Later, embryos with more than six pairs of somites show no detectable expression of Nodal adjacent to the node (Table 2). In homozygous mutant littermates at somite stages 0 to 6, expression of Nodal in the LPM was either left-sided (4/21) or right-sided (4/21), bilateral (3/21) or not detectable (10/21) by in situ hybridisation. Expression around the node was either normal (4/21 with a left bias as in the wild type), changed (three out of 21 with a right bias or equal to both sides of the node) or absent (14/21) (Table 2; Fig. 2C-E).

Similarly, expression of Leftb was altered. In wild-type and heterozygous embryos with up to seven somites, Leftb expression was detected exclusively in the left LPM (Fig. 2F). By contrast, expression of Leftb in homozygous mutant embryos was randomised ($\chi^2=0.5; 3$ df; $P=0.08$), with expression either in the left LPM (five out of 16), the right LPM (three out of 16), bilateral expression (four out of 16), or was not detected in the LPM (four out of 16) (Table 2; Fig. 2G,H).

At E8.5 Pitx2 is expressed in the head mesenchyme and in the left LPM. The left-sided expression of Pitx2 in the LPM is thought to be induced by Nodal (Shiratori et al., 2001). Similar
to the expression of *Nodal* and *Lefty*. Pitx2 expression was altered in homozygous mutant embryos with six to 10 somites but did not follow the pattern found for *Nodal* (Fig. 3A-F). This finding was further supported by the extended expression domain of the floorplate marker gene *Hnf3b* in mutant embryos at E8.5 (Fig. 2P). Therefore, we examined the morphology of the neural tube and notochord in *Dll1* mutant embryos in serial cross-sections. Although hyperplastic to a certain extent, the overall dorsoventral patterning of the neural tube was unaltered (M. H. de A. and K. Wünsch, unpublished). We found that the mutant floorplate was larger in histological preparations from E8.5 to E10.5 when compared with wild-type littermates (Fig. 3A). To further analyse these midline defects, we quantitated cell numbers in the floorplate and notochord in serial cross-sections of embryos at E9.5 and E10.5. This analysis revealed that the mutant floorplate contained significantly more cells, whereas the number of cells in the notochord was reduced (Fig. 3G,H). These data indicate that in *Dll1* mutant embryos, abnormal LR patterning is associated with defects in the development of axial structures such as floorplate and notochord.

**Expression of Notch-pathway genes during node formation**

The major source of progenitor cells for midline structures in vertebrates is the node (Kinder et al., 2001; Tam and Behringer, 1997). To investigate whether Notch signalling is required for node formation in the node itself, or whether its function may be required in a domain closely associated with the node, we analysed expression of the *Dll1* gene and other molecular factors of the Notch-signalling pathway during node formation. Interestingly, *Dll1* is expressed in a distinct domain adjacent to the node of wild-type embryos and not in the node itself (Bettenhausen et al., 1995). X-gal staining of *Dll1lacZ* heterozygous embryos at late gastrulation [E7.5, Thelier stage (TS) 11] reveals strong expression of β-galactosidase to the posterior mesoderm but excluding the node (Fig. 4A), whereas

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**Table 2. Gene expression pattern of LR-specific genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Somites</th>
<th>LPM</th>
<th>Node region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td><strong>Nodal</strong></td>
<td>+/+ or +/-</td>
<td>0-6</td>
<td>100%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>6-10</td>
<td>40%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>0-6</td>
<td>19.05%</td>
<td>19.05%</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>6-10</td>
<td>50%</td>
<td>–</td>
</tr>
<tr>
<td><strong>Lefty</strong></td>
<td>+/+ or +/-</td>
<td>4-7</td>
<td>100%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>2-6</td>
<td>100%</td>
<td>–</td>
</tr>
<tr>
<td><strong>Pitx2</strong></td>
<td>+/+ or +/-</td>
<td>6-10</td>
<td>100%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>6-10</td>
<td>20%</td>
<td>4%</td>
</tr>
</tbody>
</table>

**Ebag floorplate-specific expression**

| Ebaf | +/- or +/- | 2-6 | 100% | – | – | – | – | – | – | 6 |
| | +/- | 2-6 | – | – | – | 100% | – | – | – | 11 |

*In two individuals, *Nodal* expression was stronger at the right of the node; in one individual, *Nodal* expression was bilateral symmetric around the node.

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The major source of progenitor cells for midline structures in vertebrates is the node (Kinder et al., 2001; Tam and Behringer, 1997). To investigate whether Notch signalling is required for node formation in the node itself, or whether its function may be required in a domain closely associated with the node, we analysed expression of the *Dll1* gene and other molecular factors of the Notch-signalling pathway during node formation. Interestingly, *Dll1* is expressed in a distinct domain adjacent to the node of wild-type embryos and not in the node itself (Bettenhausen et al., 1995). X-gal staining of *Dll1lacZ* heterozygous embryos at late gastrulation [E7.5, Thelier stage (TS) 11] reveals strong expression of β-galactosidase to the posterior mesoderm but excluding the node (Fig. 4A), whereas
in homozygous embryos weak staining was found also in the node and in more anterior regions (Fig. 4B). To further analyse the involvement of Notch-signalling pathway genes in the formation and/or maintenance of the node, we analysed the expression patterns of *Dll3, Jag1, Lfng, Notch1* and *Notch2* during late gastrulation (E7.5, TS 11).

Like *Dll1*, the expression of the Notch ligand *Dll3* is restricted to the posterior mesoderm (Dunwoodie et al., 1997). No expression in the node was found (Fig. 4C). The Notch ligand *Jag1* was expressed in the mesoderm in an anterior domain complementary to the *Dll1* expression domain (Fig. 4D). At late gastrulation, the expression pattern of *Notch1* is similar to *Dll1* and *Dll3*. Transcripts are present in and adjacent to the primitive streak, in posterior ectoderm and in the mesoderm (Del Amo et al., 1992; Williams et al., 1995). No expression was detected in the node (Fig. 4E). *Notch2* is expressed in a distinct pattern with sharp boundaries (Williams et al., 1995) and was found in the node, notochord and in single stripes lateral to each side of the node (Fig. 4G). In the fly, *fringe* is thought to participate in the formation of cellular boundaries by modifying the ability of Notch to bind its ligand Delta (Cohen et al., 1997; Johnston et al., 1997). At mid gastrulation (TS 10), the mouse gene *Lunatic fringe (Lfng)* was expressed in a similar pattern like *Dll1, Dll3 and Notch1* (data not shown). At late gastrulation (TS 11), *Lfng* expression was restricted to a distinct domain lateral to the node (Fig. 4I).

Among the offspring of heterozygous intercrosses, all embryos tested (18 for each gene in three independent experiments) showed no obvious differences in the expression of *Dll3* and *Jag1* when compared with wild-type embryos (data not shown). By contrast, ~25% of the embryos revealed significant alterations in the expression of *Lfng, Notch1* and *Notch2*. In particular, *Lfng* and *Notch1* were ectopically expressed in the node and the expression in the surrounding tissues was not restricted to the characteristic domains (Fig. 4F,K). In addition, *Notch2* expression in the node was strongly reduced or patchy and expression adjacent to the node was diffuse (Fig. 4H).

Taken together, these Notch-signalling pathway genes show a distinct expression pattern surrounding and/or including the node. This pattern might be of functional relevance with respect to the morphology of the node and for the maintenance of node integrity.

**Structural abnormalities of the node in homozygous Dll1lacZ mutants**

To investigate whether the disrupted expression pattern of Notch-pathway genes in and adjacent to the node might cause abnormal node morphology in *Dll1* mutant embryos, we analysed node structures with scanning electron microscopy. In wild-type embryos at E7.5, the node has formed as a distinct structure at the apex of the embryonic cone at the anterior end of the primitive streak (Sulik et al., 1994). The mesendodermal node cells are characterised by their small surface area in comparison to the surrounding endodermal cells and a single, monociliated cell (Fig. 5B). Later, at the late headfold stage (TS 11), prior to heart looping and embryonic turning, all wild-type embryos had a symmetrical, club-shaped node with evenly distributed ciliated cells (Fig. 5C). By contrast, homozygous mutants at the late headfold stage often displayed irregularities in the node: cells with abnormal morphology disturbed the node symmetry and the regular distribution of cilia was altered (Fig. 5D). At E8.5 (TS 12), the wild-type node consists of microvilli-lined, cone-shaped cells, each with a single, motile cilium located on their ventral surface (Sulik et al., 1994) (Fig. 5E). By comparison, the mutant node contained enlarged cells of an unusual character with a smooth surface that disrupted the regular array of ciliated cells (Fig. 5F). Occasionally, we observed characteristics of cell death in the
mutant node and in cells along the future gut endoderm (data not shown). Thus, although the node is formed in homozygous Dll1lacZ mutants, its structural integrity is not maintained. The requirement of Dll1 for the formation and/or maintenance of a regular node is the earliest function associated with this gene during embryonic development described so far.

**DISCUSSION**

We describe an as yet unknown functional requirement of Notch signalling for the proper development of LR sidedness in vertebrates. In addition, the Dll1 loss-of-function phenotype is associated with defects in the development of midline structures, such as the floorplate and notochord, and an irregular morphology of the node and unusual cell types within the node. The phenotype evident during gastrulation hints to the earliest function of the Notch-signalling pathway described during vertebrate embryogenesis to date.

A complex regulatory network of genes required for the initiation, formation and maintenance of LR asymmetry of vertebrates has been discovered (Bisgrove and Yost, 2001; Capdevila et al., 2000; Hamada et al., 2002; Wood, 1997). The TGFβ family genes Nodal and Leftf, which are the earliest asymmetrically expressed genes in mouse described so far, play pivotal roles in this process. Together with the transcription factor Pitx2, they are expressed in the left lateral plate mesoderm before morphological differences between the left and right halves of the embryo are evident. The Dll1lacZ allele interferes with the expression pattern of these LR marker genes, such that their unilateral expression domain is altered and accordingly randomised in homozygous mutants. Interestingly, our data show with statistical significance, that the expression found for Pitx2 in homozygous mutants does not follow Nodal expression, although Pitx2 is a known target of Nodal (Shiratori et al., 2001). The bilateral expression of both genes might be explained by an impaired midline structure. This has been shown for several mutants in mouse and zebrafish (Bisgrove and Yost, 2001). However, this does not explain why the percentage of mutants with bilateral expression of Pitx2 is higher than with bilateral expression of Nodal. These findings suggest, that there might be an additional mechanism triggering Pitx2 expression. It has to be kept in mind that we investigated expression of Pitx2 later in development (at the 6-10 somite stage) than expression of Nodal (at the 0-6 somite stage). Nevertheless, the expression data reflect the morphological situation and randomisation of LR sidedness, suggesting that Dll1 function may act before expression of Nodal, Leftf and Pitx2.

Though laterally reversed in nearly 50% of mutant embryos, all morphologically distinct segments of the embryonic heart are present. This is consistent with the expression pattern of the Hand1 gene, which is thought to be involved in the development of specific segments during cardiogenesis (Thomas et al., 1998). As in wild-type embryos, Hand1 expression in Dll1 mutants is segment specific and independent from laterality. Taken together, these results show that Dll1 is required for proper heart looping and embryonic turning, but is not necessary for chamber specification of the developing heart.

![Fig. 4. Expression analysis of Notch-signalling pathway genes at TS11 (E7.5). Each panel shows a view of the embryo from the top (the approximate position of the node is marked by red circles) and below a lateral view of the same embryo (the position of the allantois is marked by asterisks). Anterior and posterior are marked by a and p, respectively. (A,B) X-gal staining to demonstrate the activity of β-galactosidase (lacZ) in heterozygous (Dll1+/lacZ; A) and homozygous (Dll1 lacZ/lacZ; B) mutant embryos. (C,D,E,G) Expression of delta 3 (Dll3, C), jagged 1 (Jag1, D), Notch1 (E), Notch2 (G) and lunatic fringe (Lfng, I) in wild-type embryos. (F,H,K) Although expression of Dll3 and Jag1 is not changed in homozygous Dll1lacZ mutant embryos at this stage, expression of Notch1 (5/18), Notch2 (4/18) and Lfng (4/18) among the offspring from Dll1lacZ heterozygous crosses was abnormal. Notch2 (G) and lunatic fringe (Lfng, I) in wild-type embryos. (F,H,K) Although expression of Dll3 and Jag1 is not changed in homozygous Dll1lacZ mutant embryos at this stage, expression of Notch1 (5/18), Notch2 (4/18) and Lfng (4/18) among the offspring from Dll1lacZ heterozygous crosses was abnormal.](image-url)
The Dll1 mutant is the first mouse mutant described so far with a randomised expression pattern of the LR marker genes Nodal, Leftb and Pitx2, together with a loss of Ebaf (Lefty1) expression. Other mouse mutants with laterality defects also display a strongly reduced or loss of Ebaf expression, but show invariable bilateral expression of the LR marker genes, together with thoracic left isomerism (Bisgrove and Yost, 2001), whereas the expression patterns of Nodal and Pitx2 found in Dll1 mutant embryos are in accordance with a situs ambiguous phenotype. Owing to the early lethality of Dll1 mutant embryos, it is not possible to investigate the situs of abdominal organs. No differences in the arrangement of kidney primordia both in wild-type and mutant embryos were found. In Ebaf mutants with thoracic left isomerism, the LR-marker genes are also bilaterally expressed (Meno et al., 1998). It was suggested that the asymmetrical, floorplate-specific expression of Ebaf might be required in the midline to function as a molecular barrier that prevents the expression of Nodal and Leftb in the right side of the embryo. However, the absence of Ebaf is most probably not the primary reason for the randomisation of heart looping and turning in Dll1 mutants, because in Ebaf mutants these processes are not affected (Meno et al., 1998).

Homozygous Dll1 lacZ mutants show structural abnormalities...
in midline tissues, such as an enlargement of the floorplate in combination with a decrease in the number of notochord cells. Defects in axial midline tissues are also reported from mouse mutants such as no turning, Shh$^{-/-}$ and Slt$^{-/-}$. In addition, these mutants display a combination of randomised heart looping or embryonic turning with a loss in Ebf expression (Iszraeli et al., 1999; Melloy et al., 1998; Meyers and Martin, 1999). The severe midline defects inDll1 mutant embryos are consistent with the observations that midline tissues may function as a physical barrier, which might be a prerequisite for normal development and/or maintenance of laterality in vertebrates (Klessinger and Christ, 1996; Levin et al., 1996; Lohr et al., 1997). The change in the number of floorplate and notochord cells inDll1 mutant embryos suggests that Notch signalling is involved in the specification of midline cells. Interestingly, mutations in the zebrafish homologs deltaA and deltaD cause deficiencies of cells in the midline (Appel et al., 1999; Latimer et al., 2002). In particular, deltaA mutants have fewer cells in the floorplate and an increase of cells in the notochord (Appel et al., 1999), suggesting that Notch signalling is also required in other vertebrates for the specification of midline cells.

Our analysis describes the earliest function associated with theDelta1 gene in vertebrates so far. The requirement for normal LR development in vertebrates is a novel function of Notch signalling that was not described before. However, there is no evidence to date, thatDll1 or any other molecular factor of the Notch-signalling pathway could be directly involved in the determination or maintenance of the LR axis in vertebrates. In particular, there is no description of an asymmetric expression pattern of any Notch-signalling pathway component either during early or late gastrulation in the node, in tissues adjacent to the node or in the paraxial mesoderm. All expression patterns of Notch-signalling pathway genes analysed in this study from the onset of expression at around midstreak stage (TS10, E6.5-7) until early organogenesis were symmetrical to the embryonic midline. The observed defect in the midline structure ofDll1 mutant embryos cannot fully explain the primary cause of the LR abnormalities. This led us to the hypothesis that Notch signalling might be involved in proper node development and gastrulation.

Based on known cellular movements in the node and fate maps of the node and primitive streak (Kinder et al., 2001; Sulik et al., 1994; Tam and Beddington, 1987), it is likely that the midline defects ofDll1 mutant embryos may be caused by earlier defects in the differentiation of node cells and node morphology. In fact, we observed severe morphological and cellular defects in the node ofDll1 mutant embryos. It was suggested that the shape of the node and the equal distribution of motile cilia on its ventral surface are prerequisites to generate a nodal flow, which might transport a – not yet identified – morphogen that triggers the onset of asymmetric gene expression (Nonaka et al., 1998; Okada et al., 1999). Taken together, the defects in LR-axis formation inDll1 mutant embryos may originate from a combination of altered node morphology and a distorted midline.

However, the question remains as to how Notch signalling participates in proper development of the node. It is generally known that the Notch-signalling pathway is involved in boundary formation (for a review, see Irvine and Rauskolb, 2001). We find that the early, distinct expression pattern of Notch-pathway genes at E7.5 surrounding and/or within the node (summarised in Fig. 6A) is in some way reminiscent of the expression of these genes in the wing imaginal disc ofDrosophila, where Serrate/Jagged and Delta have opposing expression domains and activation of Notch, modulated by fringe, at the wing margin is required for dorsoventral lineage restriction in the wing imaginal disc (Doherty et al., 1996; Michelli and Blair, 1999; Rauskolb et al., 1999). When Notch signalling is disrupted, cells can intermix and violate the compartment border (Michelli and Blair, 1999; Rauskolb et al., 1999). It is tempting to speculate that a somewhat similar mechanism may exist during early mouse embryogenesis and that this mechanism, by restricting the allocation of cells to the node, might be required for its proper differentiation. The loss of distinct expression boundaries of at least some Notch-pathway components in homozygousDll1lacZ mutant embryos (summarised in Fig. 6B) would lead to a softening of the compartment boundary and thus could lead to the observed defects in specification of node cells. The appearance of large, non-ciliated cells in the ventral node ofDll1 mutant embryos could be deemed to be a result of an improper cell sorting mechanism, which might be mediated by cell adhesion forces. This idea is also supported by loosening of the tightly packed cells in the ventral node ofDll1 mutant embryos. Currently, we do not have any evidence for an involvement of Delta and Notch molecules in cell adhesion during node formation, but it is well known that specific adhesive forces are required for proper gastrulation (Ip and Gridley, 2002). A relationship between cell adhesion molecules and LR development was revealed by experiments in chicken embryos, where blocking of N-cadherin function resulted in randomisation of heart looping and altered expression ofPitx2 (Garcia-Castro et al., 2000).

Interestingly, embryos homozygous for a targeted mutation of RBP-Jκ (Rbpsuh – Mouse Genome Informatics) a key downstream component of the Notch-receptor signalling-pathway, shows severe developmental abnormalities, including defective somitogenesis, neural tube defects and an incomplete rotation of the body axis (Oka et al., 1995). Although nothing is known about node defects and LR-asymmetry defects inNotch1 and Notch2 mutants, the analysis of compound mutants, homozygous for bothNotch1 and Notch2 mutant alleles could help to clarify the role of Notch signalling in node formation and in LR-development, respectively.

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