

Notch signaling regulates left–right asymmetry determination by inducing *Nodal* expression

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Generation of left–right asymmetry is an integral part of the establishment of the vertebrate body plan. Here we show that the Notch signaling pathway plays a primary role in the establishment of left–right asymmetry in mice by directly regulating expression of the *Nodal* gene. Embryos mutant for the Notch ligand *Dll1* or doubly mutant for the Notch1 and Notch2 receptors exhibit multiple defects in left–right asymmetry. Analysis of the enhancer regulating node-specific *Nodal* expression revealed the presence of binding sites for the RBP-J protein, the primary transcriptional mediator of Notch signaling. Mutation of these sites destroyed the ability of this enhancer to direct node-specific gene expression in transgenic mice. Our results demonstrate that *Dll1*-mediated Notch signaling is essential for generation of left–right asymmetry, and that the Notch pathway acts upstream of *Nodal* expression during left–right asymmetry determination in mice.

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Generation of left–right asymmetry during development is an integral part of the establishment of the vertebrate body plan (Capdevila et al. 2000; Mercola and Levin 2001; Wright 2001; Yost 2001; Hamada et al. 2002). Specification of the left–right axis requires multiple steps: (1) generation of an initial asymmetric signal in or near the embryonic node, (2) transfer of asymmetric signals from the node to the lateral plate mesoderm (LPM),

(3) induction of an evolutionarily conserved cascade of gene expression in the left LPM, and (4) transformation of these left–right asymmetric signals into morphological asymmetries of the visceral organs. In mice, generation of the initial asymmetric signal requires directional fluid flow on the ventral surface of the node (Nonaka et al. 2002). This fluid flow is generated by motile monocilia on cells of the node, and the presence of nodal cilia is conserved in other vertebrates (Essner et al. 2002). However, the mechanism by which directional fluid flow at the node specifies orientation of the left–right axis is controversial (Stern and Wolpert 2002; Tabin and Vogan 2003). In addition, the mechanism for transfer of the initial asymmetric signal from the node to the LPM is unknown.

The Notch signaling pathway is an evolutionarily conserved intercellular signaling mechanism. Mutations in Notch pathway components disrupt embryonic development in diverse multicellular organisms and cause inherited disease syndromes in humans (Artavanis-Tsakonas et al. 1999; Mumm and Kopan 2000; Allman et al. 2002; Gridley 2003). Genes of the Notch family encode large transmembrane receptors that interact with membrane-bound ligands encoded by Delta/Serrate/Jagged family genes. The signal induced by ligand binding is transmitted intracellularly by a process involving proteolytic cleavage of the receptor and nuclear translocation of the intracellular domain of the Notch family protein (Notch-IC). Once in the nucleus, Notch-IC forms a complex with the RBP-J protein, a sequence-specific DNA-binding protein that is the primary transcriptional mediator of Notch signaling. The Notch-IC/RBP-J complex then activates transcription of downstream target genes (Artavanis-Tsakonas et al. 1999; Mumm and Kopan 2000; Allman et al. 2002; Gridley 2003).

Here we demonstrate that the Notch signaling pathway plays a primary role in the establishment of left–right asymmetry in mice by directly regulating expression of the *Nodal* gene. Embryos mutant for the Notch ligand *Dll1* or doubly mutant for the Notch1 and Notch2 receptors exhibit multiple defects in left–right asymmetry. Notably, *Dll1*^{-/-} embryos do not express *Nodal* in the region around the node. Analysis of the enhancer regulating node-specific *Nodal* expression (termed the NDE) revealed the presence of binding sites for the RBP-J protein. Mutation of these sites destroyed the ability of the NDE to direct node-specific gene expression in transgenic mice. These results demonstrate that *Dll1*-mediated Notch signaling is essential for generation of left–right asymmetry, and indicate that perinodal expression of the *Nodal* gene is an essential component of left–right asymmetry determination in mice.

Results and Discussion

Laterality defects in Dll1 mutant and Notch1/Notch2 double-mutant mouse embryos

During studies on the role of the *Dll1* gene during somitogenesis (Zhang et al. 2002), we observed that some *Dll1*^{-/-} embryos (Hrabé de Angelis et al. 1997) exhibited reversed heart looping. We examined this phenotype more closely by performing scanning electron micros-

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copy on *Dll1*^{-/-} and control littermate embryos isolated at embryonic day 9.5 (E9.5). Normally, the linear heart loops from the left toward the right side (Fig. 1A). Thirty-two percent of *Dll1*^{-/-} embryos exhibited normal rightward heart looping, and 42% exhibited reversed heart looping (Fig. 1B; Supplementary Table 1). We characterized the remaining 26% of the *Dll1*^{-/-} embryos as having ventral heart looping, in which the developing right ventricle and conotruncal region of the heart was situated above the primitive left ventricle (Fig. 1C). Another morphological manifestation of left–right axis determination is the direction of embryonic turning. Wild-type embryos undergo a 180° axial rotation shortly after the initiation of heart looping. We observed defects in axial rotation in *Dll1*^{-/-} embryos. Forty-three percent of the homozygous mutant embryos underwent reversed axial rotation, whereas the remaining 57% exhibited normal axial rotation (Supplementary Table 2). These morphological analyses suggested that laterality was randomized in *Dll1*^{-/-} embryos.

We also examined embryos homozygous for a *Notch1* null mutation (Swiatek et al. 1994) and a *Notch2* hypomorphic mutation (McCright et al. 2001) for defects in heart looping and axial rotation. We did not observe any laterality defects in embryos singly homozygous for mutations in either of these genes. However, we did observe defects in both heart looping and axial rotation in

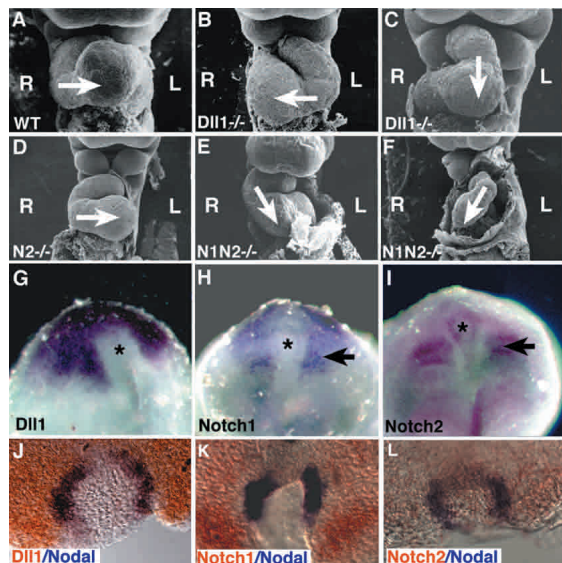


Figure 1. Heart looping defects in Notch pathway mutants. (A–F) Scanning electron micrographs of embryos at E9.5. The direction of heart looping is indicated by the arrow. The left (L) and right (R) sides of the embryo are indicated. (A) Wild-type embryo exhibiting normal right-to-left heart looping. (B) *Dll1*^{-/-} mutant embryo exhibiting reversed heart looping. (C) *Dll1*^{-/-} mutant embryo exhibiting ventral heart looping. (D) Embryo homozygous for the *Notch2*^{del1} hypomorphic allele (designated *N2*^{-/-}) exhibiting normal right-to-left heart looping. (E) *Notch1*^{-/-} *Notch2*^{-/-} double-mutant embryo exhibiting mild ventral looping. (F) Severely affected *Notch1*^{-/-} *Notch2*^{-/-} double-mutant embryo exhibiting reversed looping and cardiac hypoplasia. (G–I) Expression of Notch pathway genes around the node (asterisk). (G) *Dll1* expression. (H) *Notch1* expression. (I) *Notch2* expression. Both *Notch1* and *Notch2* are also expressed in the paraxial mesoderm (arrows). (J–L) Double-label whole-mount in situ hybridization with a digoxigenin-labeled riboprobe (blue) for *Nodal* and fluorescein-labeled riboprobes (orange) for *Dll1* (J), *Notch1* (K), or *Notch2* (L).

Notch1/Notch2 double homozygous mutant embryos (Fig. 1E,F; Supplementary Tables 1, 2), suggesting functional redundancy between the Notch1 and Notch2 proteins during left–right asymmetry determination. Laterality defects in *Notch1/Notch2* double-mutant embryos were less penetrant than in *Dll1*^{-/-} embryos, suggesting either that the hypomorphic *Notch2* allele retained partial function during left–right asymmetry determination or that other Notch family receptors also function in this process. Whole-mount in situ hybridization analyses confirmed previously reported (Bettenhausen et al. 1995; Williams et al. 1995) expression of the *Dll1*, *Notch1*, and *Notch2* genes in the region around the node in gastrulating mouse embryos (Fig. 1G–I), and double-label in situ hybridization revealed overlap between expression of the *Nodal* gene and the *Dll1*, *Notch1*, and *Notch2* genes in the region in and around the node (Fig. 1J–L).

To understand the molecular basis for the laterality defects in the *Dll1*^{-/-} mutant embryos, we examined expression of the *Nodal*, *Lefty1*, *Lefty2*, and *Pitx2* genes. These genes are expressed on the left side of the embryo, and form part of an evolutionarily conserved signaling cascade responsible for left–right morphogenesis (Capdevila et al. 2000; Mercola and Levin 2001; Wright 2001; Yost 2001; Hamada et al. 2002). At early somite stages in mice, the *Nodal*, *Lefty2*, and *Pitx2* genes all are expressed in the left LPM. In *Dll1*^{-/-} embryos, no *Nodal* or *Lefty2* expression was observed in the left LPM (Fig. 2B,F; Supplementary Tables 3, 4). *Pitx2* expression, on the other hand, was randomized in *Dll1*^{-/-} embryos. Twenty-five percent of *Dll1*^{-/-} embryos exhibited normal expression of *Pitx2* in the left LPM, 25% did not express *Pitx2* in the LPM, and 50% expressed *Pitx2* bilaterally in both the left and right LPM (Fig. 2D; Supplementary Table 3). In wild-type embryos, the *Lefty1* gene is expressed along the midline in the prospective floorplate. *Lefty1* expression is believed to serve a midline barrier function, preventing the spread of *Nodal*-induced patterning signals from the left to the right side of the embryo (Meno et al. 1998). No *Lefty1* expression was observed in *Dll1*^{-/-} embryos ($n = 9$; Fig. 2B). However, expression of the *Shh* and *Foxa2* genes was observed in the embryonic midline of *Dll1*^{-/-} embryos (Supplementary Fig. 1).

Nodal expression in the left LPM is preceded by *Nodal* expression adjacent to the node (the perinodal region; Collignon et al. 1996). We observed that at early somite stages *Nodal* expression also was absent from the perinodal region of *Dll1*^{-/-} embryos (Fig. 2F; Supplementary Table 4). In order to determine whether *Dll1*-mediated Notch signaling was required for the initiation or maintenance of node-specific *Nodal* expression, we examined *Dll1*^{-/-} embryos and littermate controls isolated at E7.5, prior to the formation of somites and the expression of *Nodal*, *Lefty2*, and *Pitx2* in the left LPM. *Nodal* was not expressed around the node in these headfold-stage mutant embryos (Fig. 2H; Supplementary Table 4), indicating that a *Dll1*-mediated Notch signal was essential for the initiation of *Nodal* expression around the node.

Przemeck et al. (2003) also recently reported laterality defects in *Dll1*^{-/-} embryos. Their results differ from ours and from those reported in the paper by Izpisua Belmonte and colleagues (Raya et al. 2003) in that they observed *Nodal* expression (in either left LPM, right LPM, or both) in approximately half of the *Dll1*^{-/-} embryos analyzed.

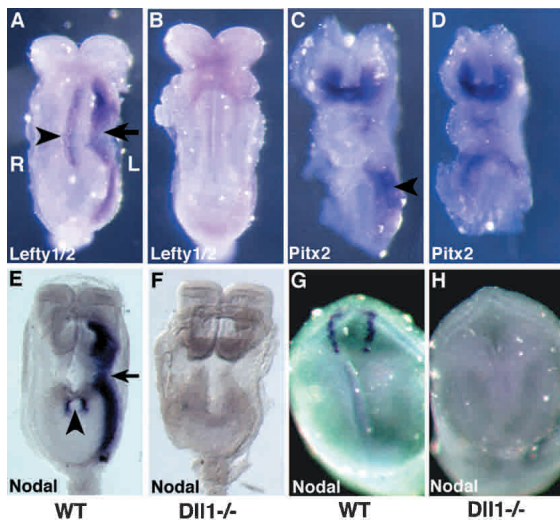


Figure 2. *Dll1*^{-/-} mutant embryos exhibit defects in the expression of left-right determinant genes. (A,B) *Lefty1/2* expression. Embryos were hybridized with an antisense riboprobe that detects both the *Lefty1* and *Lefty2* genes. (A) In the wild-type embryo, *Lefty1* is expressed along the midline in the prospective floorplate of the neural tube (arrowhead), and *Lefty2* is expressed in the left LPM (arrow). (B) In the *Dll1*^{-/-} embryo, no *Lefty1* or *Lefty2* expression is observed. Weak background staining is observed in the head of the mutant embryo. (C,D) *Pitx2* expression. (C) In the wild-type embryo, *Pitx2* is expressed in the head and in the left LPM (arrowhead). (D) In the *Dll1*^{-/-} embryo shown, *Pitx2* is expressed bilaterally in the LPM. Other *Dll1*^{-/-} mutant embryos did not express *Pitx2* in the LPM, or expressed it normally in the left LPM (see Supplementary Table 3). (E,F) *Nodal* expression at early somite stages. (E) In the wild-type embryo, *Nodal* is expressed in the node (arrowhead) and left LPM (arrow). (F) In the *Dll1*^{-/-} embryo, *Nodal* is not expressed. (G,H) *Nodal* expression in headfold-stage embryos (E7.5). (G) In the wild-type embryo, *Nodal* is expressed in the perinodal region. (H) *Nodal* is not expressed in the *Dll1*^{-/-} embryo. All images are ventral views, so that the left side of the embryo is toward the right of the panel (indicated in panel A).

They also observed *Lefty2* expression in *Dll1*^{-/-} embryos. We cannot explain the discrepancy between these results. We noted, however, that the whole-mount in situ hybridization conditions used by Przemeck et al. (2003) differed from ours. We therefore hybridized additional *Dll1*^{-/-} embryos ($n = 7$) with a *Nodal* antisense riboprobe using the in situ hybridization conditions of Przemeck et al. (2003; kindly provided by Martin Hrabé de Angelis). We did not observe *Nodal* expression in any *Dll1*^{-/-} embryos ($n = 20$) hybridized under either set of in situ hybridization conditions. It remains unclear, therefore, why the results of Przemeck et al. (2003) differ from the results reported here and in the paper by Izpisua Belmonte and colleagues (Raya et al. 2003). We should note, however, that genetic background differences exist between the two populations of *Dll1* mutant mice. The mice of Przemeck et al. (2003) were maintained on a mixed 129Sv \times C57BL/6J background, whereas our mice had been extensively back-crossed to C57BL/6J.

Nodal cilia are present and functional in *Dll1* mutant embryos

In mice, generation of the initial asymmetric signal involves a leftward fluid flow generated by the rotational movement of monocilia on the ventral surface of the

node (Capdevila et al. 2000; Mercola and Levin 2001; Wright 2001; Yost 2001; Essner et al. 2002; Hamada et al. 2002; Nonaka et al. 2002; Tabin and Vogan 2003). We established by immunofluorescence and scanning electron microscopy that monocilia were present on node cells of *Dll1*^{-/-} embryos (Fig. 3). To test whether these monocilia were functional, we examined nodal fluid flow by videomicroscopy. All *Dll1*^{-/-} embryos tested ($n = 7$) exhibited normal leftward fluid flow at the node (Supplementary Video 1). Therefore, defects in nodal flow do not appear to contribute to the laterality defects observed in *Dll1*^{-/-} embryos.

While performing videomicroscopy on these node explants, we noted that the nodes of some *Dll1*^{-/-} embryos appeared distorted, although this distortion did not affect nodal fluid flow. *Dll1*^{-/-} embryos with distorted nodes also appeared to be delayed in the formation of mesodermal somites, suggesting the possibility that the laterality defects observed in *Dll1*^{-/-} embryos were secondary to defects in somitogenesis. We therefore examined other Notch pathway mutants exhibiting defects in somite formation (Zhang et al. 2002) for morphological laterality defects, such as reversed heart looping or axial rotation. No morphological laterality defects were observed in either *Lfng*-deficient (Zhang and Gridley 1998; $n = 8$) or *Dll3*-deficient (Kusumi et al. 1998; $n = 12$) embryos, indicating that the laterality defects observed in *Dll1*^{-/-} embryos are not secondary to defects in somitogenesis.

The NDE enhancer contains RBP-J-binding sites essential for activity

Expression of the *Nodal* gene in the mouse node is regulated by a specific enhancer element termed the node-specific enhancer (NDE; Adachi et al. 1999; Norris and Robertson 1999). The NDE has been localized to a 0.8 kb-fragment located between -9.5 and -8.7 kb upstream of the *Nodal* gene (Fig. 4A; Adachi et al. 1999). Analysis

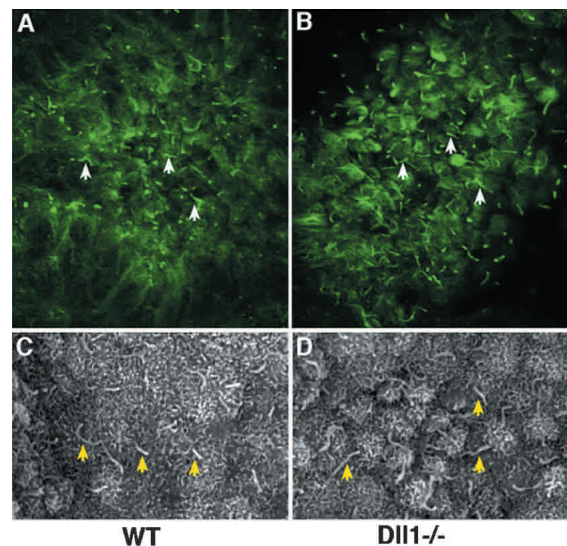


Figure 3. Presence of monocilia on node cells of *Dll1*^{-/-} mutant embryos. (A,B) Monocilia (some indicated with arrows) were detected by immunofluorescence with an antibody against acetylated tubulin. (C,D) Monocilia (arrows) were observed by scanning electron microscopy on both wild-type (C) and *Dll1*^{-/-} mutant (D) node cells.

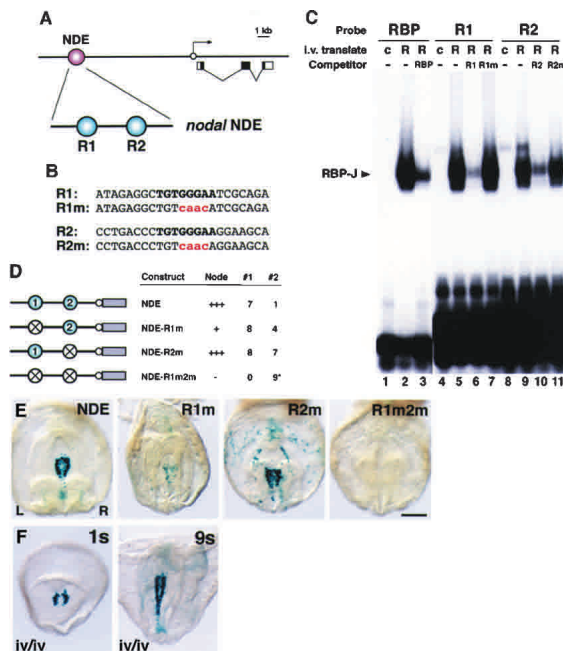


Figure 4. The *Nodal* node-specific enhancer (NDE) contains two RBP-J-binding sites essential for directing *lacZ* reporter gene expression. (A) The NDE enhancer resides in a 0.8-kb region upstream of *Nodal* coding sequences (−9.5 to −8.7 kb). Within the NDE, two motifs identical to the consensus binding sequence of RBP-J were present. The relative positions of the 5′ motif (site R1) and 3′ motif (site R2) are indicated. (B) The nucleotide sequences of sites R1 and R2 and of R1m and R2m containing four base pair substitutions (shown in red) in the RBP-J-binding consensus (shown in bold). (C) Electrophoretic mobility shift assay was performed with in vitro translated RBP-J protein (R; lanes 2,3,5–7,9–11) or control translates without RBP-J (c; lanes 1,4,8). Three labeled oligonucleotides were used: a consensus RBP-J sequence (RBP; lanes 1–3), site R1 from NDE (lanes 4–7), and site R2 from NDE (lanes 8–11). Binding reactions were performed in the absence (−) or presence of a 50-fold excess of unlabeled homologous oligonucleotides (lanes 3,6,10), or unlabeled mutated oligonucleotides R1m and R2m (lanes 7,11). (D) Summary of transient transgenic analysis of NDE constructs bearing single or double mutations in the two RBP-J-binding sites. The wild-type construct (NDE) contains the 0.8-kb NDE region linked to a *hsp68-lacZ* promoter (white circle) *lacZ* gene (gray bar) reporter cassette. The 5′ RBP-J-binding site, the 3′ RBP-J-binding site, and both RBP-J-binding sites were mutated in NDE-R1m, NDE-R2m, and NDE-R1m2m, respectively. For each construct, the level of NDE activity in the node, the number of embryos that showed NDE activity (column #1), and the number of embryos that carried the transgene but showed either only ectopic expression or no expression at all (column #2) are indicated. Four of the nine R1m2m embryos (indicated by an asterisk in column #2 of the table) showed weak, patchy expression in the center of the node, where the endogenous *Nodal* gene is not expressed. (E) Ventral views of X-gal-stained E8.2 embryos transgenic for the indicated constructs. The left (L) and right (R) sides of the axis are shown. Bar, 250 μm. (F) X-gal-stained *iv/iv* embryos transgenic for the NDE construct. One-somite (1s) and nine-somite (9s) *iv/iv* embryos exhibiting NDE activity are shown. The nine-somite embryo shown had reversed axial rotation.

of the nucleotide sequence of the NDE revealed the presence of two consensus binding sites (T/CGTGGGAA) for the RBP-J protein (Tun et al. 1994; Honjo 1996; Kato et al. 1997), the primary transcriptional mediator of the Notch signal (Fig. 4A,B). Electrophoretic mobility shift assays confirmed that these consensus RBP-J-binding sites bound recombinant RBP-J protein (Fig. 4C). Analysis of RBP-J RNA expression during mouse embryogen-

esis revealed that RBP-J is expressed ubiquitously, including in node cells that express the *Nodal* gene (Supplementary Fig. 2).

To assess the function of these RBP-J-binding sites in directing node-specific *Nodal* expression, we tested mutations of these binding sites in transgenic mouse embryos. Control experiments demonstrated that oligonucleotides containing these binding site mutations were unable to compete binding of wild-type oligonucleotides in electrophoretic mobility shift assays (Fig. 4C). As demonstrated previously, pronuclear injection of a transgene consisting of the 0.8-kb *Nodal* NDE linked to the *hsp68* promoter and β-galactosidase (*lacZ*) coding sequence (Kothary et al. 1989) gave rise to X-gal staining around the node in embryos isolated at early somite stages (Fig. 4D,E). The role of the two RBP-J-binding sites was investigated by mutating them separately or in combination. Mutation of the 5′ RBP-J-binding site (R1m) had a more severe effect on *lacZ* expression than mutation of the 3′ RBP-J-binding site (R2m). However, node-specific *lacZ* expression was lost completely when both RBP-J-binding sites were mutated (R1m2m; Fig. 4D,E).

We also examined activity of the *Nodal* NDE in embryos homozygous for the *iv* mutation, in which laterality is randomized. Previous work had demonstrated that an enhancer in the *Nodal* gene that directs expression in the left LPM (termed the asymmetric enhancer, or ASE) was responsive to the *iv* mutation (Adachi et al. 1999; Norris and Robertson 1999). However, *iv/iv* embryos transgenic for the NDE construct exhibited the same perinodal *lacZ* expression as wild-type embryos (Fig. 4F), indicating that the NDE enhancer functions independently of the *iv* mutation.

Our results demonstrate that *Dll1*-mediated Notch signaling is essential for expression of *Nodal* in the perinodal region, and that Notch signaling acts upstream of *Nodal* in left–right asymmetry determination in mice. Prior work supports the model that *Nodal* expression in the LPM is a positive regulator of subsequent *Pitx2* expression (Capdevila et al. 2000; Mercola and Levin 2001; Wright 2001; Yost 2001; Hamada et al. 2002). However, uncoupling of a strict correlation between expression of *Nodal* and *Pitx2* in the LPM has been observed previously (Meyers and Martin 1999; Constam and Robertson 2000a,b; Pennekamp et al. 2002). For example, some mouse embryos mutant for the *Pkd2* gene do not express *Nodal* in the LPM but exhibit bilateral expression of *Pitx2* in the LPM (Pennekamp et al. 2002). We observe these same patterns of expression in *Dll1*^{−/−} embryos, confirming that *Nodal* and *Pitx2* expression in the LPM need not be strictly correlated.

Our work also indicates that expression of the *Nodal* gene around the node is an essential component of left–right axis determination in mice. During the establishment of left–right asymmetry, positional information must be transferred from the node to the lateral plate mesoderm. Our results indicate that perinodal expression of the *Nodal* gene is essential for activation of asymmetric gene expression in the left LPM. A similar conclusion was reached in recent studies that used genetic strategies to selectively remove *Nodal* activity from the node (Brennan et al. 2002; Saijoh et al. 2003). These results are consistent with a model for asymmetric signal transfer in which *Nodal* protein produced in the perinodal region induces its own expression in the LPM,

although a model in which perinodal expression of *Nodal* regulates production of another molecule that induces asymmetric gene expression in the left LPM cannot be excluded. The loss of perinodal expression of the *Nodal* gene and the laterality defects observed in *Dll1* mutant and *Notch1/Notch2* double-mutant mice demonstrate that Notch signaling plays a significant and early role in regulating development of the left-right axis.

Materials and methods

Mutant mouse strains

Mutant mice used in these studies were *Dll1tm1Go* (Hrabé de Angelis et al. 1997), *Notch1in32* (Swiatek et al. 1994), *Notch2del1* (McCright et al. 2001), *LfnglacZ* (Zhang and Gridley 1998), and *Dll3pu* (Kusumi et al. 1998). Mice heterozygous for these mutant alleles were maintained by back-crossing to C57BL/6J (B6) mice. *Notch1in32*, *Notch2del1*, *LfnglacZ*, and *Dll3pu* mutant embryos were genotyped by allele-specific PCR. *Dll1tm1Go* mutant embryos were genotyped using a closely linked marker (D17Mit156) that is polymorphic between strains 129 and B6, permitting identification of the 129-derived *Dll1tm1Go* allele and the B6-derived wild-type *Dll1* allele. PCR primer sequences are available on request.

Whole-mount *in situ* hybridization and immunofluorescence

Single- and double-label whole-mount *in situ* hybridization was performed as described previously (Krebs et al. 2001). Presence of node monocilia was detected using a monoclonal antibody against acetylated tubulin (Sigma), as described (Essner et al. 2002).

Visualization of nodal flow

Embryos were dissected and cultured as described previously (Nonaka et al. 1998, 2002). Fluid flow was visualized by addition of polystyrene beads to the culture medium. Images were collected with a monochrome CCD camera. Embryos were genotyped by PCR after observation.

Electrophoretic mobility shift assay

RBP-J protein was synthesized with an *in vitro* transcription-translation coupled system (Promega). An electrophoretic mobility shift assay was performed with the *in vitro* translated RBP-J protein (Okamoto et al. 1990). Three oligonucleotides were used for the binding assay: a consensus binding sequence for RBP-J (AAACACGCCGTGGGAAAAA TTTGG) derived from the Epstein-Barr virus C promoter region (Lu and Lux 1996); the R1 site from NDE (ATAGAGGCTGTGGGAATCGCA GA); and the R2 site from NDE (CCTGACCCTGTGGGAAGGAAGCA). The mutant oligonucleotides R1m (ATAGAGGCTGTCAACATCG CAGA) and R2m (CCTGACCCTGTCAACAGGAAGCA) were used as competitors to assess binding specificity.

lacZ constructs and transient transgenic assay

For the *NDE-lacZ* construct, the 0.8-kb NDE region was subcloned into the *hsp68-lacZ* vector (Kothary et al. 1989). To generate site-directed mutant constructs, we subcloned the 0.8-kb NDE region into pKF18 (Takara), and the RBP-J-binding sites (TGTGGGAA) were mutated with oligonucleotides harboring four base substitutions (TGTCAACA). The mutated NDE fragments were similarly subcloned into the *hsp68-lacZ* vector. The resulting *lacZ* plasmids were digested with *Not I* to remove vector sequences. The *lacZ* DNA fragments were purified by gel electrophoresis and were microinjected into fertilized mouse eggs, as described (Saijoh et al. 1999). Embryos were recovered at E8.2 and examined for *lacZ* expression by X-gal staining according to standard protocols. Transgene integration was determined by PCR amplification of the *lacZ* gene.

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