RESEARCH COMMUNICATION

Notch activity induces *Nodal* expression and mediates the establishment of left–right asymmetry in vertebrate embryos

Ángel Raya,^{1,3} Yasuhiko Kawakami,^{1,3} Concepción Rodríguez-Esteban,^{1,3} Dirk Büscher,¹ Christopher M. Koth,¹ Tohru Itoh,¹ Masanobu Morita,¹ R. Marina Raya,¹ Ilir Dubova,¹ Joaquín Grego Bessa,² José Luis de la Pompa,² and Juan Carlos Izpisua Belmonte^{1,4}

¹Gene Expression Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037, USA; ²Molecular Oncology, Institut de Recerca Oncologica, Hospital Duran i Reynals, 08907 L'Hospitalet de Llobregat, Barcelona, Spain

Left-sided expression of *Nodal* in the lateral plate mesoderm is a conserved feature necessary for the establishment of normal left–right asymmetry during vertebrate embryogenesis. By using gain- and loss-of-function experiments in zebrafish and mouse, we show that the activity of the Notch pathway is necessary and sufficient for *Nodal* expression around the node, and for proper left–right determination. We identify Notch-responsive elements in the *Nodal* promoter, and unveil a direct relationship between Notch activity and *Nodal* expression around the node. Our findings provide evidence for a mechanism involving Notch activity that translates an initial symmetry-breaking event into asymmetric gene expression.

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Vertebrates display a left–right (LR) asymmetry that is most evident in the disposition of internal organs. In recent years, our understanding of the molecular mechanisms involved in LR determination during vertebrate embryogenesis has increased significantly. A genetic cascade has been proposed that positions and restricts the expression of *Nodal* on the left side of the lateral plate mesoderm (LPM; for reviews, see Burdine and Schier 2000; Capdevila et al. 2000; Mercola and Levin 2001; Hamada et al. 2002). This left-sided expression of *Nodal* is a conserved feature of all vertebrate embryos studied to date, and correlates with the normal establishment of LR asymmetry.

The expression pattern of *Nodal* during early embryo development is tightly regulated. Transgenic approaches

in mice have identified node-specific enhancer (NDE) and left-side asymmetric enhancer (ASE) elements within the *Nodal* gene (Adachi et al. 1999; Norris and Robertson 1999). Recent evidence using conditional ablation of *Nodal* gene expression in the mouse have conclusively shown that *Nodal* expression around the node is critical for the establishment of asymmetric gene expression in the left LPM, including that of *Nodal* (Brennan et al. 2002; Saijoh et al. 2003). Here we identify binding sequences for the Notch pathway effector RBPjk/CBF1/Su(H) in the NDE of the *Nodal* promoter. We also show that Notch activity is necessary and sufficient to induce *Nodal* expression around the node. Notch activity appears, therefore, to function as a key regulator of proper LR determination, as well as one of the earliest genes upstream of *Nodal* expression identified to date.

Results and Discussion

The Notch pathway includes a transmembrane receptor (Notch), the DSL ligands (*Delta* and *Serrate*/Jagged in *Drosophila* and vertebrates, *Lag-2* in *Caenorhabditis elegans*), and CSL DNA-binding protein (CBF1/RBPjk in vertebrates, Su(H) in *Drosophila*, *Lag-1* in *C. elegans*; for review, see Artavanis-Tsakonas et al. 1999). Upon binding to the DSL ligands, the cytoplasmic domain of Notch is cleaved and released, enters the nucleus, and acts as a transcription factor in conjunction with the CSL DNA-binding proteins. Thus, an experimental strategy to activate the Notch pathway is to induce the expression of the intracellular domain of Notch (Notch^{IC}). We injected one-cell stage zebrafish embryos with in vitro transcribed mRNA encoding mouse Notch^{IC}, and evaluated its effects on LR establishment. To analyze the fate of the injected mRNA, we performed in situ hybridization with a mouse-specific Notch^{IC} cRNA probe. Upon injection, patches of expression were distributed throughout the embryo ($n = 150$; Fig. 1B). To confirm that Notch^{IC} effectively activates Notch signaling in zebrafish embryos, we analyzed the expression of *hairy* and *enhancer of split 5* (*hes5*), an in vivo target of the Notch pathway (de la Pompa et al. 1997), and of *deltaC*, a zebrafish homolog of mouse *Delta-like 1* (*Dll1*; Bettenhausen et al. 1995). For this purpose, we cloned a zebrafish homolog of *hes5* (GenBank accession no. AY264404), and analyzed its expression and that of *deltaC* in control and Notch^{IC} mRNA-injected embryos. A dramatic up-regulation of both transcripts was detected in embryos injected with Notch^{IC} (Fig. 1C–F), indicating that this treatment results in effective domains of Notch activation throughout the embryo during gastrulation and somitogenesis. When embryos injected with Notch^{IC} were allowed to develop further, we observed a wide array of developmental alterations affecting the anteroposterior and dorsoventral axes. Interestingly, the LR axis was also altered in 47% of embryos ($n = 450$) injected with Notch^{IC} mRNA, as evaluated by reversal of heart looping. To confirm that the alterations in heart looping were not a consequence of other developmental alterations, we restricted our analyses to embryos not displaying evident alterations in the anteroposterior or dorsoventral axes. Also in this case the LR axis appeared randomized, with

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³These authors contributed equally to this work.

⁴Corresponding author.

E-MAIL belmonte@salk.edu; FAX (858) 453-2573.

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Figure 1. Increased Notch activity alters left-right development. (A–H) *Notch1C* (A,B), *hes5* (C,D), *deltaC* (E,F), and *cyclops* (G,H) transcripts in 12-hpf control (A,C,E,G), and *Notch1C* mRNA-injected (B,D,F,H) zebrafish embryos. Embryo views are posterior, dorsal to the top. (I,J) Transgenic zebrafish embryos expressing a heart-specific *mlc2a*-EGFP reporter were injected with *Notch1C* mRNA (J). This treatment resulted in alterations in heart looping and positioning (arrow), when compared to control embryos (I). Embryo views are ventral, anterior to the top. (K–N) Whole-mount in situ hybridization for the *Nodal*-related gene *cyclops* (K,L) and *pitx2* (M,N) revealed the appearance of ectopic transcripts for both genes in the right LPM of 24-hpf *Notch1C*-injected embryos (L,N, arrows). Embryo views are dorsal, anterior to the top. Left (L) and right (R) sides are indicated.

almost 50% of embryos (68 out of 142) displaying reversed heart looping (Fig. 1I,J).

We then analyzed the expression of genes involved in LR asymmetry, including the *Nodal*-related gene *cyclops* and *pitx2*, normally expressed only in the left LPM. Injection of *Notch1C* mRNA resulted in bilateral expression of both genes in the LPM at 20 hpf (45% and 50%, $n = 48$ and 36 for *cyclops* and *pitx2*, respectively; Fig. 1K–N), indicating that Notch activity acts early in the genetic cascade determining LR asymmetry in zebrafish embryos. To investigate the mechanism by which Notch activation results in laterality defects, we analyzed *cyclops* expression at earlier developmental stages. In 11–12 hpf embryos, *cyclops* is expressed in two distinct domains, anterior and posterior, the latter associated to the embryonic shield (Fig. 1G; Rebagliati et al. 1998). This structure has been proposed to represent the zebrafish node (Essner et al. 2002, and references therein). *Notch1C*-injected embryos displayed strong up-regulation of *cyclops* expression around the node (98%, $n = 320$). Interestingly, this expression was not perfectly symmetric in most injected embryos (Fig. 1H), indicating that *Notch1C* injection induces LR defects by creating asymmetric domains of perinodal *cyclops* expression. These results further suggest that Notch activity is able to up-regulate *cyclops* expression around the node.

To verify the evolutionary conserva-

tion of the role of the Notch pathway in LR development, we undertook a series of loss-of-function analyses in the mouse, using the two targeted mutants of the Notch pathway that display the most severe developmental phenotypes; that of the ligand *Dll1* (Hrabe de Angelis et al. 1997), and that of the effector *RBPjk* (Oka et al. 1995). After careful examination, we were able to detect reversed heart looping in around 60% of embryos homozygous for mutations in either *Dll1* (Fig. 2B,C; $n = 12$), or *RBPjk* (Fig. 2D; $n = 12$). The analysis of laterality genes revealed that *Nodal* expression was absent from the node and the left LPM in either mutant of the Notch pathway (Fig. 2E–J; $n = 7$ and 9 for *Dll1*^{−/−} and *RBPjk*^{−/−}, respectively). These results indicate that *Nodal* expression around the node depends on *Dll1*-mediated Notch activity. The finding that *Nodal* expression around the node is necessary for *Nodal* expression in the LPM is in agreement with recent evidence from experiments with node-specific *Nodal* deletion (Brennan et al. 2002; Saijoh et al. 2003). Consistent with the absence of *Nodal* expression, we could not detect *Pitx2* transcripts in the LPM of around 50% of *Dll1*^{−/−} (Fig. 2L; $n = 4$) or *RBPjk*^{−/−} (Fig. 2M; $n = 8$) mutants. Surprisingly, the remaining mutant embryos showed normal left-sided expression of *Pitx2* in the LPM, except for one *RBPjk*^{−/−} embryo, which displayed bilateral expression (data not shown). The fact that *Pitx2* expression is uncorrelated to that of *Nodal* has been previously reported for a variety of experimental conditions, and is likely to reflect the existence of additional activator(s) of *Pitx2* in the LPM (Constam and Robertson 2000; Pennekamp et al. 2002). We also analyzed the expression of *Lefty-1*, a gene also required for proper LR asymmetry (Meno et al. 1998), in *Dll1*^{−/−} and *RBPjk*^{−/−} embryos. No *Lefty-1* expression

Figure 2. Notch activity is necessary for *Nodal* expression and proper left-right determination. (A–D) Frontal views of E9.5 wild-type (A), *Dll1*^{−/−} (B,C), and *RBPjk*^{−/−} (D) embryos. Normal rightward heart looping is evident in wild-type embryos (A), while *Dll1*^{−/−} and *RBPjk*^{−/−} mutants display alterations in heart looping, ranging from incomplete looped heart (C), to leftward looping (B,D). OT, outflow tract; RV, right ventricle; LV, left ventricle; AT, atrium. (E–P) Whole-mount in situ hybridization for genes implicated in normal LR development revealed that *Nodal* transcripts were absent in the perinodal region of *Dll1*^{−/−} and *RBPjk*^{−/−} mice (E–G, arrow in E denotes presence in wild type) and also, later on, were absent in the left LPM (H–J). *Pitx2* transcripts were also absent from the left LPM (K–M; arrowhead in H and K denotes presence in wild type). Similarly, the midline and LPM domains of expression of *Lefty1* were absent in both *Dll1*^{−/−} and *RBPjk*^{−/−} mice (N–P). Embryo views are ventral, anterior to the top in E–G, and dorsal, anterior to the top in H–P. Please note that coloring reaction for in situ hybridizations was allowed to proceed longer than usual to verify the absence of signal in the mutants; hence, the increased background. Left (L) and right (R) sides are indicated.

Studies were performed in silico, in vitro, and in vivo to further investigate the nature of the genetic relationship between Notch activity and *Nodal* expression. A search for conserved regions in the noncoding sequences of the human and mouse *Nodal* genes revealed the existence of one region of high (>75%) conservation centered at -10 kb (Fig. 3A). This evolutionarily conserved region lies within the previously characterized *Nodal* NDE (Adachi et al. 1999; Norris and Robertson 1999; Brennan et al. 2002). A search for conserved regulatory elements in this region identified a consensus binding-site for Hoxc8 and two binding-sites for CBF1/RBPjk (Fig. 2B). To ascertain whether these putative binding sites are able to bind RBPjk, we performed electrophoretic mobility shift assays using a DNA probe representing nucleotides -10,261 to -9,905 in the mouse *Nodal* gene, and recombinant Su(H) produced in bacteria. The transcription factor efficiently bound to the *Nodal* promoter in vitro, as assessed by a shift in the probe's mobility. This binding could be competed with synthetic oligonucleotides representing either putative RBPjk-binding site and flanking regions, but not with oligonucleotides in which the putative recognition sites had

been mutated (Fig. 2C). These results suggest that perinodal expression of *Nodal* may be regulated by conserved promoter elements that depend directly on Notch activity.

To investigate whether these elements are functional in vivo, we utilized a transgenic approach in mice. A 355-bp fragment of the mouse *Nodal* promoter containing the RBPjk-binding sites was fused to a *lacZ*-reporter cassette to create a transgene similar to others previously shown to drive expression around the node (Adachi et al. 1999; Norris and Robertson 1999; Brennan et al. 2002). We also generated mutant versions of this construct in which the first, second, or both RBPjk-binding sites were substituted for an unrelated sequence, recognized by the endonuclease *Pme* I. Six β -galactosidase-positive embryos were obtained for the wild-type reporter construct, from a total of 10 showing transgene integration by PCR. The staining pattern in those embryos was very similar, limited to the perinodal region, recapitulating the endogenous *Nodal* expression around the node (Fig. 3D,E), consistent with previous results utilizing comparable transgenes (Adachi et al. 1999; Norris and Robertson 1999). For the 5' RBPjk-binding site mutation, no node-specific *lacZ* expression was detected in eight embryos that were positive for transgene integration by PCR (Fig. 3F). A weak, ectopic β -galactosidase staining was observed in one transgenic embryo out of seven obtained after injection of the construct mutated at the 3' RBPjk-binding site (Fig. 3G). The combined mutation resulted in a complete absence of *lacZ* expression in 15 embryos that showed transgene integration (Fig. 3H). Taken together, our results show that there are two conserved RBPjk recognition sites in the *Nodal* promoter, and that both sites are required for perinodal expression of *Nodal* in the mouse embryo.

One of the earliest determinants of LR establishment in the chick embryo is the left-sided expression of *Shh* around Hensen's node. At Hamburger-Hamilton stage 5 (HH; Hamburger and Hamilton 1951), *Shh* transcripts become asymmetrically localized on the anterior-left side of the node. Gain- and loss-of-function studies indicate that this asymmetric expression is pivotal for the subsequent left-sided *Nodal* expression in the LPM (Pagan-Westphal and Tabin 1998). Because our results show that Notch activity is immediately upstream of *Nodal* expression in the node, we reasoned that SHH might regulate *Nodal* expression by modulating Notch activity. To address this possibility, we monitored Notch activity by analyzing the expression pattern of *Dll1* [up-regulated by Notch activation in zebrafish (Fig. 1E,F) and chick embryos (data not shown)] after overexpression or down-regulation of SHH. We also analyzed the expression of *Hes5* and *Lunatic fringe* (*Lfng*), both of which are regulated by Notch activity during somitogenesis (Barrantes et al. 1999; Morales et al. 2002), as well as during gastrulation (see below). Implantation on the right side of HH3–HH4 chick embryos of SHH-soaked beads did not perturb the normal expression of *Dll1* (Fig. 4A,B; 90% of the implanted embryos, $n = 10$), *Lfng* (Fig. 4D,E; 90%, $n = 10$), or *Hes5* (100%, $n = 10$; data not shown). Conversely, implantation of beads soaked in anti-SHH blocking antibody on the left side of HH3–HH4 chick embryos did not have any effect on *Dll1* (Fig. 4A,C; 100%, $n = 10$), *Lfng* (Fig. 4D,F; 100%, $n = 10$), or *Hes5* (100%, $n = 10$; data not shown) expression. We also analyzed the expression patterns of *Dll1* and *Lfng* in

Figure 4. Notch activity during left-right determination does not depend on SHH activity. Implantation on the right side of HH3–HH4 chick embryos of SHH soaked beads (B), or of anti-SHH blocking antibody on the left side (C) did not have any effect on *Dll1* expression around Hensen's node. Similarly, *Lfng* transcripts were not altered after implanting SHH soaked beads (E) or anti-SHH antibody (F). (G–J) Whole-mount in situ hybridization in E7.5–E8.0 *Shh*^{-/-} mouse embryos did not reveal any alteration in the expression pattern of either *Dll1* or *Lfng*. Left (L) and right (R) sides are indicated. Relative position of the node is denoted by a circle.

mouse embryos homozygous for a *Shh* null mutation (Chiang et al. 1996). As with the chick experiments, the loss of *Shh* function did not induce alterations in the expression of either gene (Fig. 4G–J; data not shown; 100%, $n = 5$ each).

These results indicate that *Shh* function does not regulate Notch activity around the node. Several lines of evidence indicate that monociliated cells in the mouse node function to provide asymmetric cues upstream of the *Shh* pathway. Notch activity, in turn, does not appear to regulate the normal function of nodal cilia (Krebs et al. 2003). Therefore, we tested whether Notch activity around the node is regulated by nodal flow. For this purpose, we analyzed the expression of components of the Notch pathway in several mouse mutants with disrupted nodal cilia function. We used the expression of *Dll1* as a reporter of Notch activity, based on our finding that *Notch*^{1C} injection up-regulates *Delta*-like genes during early development of zebrafish (Fig. 1E,F) and chick (data not shown) embryos. Additionally, we used the expression of *Lfng*, because its expression is dependent on Notch activity during mouse gastrulation, as indicated by the *Lfng* down-regulation observed in either *Dll1* or RBPjk mutant mice (Fig. 5A–C).

The mouse mutation *inversus viscerum* (*iv*), characterized by randomization in LR asymmetry, disrupts the gene encoding the ciliary motor “left-right” dynein (Supp et al. 1997), and results in immotile nodal cilia (Okada et al. 1999). *Nodal* expression in the LPM of *iv* mutants is randomized (Lowe et al. 1996). We analyzed the expression of *Dll1* and *Lfng* in E7.5–E8.0 mouse embryos homozygous for the *iv* mutation, and found no obvious differences in their expression pattern when compared to wild-type littermates (Fig. 5D–E,G–H; 100%, $n = 8$ each). To confirm these results, we repeated our analyses in a different model of nodal cilia dysfunction. Embryos mutant for the microtubule-associated motor protein kinesin KIF3A display randomization of LR determination and alterations in nodal flow (Marszalek et al. 1999; Takeda et al. 1999). Again, we were un-

Figure 5. Notch activity regulates left–right asymmetry independently of nodal cilia function. *Lfng* is expressed in E7.5–E8.0 control mouse embryos as two sharp bilateral perinodal stripes (A), barely observable in *Dll1*^{−/−} (B) or *RBPjk*^{−/−} (C) embryos, indicating that *Lfng* expression depends of Notch activity during mouse gastrulation. Analysis of *Dll1* expression in E7.5–E8.0 *iv* (E) and *KIF3A*^{−/−} (F) embryos did not reveal any alteration in the expression pattern when compared to control littermates (D). Similarly, *Lfng* expression is not altered in *iv* (H) or *KIF3A*^{−/−} (I) embryos, when compared to that of wild-type (G). Relative position of the node is denoted by a circle.

able to detect any alteration in the expression of either *Dll1* (Fig. 5D,F; 100%, *n* = 7) or *Lfng* (Fig. 5G,I; 100%, *n* = 6) in *KIF3A*^{−/−} embryos. The results obtained from the analyses in both mouse mutations suggest that the mechanism by which the Notch pathway regulates LR determination does not depend on the proper function of nodal cilia.

We provide evidence for an evolutionarily conserved role of Notch signaling in the establishment of the LR axis in fish and mammals. Our results also demonstrate an absolute requirement of Notch signaling for *Nodal* expression around the node, and the need for perinodal expression of *Nodal* for its subsequent activation in the LPM (see also Brennan et al. 2002; Saijoh et al. 2003). Although it remains to be determined whether different species utilize a common mechanism to break the initial LR symmetry, a conserved feature of early normal LR determination is the left-sided expression of *Nodal* in the LPM. Our results unveil the existence of an earlier conserved feature, represented by the Notch signaling pathway, whose activity lies directly upstream of *Nodal* expression around the node, and whose normal function is critical for correct LR specification.

Materials and methods

Chick embryo culture and bead implantation

Chick embryos were explanted and grown in vitro as described (Izpisua-Belmonte et al. 1993). Beads were soaked in SHH protein or anti-SHH antibody as described (Rodríguez-Esteban et al. 1999).

Zebrafish embryo manipulation

Zebrafish embryo maintenance, culture, and RNA injection were as described (Ng et al. 2002).

In situ hybridization

After the different treatments, we processed embryos for whole-mount in situ hybridization as previously published (Izpisua-Belmonte et al. 1993; Ng et al. 2002). The different cDNAs utilized for the antisense probes utilized for mouse, chick, and zebrafish in situ hybridization have been described elsewhere. Details will be provided upon request.

Mutant and transgenic mice

A *Bgl*III fragment of the mouse *Nodal* promoter comprising nucleotides −10,261 to −9,905, and mutated versions thereof, were fused to an *hsp68* minimal promoter and the coding sequences of *lacZ*. Linearized DNA was injected into the male pronucleus of B6D2F1 fertilized oocytes. After transferring to pseudo-pregnant ICR females, embryos were recovered at E7.5–E8.5 and stained for β-galactosidase activity following standard procedures. Presence of the transgene was confirmed by PCR of yolk sac genomic DNA using primers specific for *lacZ*. *RBPjk*^{−/−}, *Shh*^{−/−}, *iv*, *Dll1*^{−/−}, and *KIF3A*^{−/−} mice have been previously reported (Oka et al. 1995; Chiang et al. 1996; Lowe et al. 1996; Hrabe de Angelis et al. 1997; Marszałek et al. 1999). Genotyping was performed following the original descriptions.

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Ángel Raya, Yasuhiko Kawakami, Concepción Rodríguez-Esteban, et al.

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