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 ligandDll1 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.

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 micro-
copy on Dll1−/− and control littermate embryos isolated at embryonic day 9.5 (E9.5). Normally, the linear heart tube 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 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 floor-plate. 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 Izpisúa 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.
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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 Izpisúa 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 × 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 Vogán 2003]. 3

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

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**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).

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**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.
of the nucleotide sequence of the NDE revealed the presence of two consensus binding sites [T/CCTGGGAA] 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 embryogenesis 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; Sajioh 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), Notch1tm2Sw (Swiatek et al. 1994), Notch2del1 (McCrIGHT et al. 2001), LfneglacZ (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. Notch1tm2Sw, Notch2del1, LfneglacZ, 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 monolalia was detected using a monoclonal antibody against acetylated tubulin (Sigma), as described (ESSNER et al. 2002).

**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 (AAACACGCCGTGGGAAAA TTTGG) derived from the Epstein-Barr virus C promoter region (Lu and LUX 1996), the R1 site from NDE (ATAGAGGCTGTTGGAATCCGCA GA), and the R2 site from NDE (CTCTGCCCTGGTGGAAAGGACCA). The mutant oligonucleotides R1m (ATAGAGGCTGTACATTG CAGA) and R2m (CCTGACCTGTTCACAGGAAGGACCA) 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 (KOTHRAY et al. 1989). To generate site-directed mutant constructs, we subcloned the 0.8-kb NDE region into pKFl8 (Takara), and the RBP-J-binding sites (TGTGGA) were mutated with mutant constructs, we subcloned the 0.8-kb NDE region into pKF18 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 (SAIJO 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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