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Reversal of left-right asymmetry induced by aberrant Nodal signaling in the node of mouse embryos

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The node at the anterior tip of the primitive streak serves as an initial generator of the left-right (L-R) axis in mammalian embryos. We now show that a small disturbance in molecular signaling at the node is responsible for the L-R reversal of visceral organs in the *inv* mutant mouse. In the node of wild-type embryos, the expression of *Nodal* and *Cerl2* (*Dand5*), which encodes an inhibitor of Nodal, is asymmetric, with the level of *Nodal* expression being higher on the left side and that of *Cerl2* expression higher on the right. In *inv/inv* embryos, however, a localized reduction in the level of *Cerl2* expression results in upregulation of the Nodal signal and a consequent induction of *Lefty* expression in the node. The ectopic expression of *Lefty1* delays the onset of *Nodal* expression in the lateral plate mesoderm. L-R asymmetry of *Cerl2* expression in the node also becomes reversed in a manner dependent on the Nodal signal. *Nodal* expression in the lateral plate mesoderm then appears on the right side, probably reflecting the balance between Nodal and Cerl2 in the node. The inhibition of *Cerl2* expression by the Nodal signal suggests a mechanism for amplification of the cue for L-R asymmetry provided by nodal flow and for stabilization of asymmetric gene expression around the node. In *inv/inv* embryos, this system may function in reverse as a result of ectopic production of Lefty, which inhibits the Nodal signal on the left side in a manner dependent on leftward nodal flow.

KEY WORDS: Left-right axis, Node, Mouse, Nodal signal, *inv*

INTRODUCTION

Establishment of the left-right (L-R) axis is fundamental for morphogenesis of visceral organs (Hamada et al., 2002; Shiratori and Hamada, 2006). The L-R axis of the mouse embryo is established by successive processes that begin with a leftward flow of fluid on the ventral surface of the node (nodal flow). Asymmetric expression of *Nodal* and *Cerl2* (*Dand5* – Mouse Genome Informatics) subsequently develops in the crown cells of the node, with the level of *Nodal* expression being higher on the left side and that of *Cerl2* expression being higher on the right. It has been suggested that Cerl2 binds Nodal and thereby inhibits its activity, with the result that more active Nodal probably emanates from the left side of the node than from the right side (Marques et al., 2004). The Nodal signal is then transmitted to the lateral plate mesoderm (LPM), where it induces *Nodal* expression (Oki et al., 2007). Positive and negative regulatory loops amplify the small difference in gene expression in the node to generate the robust expression of *Nodal* in the left LPM (Nakamura et al., 2006). After *Nodal* expression appears in the left LPM near the node, the positive regulatory loop extends the *Nodal* expression domain to the entire left LPM (Norris et al., 2002; Yamamoto et al., 2003; Nakamura et al., 2006). Conversely, Lefty1 and Lefty2, which are feedback inhibitors of the Nodal signal, prevent *Nodal* expression in the right LPM (Meno et al., 1998; Meno et al., 2001). Nodal in the left LPM then induces the expression of *Pitx2*, which encodes a transcription factor required for correct L-R morphogenesis (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Lin et al., 1999; Lu et al., 1999; Shiratori et al., 2001).

Whereas this sequence of principal events in establishment of the L-R axis has been relatively well characterized, the initial molecular mechanism by which asymmetric gene expression develops at the node remains largely unknown. This lack of knowledge is exemplified by the lack of a clear explanation for the L-R phenotype of mice with the recessive *inv* (inversion of embryonic turning) mutation (Yokoyama et al., 1993). The *inv* mutation was detected in transgenic mouse lines and results in a phenotype characterized by situs inversus and cyst formation in the kidneys. In *inv/inv* embryos, *Nodal* and *Pitx2* are expressed in the right LPM, in a reversal of the pattern seen in wild-type embryos, giving rise to the situs inversus (Collignon et al., 1996; Lowe et al., 1996; Ryan et al., 1998). The role of *inv* is evolutionarily conserved, with the corresponding human gene (*INVS*) having been shown to be responsible for infantile nephronophthisis (NPHP2) (Otto et al., 2003). The inversin (Inv) protein contains 15 tandem repeats of the ankyrin motif, two destruction boxes and two IQ motifs (Mochizuki et al., 1998; Morgan et al., 1998; Yasuhiko et al., 2001; Morgan et al., 2002). Although Inv has been shown to interact with various proteins, including calmodulin, nephrocystin (Nphp1 – Mouse Genome Informatics), Apc2, dishevelled, catenins and N-cadherin (cadherin 2 – Mouse Genome Informatics), its mechanism of action in L-R axis formation remains unknown (Yasuhiko et al., 2001; Morgan et al., 2002; Nurnberger et al., 2002; Otto et al., 2003; Simons et al., 2005). If the direction of nodal flow were rightward in the *inv/inv* embryo, the explanation for its L-R reversal phenotype would be simple. However, the direction of nodal flow in such embryos is normal, although the flow is slow and turbulent (Okada et al., 1999). The fact that L-R asymmetry is reversed despite the normal direction of nodal flow in *inv/inv* mice has been a challenge to the notion that nodal flow plays a key role in L-R determination.

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We have now examined the mechanism by which L-R asymmetry of *inv/inv* mice is reversed. We show that a small disturbance of molecular signaling at the node of *inv/inv* embryos is responsible for the L-R phenotype in a manner dependent on nodal flow. Moreover, analysis of the regulation of *Cerl2* expression suggests the presence of an innate mechanism for amplification and stabilization of L-R asymmetry at the node.

MATERIALS AND METHODS

Mutant mice

All mutant mice were maintained by backcrossing to the FVB/N strain. Data were obtained from mice of at least the N3 generation after confirmation that the pattern of *Nodal* expression in *inv/inv* embryos was identical to that apparent on the original FVB/N background. The number of backcrosses for each genotype studied was N5 to N11 for *Cerl2* and *inv;Cerl2* mutants, N5 to N10 for *Lefty1* and *inv;Lefty1* mutants, N4 to N9 for *Cryptic* (*Cfc1* – Mouse Genome Informatics) and *inv;Cryptic* mutants and N6 to N7 for the *inv;iv* double mutant. Staining of embryos harboring the *Lefty2-lacZ* transgene with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was performed on the N3 generation. Genotyping of mice and embryos was performed by PCR analysis of genomic DNA isolated from the tail or yolk sac.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed according to standard procedures. For two-color analysis, embryos were subjected to simultaneous hybridization with digoxigenin- and fluorescein-labeled probes. Both probes were successively detected with alkaline phosphatase-conjugated antibodies. The first color was developed with NBT/BCIP (Roche). After inactivation of alkaline phosphatase at 70°C, the second antibody was applied and the associated color was developed with INT/BCIP (Roche); this brownish red color can be eliminated by exposure to methanol. Mutant embryos were always processed with control embryos, and the color was developed with the same stop time for comparison. Photographs of the stained embryos were taken with a digital camera (Olympus D12 or Nikon DS-2Mv) attached to a dissecting microscope (Leica). For photographs of the node, the embryo was carefully oriented in 80% glycerol solution so that the crown cells on the left and right sides were positioned in the same horizontal plane.

Quantitative analysis of gene expression

Cerl2 expression detected by whole-mount in situ hybridization was subjected to quantitative analysis. Images were processed with the use of Photoshop (Adobe) software. The stained areas in the node were selected with the 'Magic Wand' tool and snapshots were taken with the use of 'Grab' on Mac OSX. The images were then converted to grayscale and inverted. Statistical information for the selected areas was displayed in 'Histogram Palette', and 'Mean' and 'Pixels' were recorded. 'Mean' represents the average intensity value. The 'Mean' of the background color in the center of the node was subtracted from that of *Cerl2* staining in each sample. The product of 'Mean' and 'Pixels' was regarded as a measure of the relative level of expression. The value of R/L (when R>L) or L/R (when L>R) for staining in each embryo was plotted on the *y*-axis of a graph, with *y*=1 corresponding to L=R.

Histograms of two-color staining for *Cerl2* and *Nodal* expression were also obtained with the use of Photoshop. In brief, the stained areas in the node were selected with the 'Rectangular Marquee' tool. Composite histograms of the RGB color channels displayed in 'Histogram Palette' were recorded with the use of 'Grab' on Mac OSX.

Whole-embryo culture

Embryos were collected at the late-bud to headfold stage, transferred to Hepes-buffered Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, and cultured until the four-somite stage in 50 ml tubes containing 25% DMEM and 75% rat serum in the presence of 40 μM SB431542 (Sigma) or vehicle alone (0.15% dimethyl sulfoxide). The tubes were rotated in a humidified incubator containing 5% CO_2 and 95% air.

RESULTS

Reversed asymmetry of *Cerl2* expression in *inv/inv* embryos

Nodal is expressed in the right LPM, rather than the left LPM, in *inv/inv* embryos (Collignon et al., 1996; Lowe et al., 1996). Given that *Nodal* expression in the LPM is induced by Nodal from the node (Brennan et al., 2002; Saijoh et al., 2003; Oki et al., 2007), we first examined the expression of *Nodal* and *Cerl2*, which encodes a Nodal inhibitor, in the node. In wild-type embryos, expression of *Nodal* and *Cerl2* becomes asymmetric from the two-somite stage, with that of *Nodal* being stronger on the left side (Fig. 1A,B; see Fig. 3A,C,E) and that of *Cerl2* stronger on the right (Fig. 1B) (Collignon et al., 1996; Lowe et al., 1996; Marques et al., 2004). In *inv/inv* embryos, however, *Nodal* expression was symmetric (22 of 25 embryos at the two- to seven-somite stages) (Fig. 1B; see Fig. 3B,D,F). Furthermore, *Cerl2* expression was found to be equal on both sides until the two-somite stage but was reversed from the three-somite stage, being stronger on the left side (Fig. 1B; see Fig. 6E). These results suggested that, in *inv/inv* embryos, active Nodal is initially distributed symmetrically in the node, and that the crown cells on the right side subsequently produce more active Nodal than those on the left as a result of the reversed pattern of *Cerl2* expression.

To test whether the reversal of *Cerl2* expression is responsible for the right-sided expression of *Nodal* in the LPM of *inv/inv* embryos, we analyzed *inv* and *Cerl2* double-mutant mice. If the balance between Nodal and Cerl2 at the node is a key determinant of the sidedness of *Nodal* expression in the LPM, the expression pattern of *Nodal* in the LPM of *inv/inv;Cerl2*$^{-/-}$ embryos would be expected to be subject to the status of *Nodal* expression in the node. During the course of this study, we noticed that the phenotype of *inv/inv* appears to depend on the mouse background. For example, many *inv/inv* embryos (5/7) on the FVB/129 hybrid background expressed *Pitx2*, a direct target gene of Nodal signaling, in both left and right LPM (Fig. 2A). We therefore performed all our analyses on animals with the FVB/N background (the original background of *inv*). As shown previously (Marques et al., 2004), *Cerl2*$^{-/-}$ embryos on the FVB/N background expressed *Nodal* in the left or both left and right LPM (Fig. 2A,B). By contrast, *Nodal* expression was completely randomized in *inv/inv;Cerl2*$^{-/-}$ embryos (Fig. 2A). Consistent with this expression pattern, *Pitx2* expression was also found to be randomized in *inv/inv;Cerl2*$^{-/-}$ embryos (Fig. 2A,C). This randomization correlates with the symmetric expression of *Nodal* in the node of *inv/inv* embryos (see Fig. 7C for model). These results suggested that the reversal of L-R asymmetry in *inv/inv* embryos is probably attributable to the combination of the reversed expression pattern of *Cerl2* and the symmetric expression of *Nodal* in the node.

Ectopic *Lefty* expression at the node of *inv/inv* embryos

Lefty1 and Lefty2 function as feedback inhibitors of Nodal (Meno et al., 1999; Meno et al., 2001) and contribute to initial L-R determination characterized by the unilateral expression of *Nodal* in the left LPM. We therefore examined the expression of both *Lefty1* and *Lefty2* around the node of *inv/inv* embryos. In wild-type embryos, *Lefty1* expression in the floorplate extended anteriorly from the region abutting the node at around the three-somite stage, whereas its expression in the node was detected only after the appearance of *Nodal* expression in the left LPM (*n*=29 embryos at the one- to four-somite stages) (Fig. 1A; Fig. 3A,C). In *inv/inv* embryos, however, ectopic expression of *Lefty1* was observed on the posterior side of the node, and it spread into the node before the

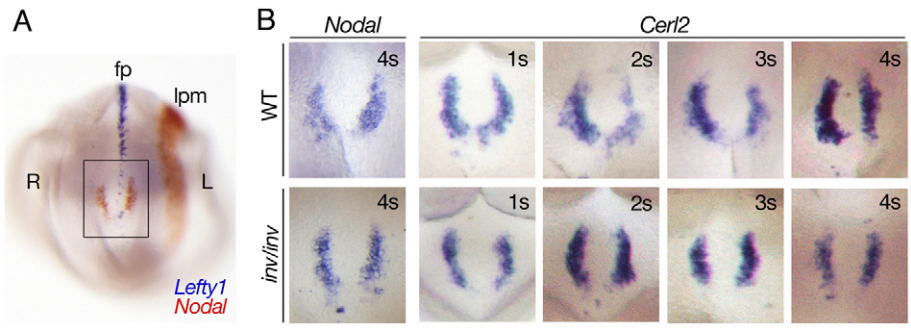

Fig. 1. Reversal of *Cerl2* expression in the node of *inv/inv* embryos. (**A**) Distal view of a wild-type embryo at the four-somite stage expressing *Nodal* (red) and *Lefty1* (blue). The anterior side is at the top. The box indicates the node region, and all images of the node in this paper are oriented in this manner. (**B**) *Nodal* and *Cerl2* expression in the node of wild-type and *inv/inv* embryos. The numbers in each panel indicate the somite stage (s). fp, floorplate; lpm, lateral plate mesoderm; WT, wild type.

onset of *Nodal* expression in the LPM (n=14 embryos at the one- to four-somite stages) (Fig. 3B,D). We also confirmed the ectopic expression of *Lefty1* in *inv/inv* embryos on the B6/129 background (data not shown). Similar results were obtained for *Lefty2*. Whereas *Lefty2* was expressed at a low level in the floorplate of wild-type embryos (n=7 at the two- to four-somite stages), its expression was markedly increased on the posterior side of the node of *inv/inv* embryos (n=10 at the one- to three-somite stages) (Fig. 3E,F). We next examined which cell types in the node region ectopically express *Lefty* genes in *inv/inv* embryos. Frontal sections perpendicular to the node region showed that *Lefty1* was expressed in cells at the midline of the dorsal layer of the node, in the crown cells and endoderm cells at the posterior of the node, and in the mesoderm and ectoderm cells adjacent to the posterior of the node (Fig. 3G,H). Expression of *Lefty2* was detected in the same cell types as *Lefty1*, although it was restricted to the posterior side of the node (Fig. 3I).

Increased Nodal signaling in *inv/inv* embryos

Lefty1/2 expression in the midline is induced by the Nodal signal (Bamford et al., 2000; Saijoh et al., 2000; Meno et al., 2001; Yamamoto et al., 2003). The ectopic expression of *Lefty* genes in *inv/inv* embryos therefore suggested that the level of the Nodal signal was increased in the corresponding regions. To confirm this suggestion, we performed two sets of experiments. First, we introduced a *Lefty2-lacZ* transgene (line E38) into *inv* mutant mice. This transgene detects the Nodal signal in the left LPM and floorplate (Saijoh et al., 1999). At the three- to four-somite stages, staining with X-gal revealed a low level of *lacZ* expression in the node of wild-type or *inv/+* embryos (n=5) (Fig. 4A; data not shown). However, in *inv/inv* embryos at the same stages, the staining was increased, especially in the posterior region of the node (n=3) (Fig. 4B), indicative of an increase in the level of the Nodal signal. Second, we examined the effect of SB431542, a specific inhibitor of Alk4 (Acvr1b – Mouse Genome Informatics) (a type I receptor for Nodal), on *Lefty1/2* expression in the node of *inv/inv* embryos in culture. The increased expression of *Lefty1/2* in *inv/inv* embryos was also apparent in whole-embryo culture (Fig. 4C). Addition of SB431542 to the culture medium at various concentrations revealed that *Lefty* expression was substantially inhibited at 10 μM (4/4 embryos) whereas it was lost (3/5 embryos) or apparent in only a few cells (2/5 embryos) at 20 μM (data not shown). At 40 μM, SB431542 completely eliminated *Lefty* expression in *inv/inv* embryos (Fig. 4D). These results thus indicated that ectopic *Lefty* expression is dependent on the Nodal signal, which may be increased in the node of *inv/inv* embryos.

Theoretically, the increase in the level of the Nodal signal in *inv/inv* embryos might be attributable to upregulation of components of the Nodal signaling pathway or to downregulation of inhibitors of Nodal signaling. We first examined whether the expression of

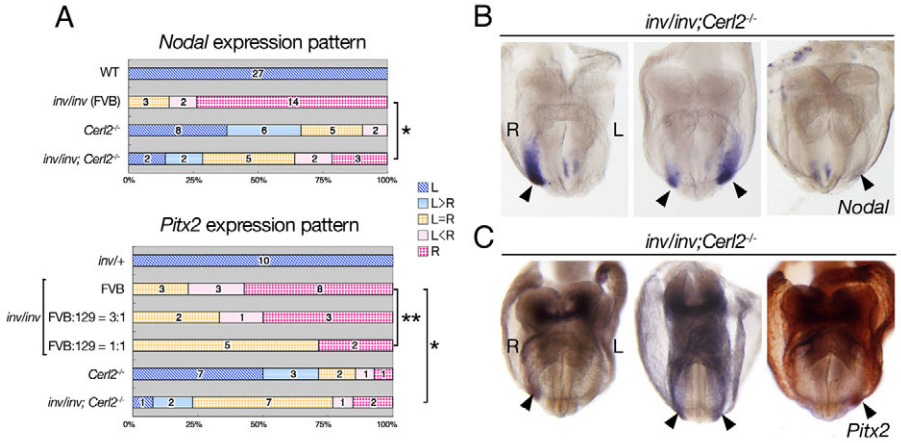

Fig. 2. Randomized L-R asymmetry in *inv/inv;Cerl2*$^{-/-}$ embryos. (**A**) Expression patterns of *Nodal* and *Pitx2* in the LPM of mutant embryos. The numbers of embryos showing each pattern are indicated within the bars. The bilateral pattern is further classified (L>R, L=R, L<R) according to the relative levels on the left and right. The expression patterns for *inv/inv* embryos were examined on the FVB/N and FVB/129 hybrid backgrounds. **P<0.05 (χ^2 test) for comparison of symmetric (L=R) or right-dominant (R and L<R) patterns between the indicated backgrounds; *P<0.01 (χ^2 test) for comparison of left-dominant (L and L>R), symmetric (L=R), or right-dominant (R and L<R) patterns between the indicated genotypes. (**B**,**C**) Anterior view of randomized expression of *Nodal* (B) and *Pitx2* (C) in *inv/inv;Cerl2*$^{-/-}$ embryos. Arrowheads indicate expression in the LPM. WT, wild type.

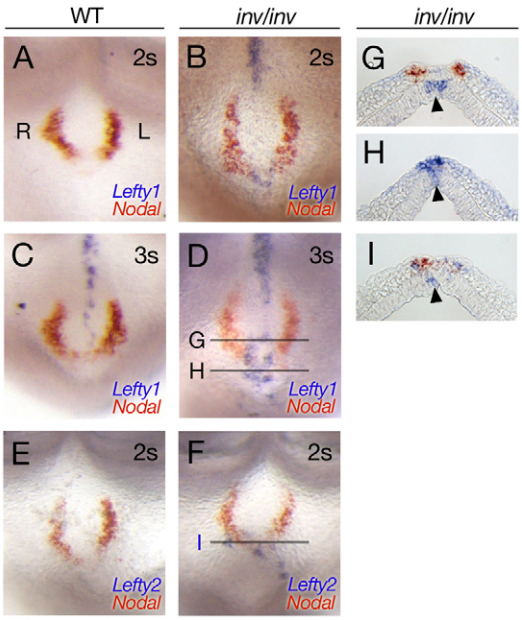

Fig. 3. Ectopic expression of *Lefty* genes in the node of *inv/inv* embryos. (**A-F**) Expression of *Lefty1* (blue) (A-D) and *Lefty2* (blue) (E,F) as well as that of *Nodal* (red) in wild-type (A,C,E) and *inv/inv* (B,D,F) embryos at the indicated somite stages (s). (**G-I**) Frozen sections at the indicated levels of the node region of the *inv/inv* embryos shown in D and F. The ventral side is at the top. The arrowheads indicate *Lefty* expression in the dorsal layer of the node (G,I) and the anterior primitive streak (H). WT, wild type.

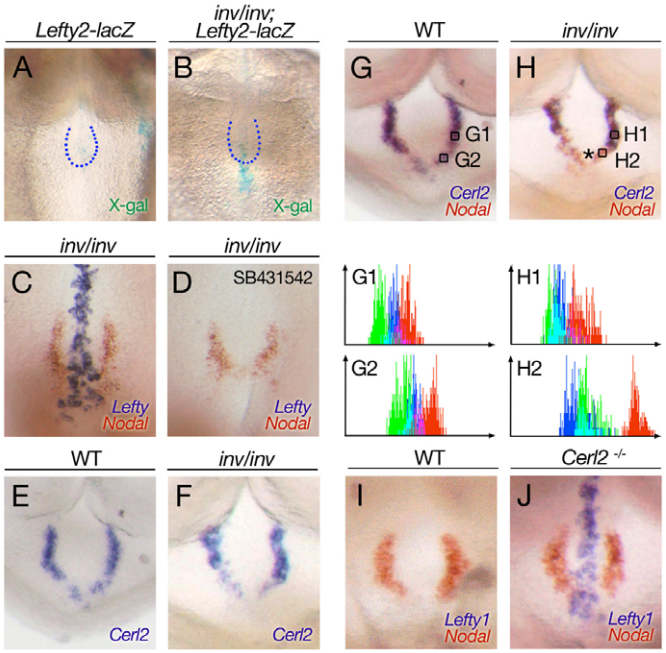

Fig. 4. Upregulation of the Nodal signal as a result of a local decrease in *Cerl2* expression in the node of *inv/inv* embryos. (**A**,**B**) X-gal staining of wild-type (A) and *inv/inv* (B) embryos harboring the *Lefty2-lacZ* transgene at the four-somite stage. Blue dots indicate the node. (**C**,**D**) Expression of *Lefty1/2* (blue) and *Nodal* (red) in *inv/inv* embryos cultured in the absence (C) or presence (D) of SB431542. (**E**,**F**) Expression of *Cerl2* in wild-type (E) and *inv/inv* (F) embryos at the late-headfold stage. (**G**,**H**) Simultaneous detection of *Nodal* (red) and *Cerl2* (blue) expression in the embryos shown in E and F, respectively. The asterisk indicates that red staining is more prominent in the posterior node region of the *inv/inv* embryo than in that of the wild-type embryo. Panels G1, G2, H1 and H2 show composite histograms of the RGB color channels for the boxed areas in G and H. Each histogram represents the number of pixels (*y*-axis) at each luminance value (*x*-axis). RGB values are shown in red, green and blue, respectively. The R (red) signal is prominent in the posterior node region of the *inv/inv* embryo (H2). (**I**,**J**) Expression of *Nodal* (red) and *Lefty1* (blue) in wild-type (I) and *Cerl2*$^{-/-}$ (J) embryos at the two-somite stage. WT, wild type.

Cryptic or *Gdf1*, both of which encode components of the Nodal signaling pathway, might be increased. Both genes were found to be normally expressed in the node of *inv/inv* embryos (data not shown). We next focused on the early expression of *Cerl2*. At around the onset of somitogenesis in wild-type embryos, *Cerl2* is expressed in a horseshoe pattern, with the expression level being lowest in the posterior domain (Fig. 1B; Fig. 4E) (Marques et al., 2004). In *inv/inv* embryos, however, *Cerl2* expression in the posterior domain was slightly weaker than that in wild-type embryos until the two-somite stage (Fig. 1B; Fig. 4F). Double staining for *Nodal* and *Cerl2* expression indicated that the expression of *Nodal* was markedly greater than that of *Cerl2* in the posterior region of the node of *inv/inv* embryos but not in that of wild-type embryos (n=15, wild type or *inv*/+; n=11, *inv/inv*) (Fig. 4G,H). Composite histogram analysis of color components also showed that the luminance value of staining for *Nodal* expression was markedly higher in the posterior domain than in the lateral domain of the node in *inv/inv* embryos (Fig. 4H1,H2), whereas such was not the case for wild-type embryos (Fig. 4G1,G2). These results suggested that the local downregulation of *Cerl2* expression might be responsible for increased Nodal activity and thereby for the ectopic expression of *Lefty* genes in *inv/inv* embryos. This notion was also supported by the pronounced expression of *Lefty1* that was observed in and on the posterior side of the node in 10 of 11 *Cerl2*$^{-/-}$ embryos compared with that in wild-type embryos (Fig. 4I,J). Together, these results suggested that a local decrease in the level of *Cerl2* expression leads to the ectopic and increased expression of *Lefty* genes in *inv/inv* embryos.

Ectopic Lefty delays *Nodal* expression in the LPM of *inv/inv* embryos

To determine whether the ectopic expression of *Lefty* genes contributes to development of the L-R phenotype of *inv/inv* mice, we analyzed *inv* and *Lefty1* double-mutant embryos. In *inv/inv* embryos, the onset of *Nodal* expression in the LPM was delayed and the sidedness of the expression was reversed compared with wild-type embryos (Fig. 5A,B,F,G). We previously showed that *Nodal* is ectopically expressed in the anterior right LPM of *Lefty1*$^{-/-}$ embryos on the B6/129 hybrid background, resulting in left isomerism of visceral organs, with only one-quarter of the *Lefty1*$^{-/-}$ embryos surviving to weaning age (Meno et al., 1998). However, we found that the phenotype of *Lefty1*$^{-/-}$ mice depends on the genetic background. On the FVB/129 hybrid background, intercrosses of *Lefty1*$^{+/-}$ mice gave rise to *Lefty1*$^{-/-}$ animals in approximately the expected Mendelian ratio at weaning (20 of 85 animals, or 23.5%). After further backcrossing to the FVB/N background, *Nodal* expression in *Lefty1*$^{-/-}$ embryos became normal (Fig. 5C,H), probably because upregulated expression of *Lefty2* in the floorplate

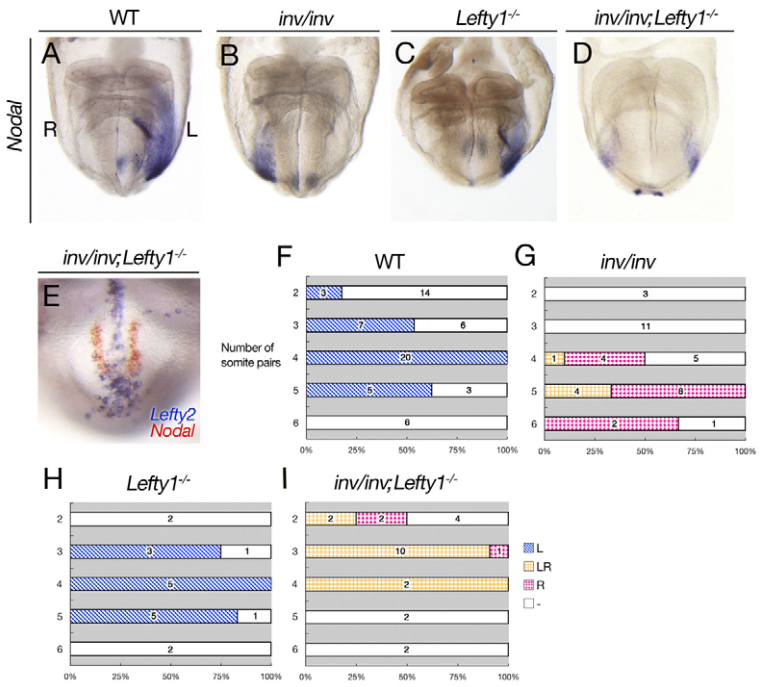

Fig. 5. Contribution of *Lefty1* to development of the L-R phenotype of *inv/inv* mice. (**A-D**) Representative patterns of *Nodal* expression in wild-type (A), *inv/inv* (B), *Lefty1*$^{-/-}$ (C) and *inv/inv;Lefty1*$^{-/-}$ (D) embryos. (**E**) Extensive expression of *Lefty2* (blue) in the node and floorplate of an *inv/inv;Lefty1*$^{-/-}$ embryo at the three-somite stage. *Nodal* expression (red) is also shown. (**F-I**) Timecourse and pattern of *Nodal* expression in wild type (F), *inv/inv* (G), *Lefty1*$^{-/-}$ (H), and *inv/inv;Lefty1*$^{-/-}$ (I) embryos. The *y*-axis indicates somite stage. White bars (–) represent embryos without *Nodal* expression in LPM, meaning that *Nodal* expression has not yet started or has already disappeared. WT, wild type.

compensated for the loss of *Lefty1* (Meno et al., 1998). Remarkably, most (14 of 17) *inv/inv;Lefty1*$^{-/-}$ embryos showed bilateral *Nodal* expression in the LPM, despite the upregulation of *Lefty2* expression (Fig. 5D,E,I). The expression on both sides was apparent in the distal portion at early somite stages and extended from the anterior to the posterior LPM, suggesting that it was bilaterally induced by Nodal from the node (Fig. 5D). Furthermore, the onset of *Nodal* expression in LPM of the double mutant was as early as that apparent in wild-type embryos (Fig. 5F,I), indicating that the delay in *Nodal* expression in *inv/inv* embryos is attributable to the ectopic expression of *Lefty1* in the node. Given that the timing of *Nodal* expression in LPM was advanced in the double mutant compared with that in *inv/inv* embryos, it is likely that *Nodal* was bilaterally expressed in the LPM as a result of the symmetric expression of *Cerl2* in the node by the two-somite stage (Fig. 1B; see Fig. 6E and Fig. 7A,B).

Asymmetry of *Cerl2* expression depends on the Nodal signal in *inv/inv* embryos

The observation that *Lefty* genes are ectopically expressed in *inv/inv* embryos prompted us to explore the possibility that *Cerl2* expression is regulated by the Nodal signal. We cultured *inv* mutant embryos in the absence or presence of SB431542 and then examined *Cerl2* expression. SB431542 had no effect on the expression of *Cerl2* in wild-type or *inv/+* embryos (Fig. 6A,C; data not shown). Unexpectedly, *Cerl2* expression was decreased and symmetric in *inv/inv* embryos (4/4) cultured without the drug (Fig. 6B). Consistent with this symmetry of *Cerl2* expression, *Pitx2* was bilaterally expressed in the left and right LPM of *inv/inv* embryos (7/8, or 88%), whereas it was normally expressed in the left LPM of wild-type (5/5) or *inv/+* (10 of 10) embryos (see Fig. S1 in the supplementary material). Despite this phenotypic change in *inv/inv* embryos, the addition of SB431542 restored *Cerl2* expression in these embryos (5/7, or 71%) to a level similar to that in wild-type or *inv/+* embryos (Fig. 6D), suggesting that *Cerl2* expression is inhibited by the Nodal signal.

To ascertain whether *Cerl2* expression is regulated by the Nodal signal in utero, we examined *Cerl2* expression in *inv/inv;Cryptic*$^{-/-}$ embryos. Cryptic functions as a cofactor in Nodal signaling, and *Cryptic*$^{-/-}$ embryos lack *Lefty* expression as a result of loss of the Nodal signal (Fig. 6F,G) (Yan et al., 1999). To assess the difference in *Cerl2* expression between the left and right sides, we performed a densitometric evaluation of photographs of embryos stained for *Cerl2* transcripts. The pattern of *Cerl2* expression in *inv/inv;Lefty1*$^{-/-}$ embryos was the same as that in *inv/inv* embryos (Fig. 6E), possibly because *Lefty2* expression was upregulated in the double mutant (Fig. 5E). *Cerl2* expression in *Cryptic*$^{-/-}$ embryos (8/8) was found to be normal (Fig. 6H). Importantly, *Cerl2* expression in *inv/inv;Cryptic*$^{-/-}$ embryos became almost symmetric (Fig. 6E,I), a pattern different from that apparent in wild-type or *inv/inv* embryos (see Fig. 7D for model), indicating that *Cerl2* expression is regulated by the Nodal signal. These results thus suggested that the Nodal signal is probably inhibited in the crown cells on the left side in *inv/inv* embryos, thus resulting in the reversed asymmetry of *Cerl2* expression.

Finally, we addressed whether leftward nodal flow contributes to the L-R reversal phenotype of *inv/inv* embryos by generating the *inv/inv;iv/iv* double mutant. The cilia on the ventral side of the node are immotile in *iv/iv* embryos, resulting in the inability of these embryos to generate nodal flow (Okada et al., 1999). The expression of *Lefty* genes was increased in *inv/inv;iv/iv* embryos (Fig. 6K), compared with that in *inv/+;iv/iv* embryos (Fig. 6J), as it was in *inv/inv* embryos. Notably, *Pitx2* expression was randomized in *inv/inv;iv/iv* embryos (left-sided, right-sided, or bilateral in 2/12, 3/12 and 7/12 embryos, respectively) (Fig. 6L) as well as in *iv/iv* embryos (2/12, 4/12 and 6/12, respectively; data not shown) (Yoshioka et al., 1998), indicating that the *inv* phenotype is dependent on nodal flow.

On the basis of our results, we propose a mechanism for the generation of asymmetry in the node (Fig. 7A). In this model, nodal flow triggers the initial asymmetric expression of *Nodal* and *Cerl2* in the crown cells of the node of wild-type embryos in a manner

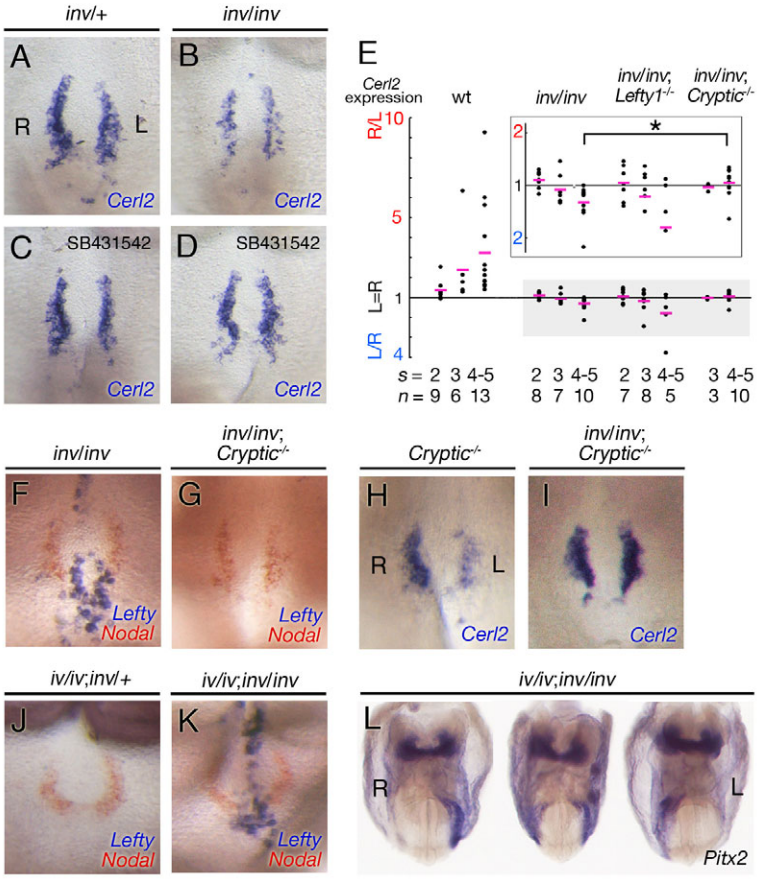

Fig. 6. Reversal of the expression of *Cerl2* in *inv/inv* embryos depends on the Nodal signal in the node. (**A-D**) Expression of *Cerl2* in *inv/+* (A,C) and *inv/inv* (B,D) embryos cultured in the absence (A,B) or presence (C,D) of SB431542. (**E**) Difference in the level of *Cerl2* expression between the left and right sides of embryos of the indicated genotypes. The R/L or L/R ratio was determined for the indicated numbers (*n*) of embryos of each genotype at the indicated somite stages (*s*). The mean value of each distribution is indicated by the magenta bar. The shaded region is shown in the inset with an enlarged scale of the *y*-axis. **P*<0.05 (Student's *t* test). (**F**,**G**) Expression of *Lefty1/2* (blue) and *Nodal* (red) in *inv/inv* (F) and *inv/inv;Cryptic*$^{-/-}$ (G) embryos at the three-somite stage. (**H**,**I**) Expression of *Cerl2* in *Cryptic*$^{-/-}$ (H) and *inv/inv;Cryptic*$^{-/-}$ (I) embryos at the four-somite stage. (**J**,**K**) Expression of *Lefty1/2* (blue) and *Nodal* (red) in *iv/iv;inv/+* (J) and *iv/iv;inv/inv* (K) embryos at the two-somite stage. (**L**) Representative patterns of *Pitx2* expression in *iv/iv;inv/inv* embryos. wt, wild type.

dependent on the Inv protein. Nodal in the node not only induces expression of its own gene (Norris et al., 2002) but also downregulates that of the gene for its inhibitor, Cerl2. Such a relation would result in amplification of an initial small difference and stabilize the asymmetric expression of *Nodal* and *Cerl2* in the node, ensuring *Nodal* expression in the left LPM (Nakamura et al., 2006; Oki et al., 2007). In *inv/inv* embryos, the symmetric expression of *Nodal* and a local downregulation of *Cerl2* expression allow the Nodal signal to induce *Lefty1* expression in the node. The asymmetry of *Cerl2* expression is then reversed, because the Nodal signal is probably inhibited on the left side in a manner dependent on nodal flow. Ectopic Lefty in the ventral layer of the node is a candidate mediator of the asymmetric inhibition of Nodal signaling, based on the assumption that it is transported by leftward nodal flow and is able to prevent Nodal from inhibiting *Cerl2* expression of the left side of the crown cells (Fig. 7B). More active Nodal is therefore produced on the right side, which is sensed by the right LPM and results in its expression of *Nodal* before that in the left LPM.

DISCUSSION

Establishment of the L-R axis is mediated by sequential events that amplify a small difference into a robust one. The initial L-R cue generated by nodal flow results in asymmetric gene expression around the node and subsequent transfer of information to the left LPM. Nodal produced around the node is required for the induction of *Nodal* expression in the left LPM (Brennan et al., 2002; Saijoh et al., 2003). In addition, it has been suggested that Nodal diffuses from the node to the LPM and directly induces its own expression there (Oki et al., 2007). We recently revealed that Nodal-Lefty signaling contributes to the L-R decision on the side of the LPM that expresses *Nodal* (Nakamura et al., 2006). Such a system, which we designated 'SELI' (self-enhancement and lateral inhibition), explains how a slight difference is amplified into the robust and unilateral expression of *Nodal* in the LPM. In the present study, through an analysis of *inv* mutant mice, we have shown that the mechanism responsible for generation of the L-R difference in the amount of active Nodal around the node is key to an understanding of the initial events in L-R axis formation.

The amount and activity of Nodal produced by the crown cells of the node are thought to be regulated by several mechanisms. First, Nodal requires GDF1 as a binding partner to exert its full activity (Tanaka et al., 2007). *Gdf1* is expressed in the crown cells of the node and the LPM, and *Gdf1* mutant mice lack *Nodal* expression in the left LPM (Rankin et al., 2000). Second, Cerl2 produced by the crown cells of the node inhibits Nodal, probably by binding to it (Marques et al., 2004). Finally, the expression of *Nodal* and *Cerl2* is differentially regulated on the left and right sides, with *Nodal* expression being stronger on the left side and *Cerl2* expression stronger on the right (Collignon et al., 1996; Lowe et al., 1996; Pearce et al., 1999; Marques et al., 2004). Although a method to detect the activity of Nodal secreted from the node remains to be developed, such a pattern of regulation probably generates more active Nodal on the left side than on the right. This notion is supported by the observation that *Lefty1* expression, which is induced by Nodal (Yan et al., 1999; Yamamoto et al., 2003), is left-sided in the dorsal layer of the node of wild-type embryos, whereas it is bilaterally upregulated in the node of *Cerl2*$^{-/-}$ embryos.

The inhibition of Nodal by Cerl2 in the node region appears to be fundamental to L-R determination. About half of *Cerl2*$^{-/-}$ embryos express *Nodal* and *Pitx2* bilaterally in the LPM, suggesting that

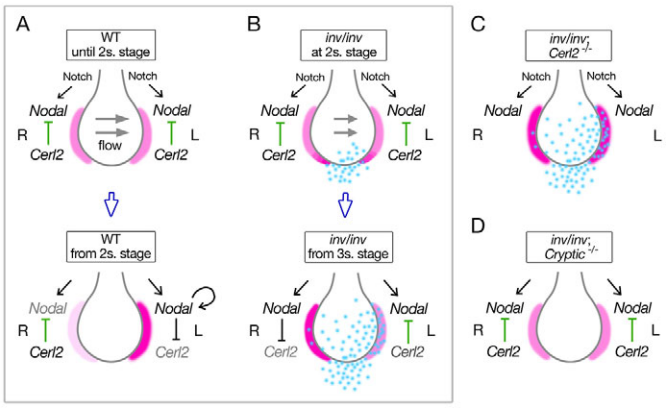

Fig. 7. Model for generation of molecular asymmetry in the node. Models are shown for (**A**) wild-type, (**B**) *inv/inv*, (**C**) *inv/inv;Cerl2*$^{-/-}$ and (**D**) *inv/inv;Cryptic*$^{-/-}$ embryos. The node region is viewed from the ventral side. The putative amount of active Nodal in the region of the crown cells of the node, as determined by the balance between *Nodal* and *Cerl2* expression, is represented by the intensity of the purple color. Diffusion of Nodal and Lefty within the embryos is not depicted for simplicity. Gray lettering for *Nodal* or *Cerl2* indicates weakened expression. Blue dots represent a putative Lefty gradient on the surface of the node of *inv/inv* or *inv/inv;Cerl2*$^{-/-}$ embryos. Black and green lines indicate genetic relationship and protein action, respectively. WT, wild type.

increased Nodal activity on the right side of the node induces *Nodal* in the right LPM (Marques et al., 2004). However, the remaining half of *Cerl2*$^{-/-}$ embryos normally express *Nodal* and *Pitx2* in the left LPM. Given that the asymmetry of *Nodal* expression in the node is maintained in *Cerl2*$^{-/-}$ embryos (Marques et al., 2004), the subtle difference in the amount of Nodal is likely to be amplified by the SELI system to result in the expression of *Nodal* in the left LPM (Nakamura et al., 2006). The asymmetry of *Cerl2* expression might be sufficient to determine the sidedness of *Nodal* expression in the LPM, even if *Nodal* is symmetrically expressed in the node. In *inv/inv* and *inv/inv;Lefty1*$^{-/-}$ embryos, which express *Nodal* symmetrically in the node, *Nodal* expression in the LPM corresponded to the asymmetry of *Cerl2* expression immediately before the onset of *Nodal* expression in the LPM. Furthermore, the absence of *Cerl2* in *inv/inv* embryos gave rise to the randomized expression of *Nodal* and *Pitx2*, probably reflecting the symmetric expression of *Nodal* in the node. Mutant mice with a modified cis-element of *Nodal* as well as transgenic mice that express *Nodal* symmetrically in the node were found to manifest normal *Nodal* expression in the left LPM (Brennan et al., 2002; Saijoh et al., 2003). Although *Cerl2* expression was not examined in these mice, their phenotype might be explained if the asymmetry of *Cerl2* expression was normal.

Then, how does the balance between *Nodal* and *Cerl2* expression in the node determine the side of the LPM that expresses *Nodal*? We have previously shown that Myc epitope-tagged Nodal is detected in the area immediately external to the apical and basolateral membranes of crown cells of the node expressing this protein, suggesting that Nodal is secreted from both membrane regions (Oki et al., 2007). We also showed that Nodal secreted from the basolateral membrane of the crown cells may diffuse along the internal route to the LPM. The LPM on the same side as the crown cells that secrete the larger amount of active Nodal may therefore first experience a level of the Nodal signal sufficient to induce *Nodal* expression. Once *Nodal* is expressed in the LPM, the induction of *Lefty* gene expression results in inhibition of *Nodal* expression on the contralateral side by the SELI system (Nakamura et al., 2006). Visualization of the diffusing Nodal and Lefty proteins in the embryo will be required for a full understanding of these processes, but as yet we have not been able to achieve such visualization.

If the generation of a subtle but consistent asymmetry in Nodal activity around the node is key to L-R patterning, then how are the expression patterns of *Nodal* and *Cerl2* established? An enhancer located in the upstream region of *Nodal* drives bilateral expression in the node, which is activated by Notch and its ligand delta-like 1 (Dll1) (Krebs et al., 2003; Raya et al., 2003). It has been suggested that the left-biased expression of *Nodal* in the node is generated by a positive feedback loop mediated by Nodal and the intronic enhancer ASE (Brennan et al., 2002; Norris et al., 2002). This suggestion seems reasonable, because if there is less Cerl2 on the left side of the node then the expression of *Nodal* on the left is probably augmented by the Nodal positive loop. We have also now revealed that *Cerl2* expression in the node of *inv/inv* embryos is suppressed by the Nodal signal. First, *Cerl2* expression in *inv/inv* embryos was increased by exposure to SB431542, a specific inhibitor of Alk4. Second, the asymmetry of *Cerl2* expression was lost in *inv/inv;Cryptic*$^{-/-}$ embryos. Given that *Cerl2* expression in *inv/inv* embryos is stronger on the left side, the Nodal signal would be expected to be lower in the crown cells on the left side in *inv/inv* embryos. The inhibition of *Cerl2* expression by the Nodal signal would be expected to be a reliable mechanism for generating asymmetry in the net amount of active Nodal in wild-type embryos. After the slight asymmetry in the expression of *Nodal* and *Cerl2* is established in the node, the difference is further amplified because the greater the extent of Nodal signaling on the left side, the more the expression of *Cerl2* is suppressed. This pattern of regulation probably stabilizes the L-R difference in the node sufficiently to allow induction of *Nodal* expression in the left LPM by the SELI system.

With regard to *Cerl2*, cis-regulatory elements for expression of the gene in the node have not been identified to date. In the present study, we found that the loss of Inv resulted in downregulation of *Cerl2* expression on the posterior side of the node, symmetry of *Cerl2* expression at the two-somite stage, and reversal of the normal asymmetry of *Cerl2* expression at later stages. The local diminution of *Cerl2* expression at the posterior side of the node suggests that the Inv protein may contribute to a signaling pathway responsible for the upregulation of *Cerl2* expression. Here, we propose that *Cerl2* expression in the node of wild-type embryos is regulated by an Inv-dependent mechanism and the Nodal signal. The crown cells of the node on the left side may sense nodal flow by an unknown mechanism dependent on the Inv protein and downregulate *Cerl2* expression in response. The Nodal signal on the left side further inhibits *Cerl2* expression in a manner that does not require Inv. In *inv/inv* embryos, the Inv-dependent pathway that generates asymmetric *Cerl2* expression is absent, but the inhibitory effect of the Nodal signal on *Cerl2* expression is still operative. The Nodal signal is probably lower on the left side in *inv/inv* embryos, which eventually results in reversal of the asymmetric expression of *Cerl2*. This model explains why the regulation of *Cerl*2 expression by the Nodal signal appeared only in *inv/inv* embryos, as revealed by inhibition of the Nodal signal either by the addition of SB431542 or by the loss of Cryptic. Disturbance of the Nodal signal alone may not affect *Cerl2* expression, because the Inv-dependent pathway is sufficient to establish the asymmetry of *Cerl2* expression.

Our results indicate that the L-R reversal phenotype of *inv/inv* embryos is attributable to complex events that include a delay in *Nodal* expression in the LPM and the reversal of asymmetric *Cerl2*

expression. Ectopically expressed *Lefty1* in the node was shown to delay the onset of *Nodal* expression in the LPM, probably by diffusing bilaterally and inhibiting the Nodal signal in the LPM. The delay in *Nodal* expression in the LPM seems to be a prerequisite for the L-R reversal phenotype of *inv/inv*, given that *Nodal* was expressed bilaterally in the LPM of *inv/inv;Lefty1*$^{-/-}$ embryos, in which the timing of *Nodal* expression was normal. Ectopic expression of *Lefty* genes was detected in the crown cells on the posterior side of the node in addition to the floorplate and the dorsal layer of the node. Given that the crown cells face the yolk sac cavity, apically secreted Lefty may spread and be conveyed toward the left side by nodal flow, resulting in inhibition of the Nodal signal in the crown cells on the left side. This scenario would account for the timecourse of *Cerl2* expression in *inv/inv* embryos. The prevention of asymmetric regulation of *Cerl2* expression by the loss of Inv is followed by the inhibition of Nodal by Lefty on the left side, eventually resulting in the reversal of asymmetric *Cerl2* expression. Determination of whether *Lefty* genes are actually required for reversal of the asymmetry of *Cerl2* expression in *inv/inv* embryos will require the generation and characterization of *inv* mutants lacking both *Lefty1* and *Lefty2* expression (such as *inv/inv;Lefty1*$^{-/-}$*;Lefty2*$^{\Delta ASE/\Delta ASE}$ embryos).

In conclusion, we have addressed the longstanding mystery of the *inv* phenotype. Our results suggest that the L-R reversal in *inv/inv* embryos results from an aberration of the mechanism responsible for generating molecular asymmetry in the node: *Nodal* expression thus becomes symmetric, and the asymmetry of *Cerl2* expression is reversed at later stages, resulting in the production of more active Nodal on the right side than on the left. Furthermore, ectopic Lefty, produced as a result of a local downregulation of *Cerl2* expression, suppresses *Nodal* expression in the LPM. The LPM on the right may therefore receive the Nodal signal at a level sufficient to override suppression by Lefty earlier than that on the left, resulting in *Nodal* expression in the right LPM.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/23/3917/DC1

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