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Node and midline defects are associated with left-right development in *Delta1* mutant embryos

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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

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.

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.

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; Frisen 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 *Dll1*^{lacZ} 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) 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 $CaCl_2$; washed several times in the same solution without glutaraldehyde; fixed for 2 hours with 2% OsO_4; 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 CO_2 by a routine procedure and sputter-coated (K575 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 *Dll1*$^{+/lacZ}$ heterozygous crosses

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

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 *Dll1*^{lacZ} allele. Genotypes were controlled by yolk-sac PCR as described previously (Hrabé de Angelis et al., 1997).

*In three out of 33 homozygous *Dll1*^{lacZ} mutant embryos at E9.5, determination of the tail position was not possible because of the severity of the phenotype.

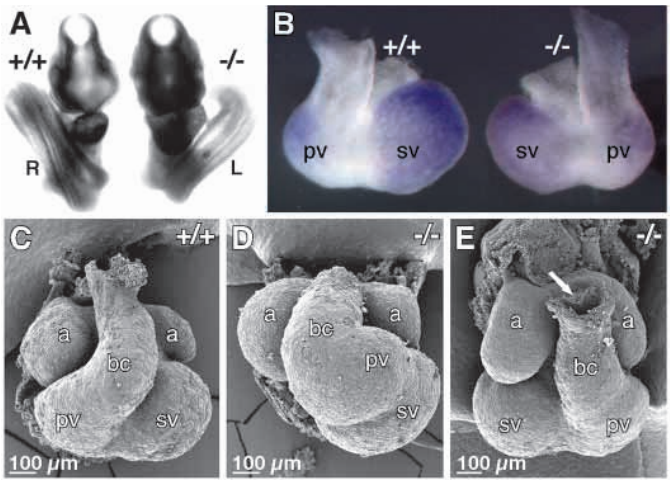

Fig. 1. Tail placement in E9.5 and heart morphology in E10.5 embryos. (A) As a result of an anti-clockwise rotation, the tail lies at the right body side of the wild-type embryo (+/+). In half of the homozygous *Dll1*^{lacZ} mutants (–/–), the tail was left-sided or abnormal (three out of 33), due to the randomisation of the direction of axial rotation. (B) Expression of *Hand1* in the wild-type heart (+/+) is seen in the future systemic ventricle (sv) and on the outside of the future pulmonary ventricle (pv). In mirror-imaged mutant hearts (–/–), *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.

RESULTS

Randomisation of heart looping and embryonic turning in homozygous *Dll1*^{lacZ} 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 of 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 *Dll1*^{lacZ} 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 *Dll1*^{lacZ} 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 *bulbus cordis*, the future left and right ventricles, were present, including the beginning bifurcation into branchial arteries (Fig. 1E).

To confirm the anatomical reversion towards mirror-imaged hearts at the molecular level, we analysed the expression of the bHLH transcription factor *Hand1* (also called *eHAND*) (Srivastava et al., 1995). *Hand1* expression starts during pre-implantation and is subsequently restricted to the developing heart and to neural-crest derivatives (Firulli et al., 1998; Riley et al., 1998). In looped wild-type hearts at E10.5, *Hand1* is specifically expressed in a large domain in the proampulla (future left or systemic ventricle) and in a restricted region of the metampulla (future right or pulmonary ventricle, Fig. 1B, left) (Thomas et al., 1998). According to the mirror-imaged morphology in mutant hearts, we found that *Hand1* is expressed in a large domain reminiscent to the expression in the proampulla of normal heart primordia on the right side and in a restricted domain on the left side comparable with the expression in the right pulmonary ventricle of wild-type embryos (Fig. 1B, right). Taken together, these data suggest

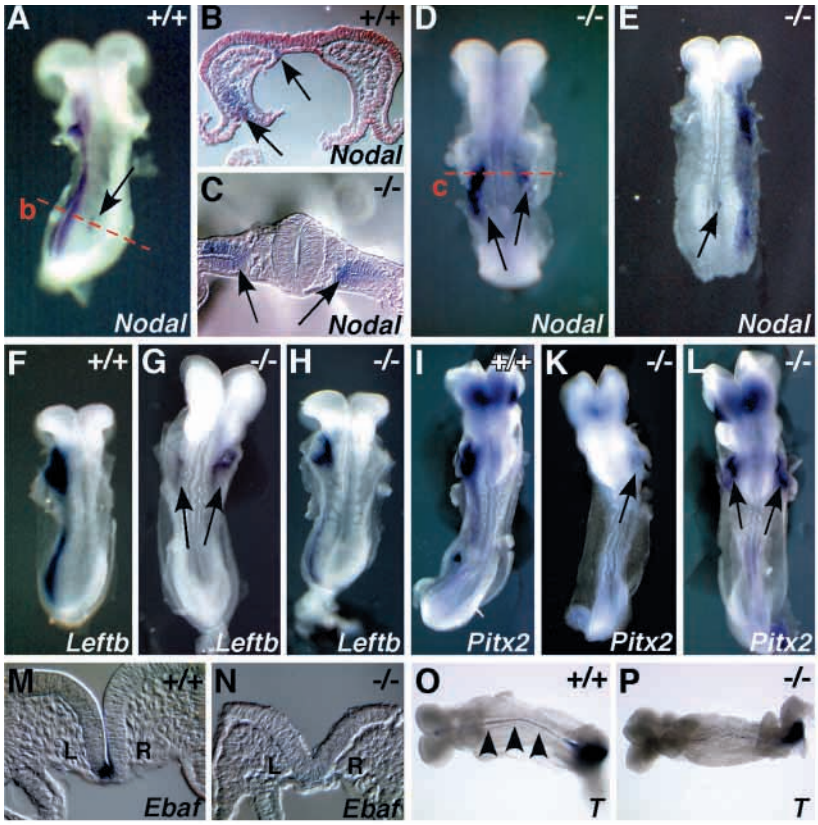

Fig. 2. Analysis of LR-asymmetric gene expression at E8.5 (A-N). (A-E) Expression of *Nodal* at the 0-6 somite stage. In wild-type embryos (A) *Nodal* is expressed in the left lateral plate mesoderm (LPM) and lateral to the node (arrow). (B) Cross-section of the embryo in A; arrows indicate *Nodal* expression in the left LPM and lateral to the node. In homozygous *Dll1*^{lacZ} 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 *Dll1*^{lacZ} 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 *Dll1*^{lacZ} 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 *Ebaf* at the two to six somite stage. In wild-type embryos (M), expression of *Ebaf* was found in the left half of the floorplate, whereas in homozygous *Dll1*^{lacZ} embryos (N), *Ebaf* 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 *Dll1*^{lacZ} 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*, *Ebaf* (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*, *Ebaf*, *Leftb* and *Pitx2*) we performed whole-mount RNA in situ-hybridisation in *Dll1* mutant embryos.

In wild-type (*Dll1*$^{+/+}$) and heterozygous mutant embryos (*Dll1*$^{+/lacZ}$) 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 (χ^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

Table 2. Gene expression pattern of LR-specific genes

Gene	Genotype	Somites	LPM				Node region			Total number
			Left	Right	Bilateral	Absent	Normal	Changed*	Absent	
Nodal	+/+ or +/–	0-6	100%	–	–	–	100%	–	–	10
		6-10	40%	–	–	60%	–	–	100%	5
	–/–	0-6	19.05%	19.05%	14.3%	47.6%	19.05%	14.3%	66.7%	21
		6-10	50%	–	–	50%	–	–	100%	2
Leftb	+/+ or +/–	4-7	100%	–	–	–				8
	–/–	4-7	31.2%	18.8%	25%	25%				16
Pitx2	+/+ or +/–	6-10	100%	–	–	–				12
	–/–	6-10	20%	4%	60%	16%				25
			Ebaf 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.

to the expression of *Nodal* and *Leftb*, *Pitx2* expression was altered in homozygous mutant embryos with six to 10 somites but did not follow the pattern found for *Nodal* (χ^2=44,7; df=3; $P \geq 0$). *Pitx2* was expressed either in the left LPM (five out of 25), in the right LPM (one out of 25), in the left and right LPM (15/25), or was absent from the LPM (four out of 25). In heterozygous and wild-type embryos, *Pitx2* expression was detected only in the left LPM as expected (Fig. 2I-L; Table 2).

It was suggested that expression of *Ebaf* (*Lefty1*) in the left half of the floorplate may be required for midline structures to prevent, for example, expression of left-sided genes on the right side of the embryo (Meno et al., 1997; Meno et al., 1998). Because we observed the expression of marker genes for left LPM also on the right, we analysed expression of *Ebaf* in mutant embryos. Although *Ebaf* was expressed in the left half of the floorplate of wild-type and heterozygous embryos at the two to six somite stage, *Ebaf* expression was not detected by in situ hybridisation in homozygous mutant embryos (0/11) (Fig. 2M,N; Table 2).

The loss of the unilateral expression domains of *Nodal*, *Leftb*, *Ebaf* and *Pitx2* is in accordance with the observed situs ambiguous phenotype in homozygous *Dll1*^{lacZ} knockout mutants. The expression analysis demonstrates that Dll1 function is required for the consistent asymmetrical expression of these marker genes.

Altered development of midline structures

It has been suggested that the lack of intact midline structures such as floorplate and notochord may cause defects in LR asymmetry (Dufort et al., 1998; Izraeli et al., 1999; King et al., 1998; Melloy et al., 1998). Although it is not clear how embryonic midline structures are functionally involved in the determination and/or maintenance of LR sidedness, it has been argued that axial structures might function as physical barrier for unilateral signals. To investigate whether the defects in LR asymmetry are accompanied by defects in the embryonic midline of *Dll1* mutants, we analysed the morphology of the floorplate and notochord, and examined the expression of essential midline markers.

In wild-type embryos, the axial marker gene *T* (brachyury) is expressed from early gastrulation onwards in the primitive streak and in the developing notochord (Wilkinson et al., 1990) (Fig. 2O). By contrast, in homozygous *Dll1*^{lacZ} mutant embryos *T* expression was present in the primitive streak, but strongly reduced and often absent along the notochord 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 hyperplasic 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-F). This finding was further supported by the extended expression domain of the floorplate marker gene *Hnf3b* in mutant embryos at E8.5 (Fig. 3A,B). In addition to the floorplate defect, we observed regional abnormalities in the morphology of the notochord (Fig. 3B). In some regions along the AP axis, which coincide with absent *T* expression, presumptive notochord cells appeared rather as a sheet tightly associated with the dorsal primitive gut endoderm (Fig. 3B) when compared with the rod-like shape in the wild-type embryo (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 *Dll1*^{lacZ} heterozygous embryos at late gastrulation [E7.5, Theiler stage (TS) 11] reveals strong expression of β-galactosidase to the posterior mesoderm but excluding the node (Fig. 4A), whereas

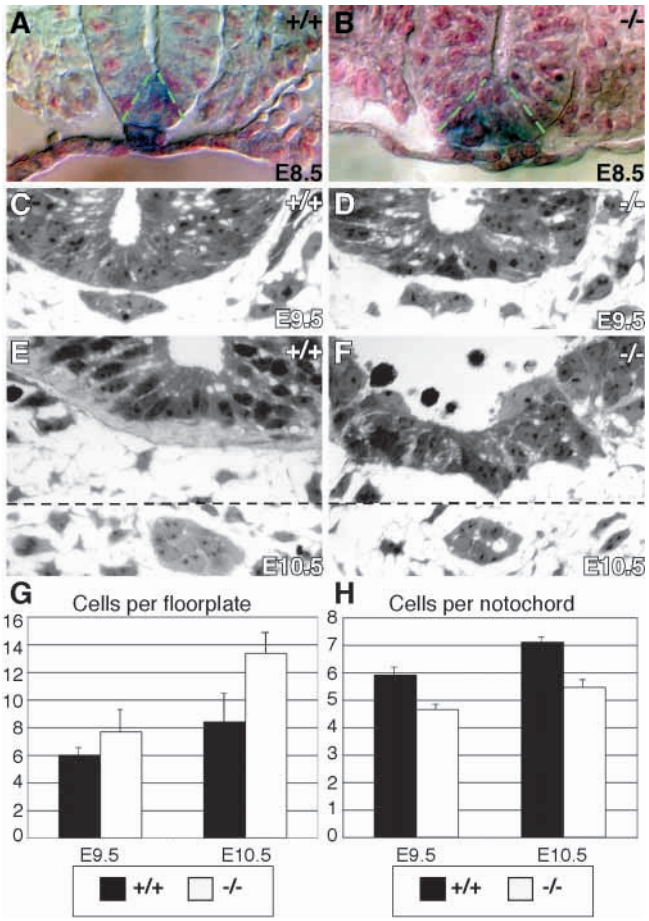

Fig. 3. Analysis of midline morphology. (A,B) *Hnf3b* expression at E8.5. Sections of plastic embedded embryos after whole-mount RNA in situ hybridisation. The floorplate specific expression domain (green brackets in A and B) in homozygous *Dll1*^{lacZ} mutant embryos (B) was enlarged in comparison with wild-type embryos (A). Note the sheet-like notochord morphology in B, which appeared regionally along the AP axis. (C-H) Comparison of cell numbers between wild type and mutant floorplate and notochord at E9.5 and E10.5. (C-F) Semi-thin cross-sections of the neural tube just posterior to the forelimb-bud level of wild-type (C,E) and homozygous *Dll1*^{lacZ} mutant embryos (D,F). (G,H) Quantitative analysis of serial cross sections of five individuals each per age and genotype. Homozygous *Dll1*^{lacZ} mutant embryos have significantly more cells in the floorplate (G) and fewer cells in the notochord (H) when compared with wild-type embryos (P<0.0007 for the floorplate, P<0.0005 for the notochord). Broken line indicates that parts of the picture were removed due to space restrictions.

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 *Dll1*^{lacZ} 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, central cilium on each cell (Fig. 5A). Approximately 25% of the offspring from *Dll1*^{lacZ} heterozygous intercrosses displayed morphological changes in the node. These are evident as rupturing of the surface, bulging of cells and loss of monociliated cells (Fig. 5B). Later, at the late headfold stage (TS 11), prior to heart looping and embryonic turning, all wild-type embryos analysed 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 *Dll1*^{lacZ} 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 *Leftb*, 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 *Dll1*^{lacZ} 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* was 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*, *Leftb* 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.

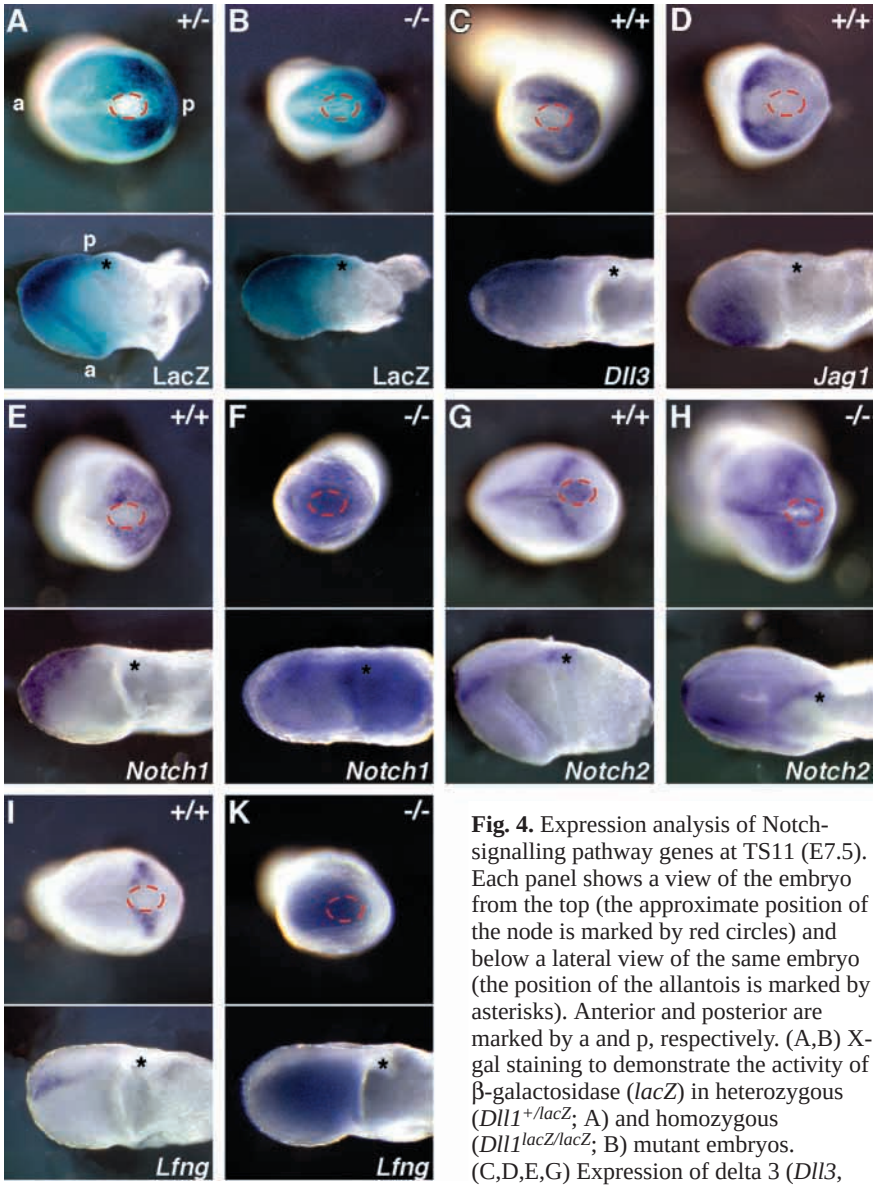

Fig. 4. Expression analysis of Notch-signalling pathway genes at TS11 (E7.5). Each panel shows a view of the embryo from the top (the approximate position of the node is marked by red circles) and below a lateral view of the same embryo (the position of the allantois is marked by asterisks). Anterior 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 *Dll1*^{lacZ} mutant embryos at this stage, expression of *Notch1* (5/18), *Notch2* (4/18) and *Lfng* (4/18) among the offspring from *Dll1*^{lacZ} heterozygous crosses was abnormal.

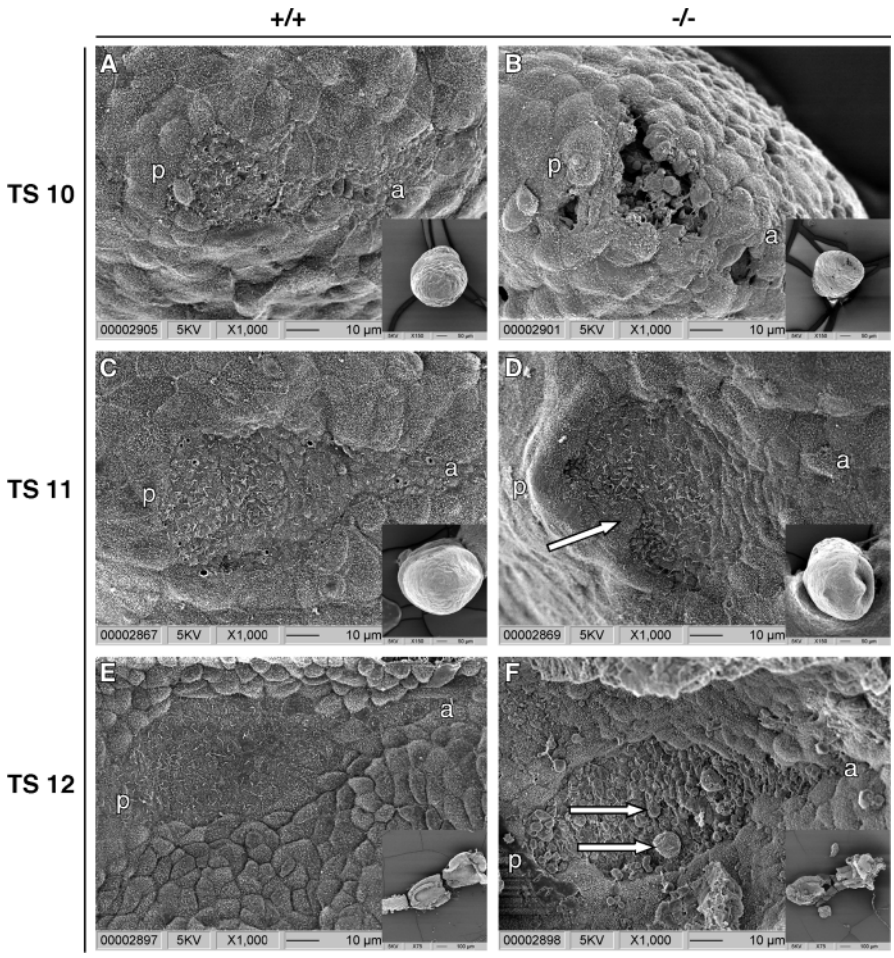

Fig. 5. Scanning electron micrographs (SEM) of the mouse node from TS10 to TS12 (E7.5-E8.5). Insets show the corresponding embryo at lower magnification. Anterior and posterior are marked by a and p, respectively. (A,B) At TS10, the node is established at the apex of the embryonic cone. The wild-type node (A) consists of small mesendodermal cells each with a prominent single cilium. In homozygous *Dll1*^{lacZ} mutants (B), morphological disturbances are evident as rupturing of the surface, bulging of cells and loss of monociliated cells. (C,D) The node at TS11. Wild-type embryos (C) have a club-shaped node with evenly distributed ciliated cells. Homozygous *Dll1*^{lacZ} mutants have a deformed node, where abnormal cell masses adjacent to the node disrupt the node symmetry (arrow in D). (E,F) SEM of the ventral mouse node from embryos at the six-somite stage (TS 12). The wild-type node (E) consists of microvilli-lined mesendodermal cells each with a cilium, whereas the homozygous *Dll1*^{lacZ} mutant node contained in addition enlarged cells without cilia (arrows in F).

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

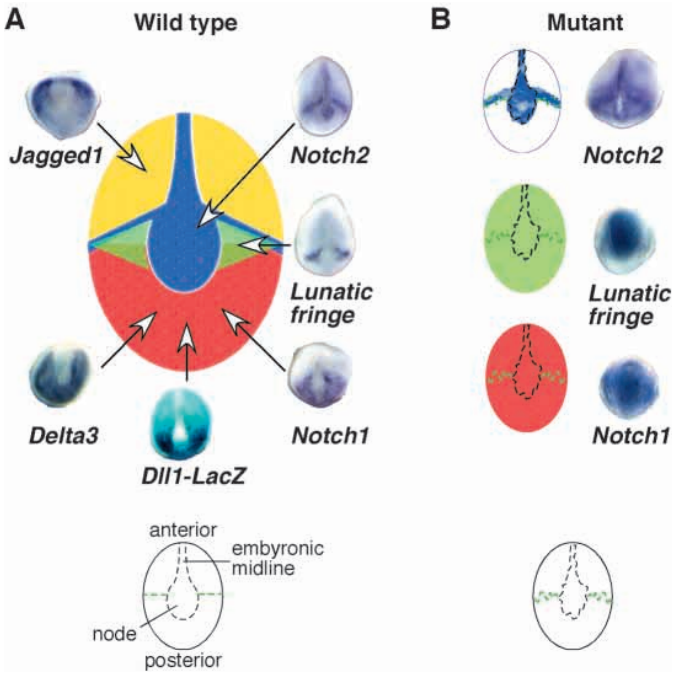

Fig. 6. Scheme of Notch-signalling pathway gene expression pattern at TS11 in wild-type (A) versus mutant (B) embryos. Details are given in the text. Hypothesised molecular boundaries are indicated as broken green lines, morphological boundaries of the node and embryonic midline as broken black lines.

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 *Sil*$^{-/-}$. In addition, these mutants display a combination of randomised heart looping or embryonic turning with a loss in *Ebaf* expression (Izraeli et al., 1999; Melloy et al., 1998; Meyers and Martin, 1999). The severe midline defects in *Dll1* 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 in *Dll1* 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 the *Delta1* 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, that *Dll1* 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 of *Dll1* 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 of *Dll1* 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 of *Dll1* 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 in *Dll1* 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 of *Drosophila*, 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; Micchelli and Blair, 1999; Rauskolb et al., 1999). When Notch signalling is disrupted, cells can intermix and violate the compartment border (Micchelli 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 homozygous *Dll1*^{lacZ} 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 of *Dll1* 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 of *Dll1* 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 of *Pitx2* (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 in *Notch1* and *Notch2* mutants, the analysis of compound mutants, homozygous for both *Notch1* 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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