Supplementary Materials for

Role of Ca$^{2+}$ transients at the node of the mouse embryo in breaking of left-right symmetry

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The PDF file includes:

Figs. S1 to S10

Other Supplementary Material for this manuscript includes the following:

(available at advances.sciencemag.org/cgi/content/full/6/30/eaba1195/DC1)

Movies S1 to S20
Figure S1. Transgene does not affect organ laterality, ciliary density or length. 

(A, F) Pups obtained from mating between wildtype and transgene-positive parents. Arrowheads show the position of the stomach (milk spot). ID numbers used for genotyping in (B, G) are indicated. Pups indicated with red and white ID numbers are positive and negative for the transgene, respectively. Note that all pups have the stomach on the left side regardless of their genotype. Scale bar: 1 cm. (B, G) PCR genotyping with GCaMP6 primers. (C, H) Orientation of the heart apex and the pattern of lung lobation are shown for representative pups. Numbers of the pups examined and the proportion of pups showing normal laterality are indicated for each group. Note that all pups show the normal laterality. Sale bar: 3 mm. (D, I) Immunofluorescence image of the node from transgenic (Tg positive) and nontransgenic (Tg negative) embryos. Anti-acetylated (Ac-) tubulin and anti-GFP antibodies were used. The length and density of cilia were indistinguishable between Tg positive and negative embryos. Scale bar: 20 µm. (E, J) Comparison of the length of node cilia from transgenic and nontransgenic embryos. No significant difference was observed between two groups (Mann-Whitney U test.). n indicate the numbers of cilia examined. Photo by Katsutoshi Mizuno, first author.
Figure S2. Properties of ciliary Ca\textsuperscript{2+} transients.
(A), Percentages of cilia with Ca\textsuperscript{2+} transients on the left (L) or right (R) side of the node of control (wild-type plus heterozygous), Pkd2\textsuperscript{−/-}, and iv/iv mouse embryos at the late-
headfold to three-somite stage. The \( n \) values indicate the numbers of embryos analyzed.

**\( p < 0.01 \); n.s., not significant (Student’s paired \( t \) test). (B), Total number of ciliary \( \text{Ca}^{2+} \) transients on the left or right side of the node in control, \( Pkd2^{-/-} \), and \( iv/iv \) mouse embryos. **\( p < 0.01 \), n.s., not significant (Wilcoxon signed-rank test). (C), Peak intensity of ciliary \( \text{Ca}^{2+} \) transients in control embryos. n.s., not significant (Mann-Whitney U test). (D), Duration of ciliary \( \text{Ca}^{2+} \) transients on the left and right sides of the node in control embryos. n.s., not significant (Mann-Whitney U test). (E), Relation between the duration and frequency of ciliary \( \text{Ca}^{2+} \) transients in control embryos. Each dot indicates the values for one cilium. (F), Circular plot of percentages of cells with ciliary \( \text{Ca}^{2+} \) transients in each region of the node for embryos of the indicated genotypes. The \( n \) values indicate the numbers of embryos analyzed.
Figure S3. Properties of cytoplasmic Ca$^{2+}$ transients. (A), Percentages of crown cells with cytoplasmic Ca$^{2+}$ transients on the left or right side of the node of control (wild-type plus heterozygous), *Pkd2*<sup>−/−</sup>, iv/iv, and *Kif3a*<sup>−/−</sup> embryos at the late-headfold to three-
somite stage. The \( n \) values indicate the numbers of embryos analyzed. \( *p < 0.05, \text{n.s.} \) (Student’s paired \( t \) test). (B), Total number of cytoplasmic \( \text{Ca}^{2+} \) transients on the left or right side of the node of control, \( Pkd2^{-/-}, iv/iv \), and \( Kif3a^{-/-} \) embryos. \( *p < 0.05, \text{n.s., not significant} \) (Wilcoxon signed-rank test). (C), Peak intensity of cytoplasmic \( \text{Ca}^{2+} \) transients in control embryos. \( \text{n.s.}, \text{not significant} \) (Mann-Whitney U test). (D), Duration of cytoplasmic \( \text{Ca}^{2+} \) transients on the left and right sides of the node in control embryos. \( **p < 0.01 \) (Mann-Whitney U test). (E), Relation between the duration and frequency of cytoplasmic \( \text{Ca}^{2+} \) transients in control embryos. Each dot indicates the values for one cell. (F), Percentages of cells with cytoplasmic \( \text{Ca}^{2+} \) transients in each region of the node for embryos of the indicated genotypes. The \( n \) values indicate the numbers of embryos analyzed.
Figure S4. Relation between ciliary and cytoplasmic Ca$^{2+}$ transients.

(A), A cell showing highly synchronized cytoplasmic and ciliary Ca$^{2+}$ transients at the node of an embryo expressing ciliary 5HT$_6$-GCaMP6 (green) and cytoplasmic RGECO1 (red). The white box contains the cell analyzed in (B) through (D). Scale bar, 25 µm. (B), Time-averaged fluorescence image of the cell analyzed in (C) and (D). The cell body and the cilium are outlined by the white and blue dotted lines, respectively. Scale bar, 5 µm. (C), Images of cytoplasmic and ciliary Ca$^{2+}$ transients. The time interval between consecutive frames is 0.25 s. Color scales indicate the $F/F_0$ value. Scale bars, 5 µm. (D), Time course of $F/F_0$ for ciliary GCaMP6 and cytoplasmic RGECO1. (E), A cell showing Ca$^{2+}$ transients in the cilium but not in the cell body at the node. The white box indicates the cell analyzed in (F) through (H). Scale bar, 25 µm. (F), Time-averaged image of the cell analyzed in (G) and (H). Scale, 5 µm. (G), Images of a ciliary (GCaMP6) Ca$^{2+}$ transient occurring in the absence of a cytoplasmic (RGECO1) Ca$^{2+}$ transient. The
interval between consecutive frames is 0.25 s. Color scales indicate the $F/F_0$ value. (H), Time course of $F/F_0$ for ciliary GCaMP6 and cytoplasmic RGECO1.
**Figure S5. Properties of apically restricted and nonrestricted cytoplasmic Ca\(^{2+}\) transients, and ER in crown cells.** (A), Mean spike frequency of apically restricted and nonrestricted cytoplasmic Ca\(^{2+}\) transients on the left and right sides of the node for embryos of the indicated genotypes. The \(n\) values indicate the numbers of embryos analyzed. \(*p < 0.05\), \(* * *p < 0.001\), n.s., not significant (Mann-Whitney U test). (B), An image of the node stained with ER-tracker. (C), Magnified image of cells in the boxed region in (B). Apical region of cells showed strong signals. See Video S11. (D), Immunofluorescence staining of a mouse embryo at the one-somite stage for the ER protein PDI and acetylated-tubulin. Top: an entire image of node regions. Bottom: boxed areas on top were further observed with Airyscan (Zeiss). Arrowheads indicate the base of cilia. PDI protein is abundant at the apical region of crown cells. (E)–(J), FIB-SEM images of the node of a mouse embryo. CA, cavity of the node. The boxed region in (E) is enlarged in (F). White lines in (F) indicate the planes in (G–J). Opened arrowheads indicate ER structures. A black arrowhead indicates a cilium. *, ** indicate the corresponding direction in (F). See Video S12. (K)-(P) FIB-SEM images of the node from another mouse embryo are shown similarly.
Figure S6. Effects of various inhibitors on cytoplasmic Ca$^{2+}$ transients in crown cells at the node of mouse embryos. (A), Percentages of cells showing cytoplasmic Ca$^{2+}$ transients in embryos treated with the indicated reagent. The $n$ values indicate the numbers of embryos analyzed. (B), Total spike number for cytoplasmic Ca$^{2+}$ transients in embryos shown in (A). (C), Mean frequency of cytoplasmic Ca$^{2+}$ transients in embryos treated with 100 µM nifedipine. (D), Circular plot of the mean spike frequency for cytoplasmic Ca$^{2+}$ transients in embryos shown in (C). (E), Circular plots of the percentage of cells with cytoplasmic Ca$^{2+}$ transients in embryos shown in (A). (F), PIV analysis of nodal flow in embryos treated with the indicated reagent for 1 h. Small arrows indicate the direction and the velocity of the flow. The relative color scale indicates the magnitude of flow velocity. The leftward flow is largely maintained in these embryos. (G), Mouse embryos with Nodal-LacZ BAC transgene were cultured from the early headfold stage to 4~7 somite stage with the indicated reagent. Left-sided Nodal expression in the LPM was lost by GdCl$_3$ and U-73122. (H), Percentages of embryos showing Nodal-LacZ expression in the left LPM. The numbers of embryos examined are indicated. *$p < 0.05$, **$p < 0.01$, and n.s., not significant in (A, C, B, H).
Figure S7. L-R asymmetric Nodal activity at the node is maintained in Cacna1c mutant embryos. The expression pattern of the ANE-LacZ transgene in heterozygous or homozygous Cacna1c mutant embryos harboring a mutant allele lacking either exon 2 (A) or exons 14 and 15 (B) was revealed by staining with the LacZ substrate X-gal. L-
R asymmetric Nodal activity at the node is maintained in the homozygous mutant embryos. The numbers of embryos examined are indicated.
Figure S8. Effects of thapsigargin on cytoplasmic and ciliary Ca\(^{2+}\) transients.

(A), Percentage (mean ± s.e.m. values) of crown cells on the left or right side of the node showing cytoplasmic Ca\(^{2+}\) spikes (RGECO1 fluorescence) in embryos at the late-headfold to three-somite stage exposed to DMSO vehicle or 1 µM thapsigargin (Thaps). *p < 0.05, n.s., not significant (Student’s paired t test). The n values indicate the numbers of embryos analyzed. (B), Percentage (mean ± s.e.m. values) of immotile cilia showing Ca\(^{2+}\) transients in embryos treated with DMSO or 1 µM thapsigargin. *p < 0.05, Student’s paired t test. The n values indicate the numbers of embryos analyzed. (C), Duration of ciliary Ca\(^{2+}\) transients in embryos treated with DMSO or 1 µM thapsigargin. The n values
indicate the numbers of embryos analyzed. *p < 0.05, (Mann-Whitney U test). (D), (E), Relation between the duration and mean frequency of ciliary Ca^{2+} transients in embryos treated with DMSO (D) or 1 µM thapsigargin (E).
Figure S9. Embryos cultured in DF10 medium fail to develop proper L-R patterning

(A, B), Mouse embryos between the late-headfold and three-somite stage were cultured in DF10 medium for >1 h, and they were examined for percentages of crown cells with ciliary Ca\(^{2+}\) transients (A) and total number of ciliary Ca\(^{2+}\) transients (B) on the left or right side of the node, respectively. The \(n\) values indicate the numbers of embryos analyzed. n.s., not significant. (C), Embryos at the early headfold stage with (LacZ\(^{+}\)) or without (LacZ\(^{-}\)) Nodal-LacZ BAC transgene were cultured in 75% rat serum/DMEM for ~16 h. These embryos developed to the 6~7 somite stage after the culture. L-R patterning was examined by X-Gal staining. Three transgenic embryos all maintained Nodal expression on the left LPM. (D), Magnified views of the transgenic embryo shown with
the asterisk in (C). (E) Embryos at the early headfold stage with or without *Nodal*-LacZ BAC transgene were cultured in DF10 medium for ~16 h. Embryos from two different experiments are shown. (F), Magnified images of the four embryos shown with the asterisk in (E). These embryos remained at the early headfold stage (the leftmost column) or only developed to the 1–2-somite stage. Asymmetric *Nodal* expression in LPM was lost in all embryos.
Figure S10. Expression of Cerl2 and Nodal in parvalbumin transgenic embryos. (A) in situ hybridization analysis for Cerl2 mRNA in control (Pvalb negative) and embryos expressing parvalbumin in crown cell cilia (Pvalb positive) at three different stages, from the four-somite (4-so) stage to the six-somite (6-so) stage. Note that establishing L-R asymmetry of Cerl2 mRNA is delayed by ciliary parvalbumin. See the text for detail. Scale bar, 100 µm. (B), Whole-mount in situ hybridization analysis of Nodal expression
in control embryos and in embryos expressing ciliary parvalbumin. Arrowheads indicate left-sided *Nodal* expression in the LPM. (C), Percentages of embryos with or without *Nodal* expression in the LPM. (D), Immunohistofluorescence staining of parvalbumin transgenic (Pvalb positive) embryos at the 4-somite stage (4-so.) to 6-somite (6-so.) for acetylated (Ac)–tubulin and parvalbumin. Scale bar, 20 µm.
Legends to supplementary videos

Video. S1. The node of control embryo with ciliary GCaMP6 and mCherry expression.
The node of a *Pkd2*+/- mouse embryo expressing GCaMP6 (green fluorescence, middle) and mCherry (red fluorescence, left) in immotile cilia of crown cells. This embryo is also shown in Figure 1b. GCaMP6 fluorescence images were also processed with the “royal” lookup table (LUT) of ImageJ (right). Images were captured at 4 frames per second (fps) and are played at 30 fps. Time stamps represent minutes:seconds. Scale bar, 20 µm. A, anterior; P, posterior; L, left; R, right.

Video. S2. The node of *Pkd2*−/− embryo with ciliary GCaMP6 and mCherry expression.
The node of a *Pkd2*−/− mouse embryo expressing GCaMP6 (green fluorescence, middle) and mCherry (red fluorescence, left) in immotile cilia of crown cells. GCaMP6 fluorescence images were also processed with the “royal” lookup table (LUT) of ImageJ (right). Images were captured at 4 fps and are played at 30 fps. Time stamps represent minutes:seconds. Scale bar, 20 µm.

Video. S3. The node of iv/iv embryo with ciliary GCaMP6 and mCherry expression.
The node of an iv/iv mouse embryo expressing GCaMP6 (green fluorescence, middle) and mCherry (red fluorescence, left) in immotile cilia of crown cells. GCaMP6 fluorescence images were also processed with the “royal” lookup table (LUT) of ImageJ (right). Images were captured at 4 fps and are played at 30 fps. Time stamps represent minutes:seconds. Scale bar, 20 µm.

Video. S4. The node of control embryo with ciliary GCaMP6 and cytoplasmic RGECO1 expression.
The node of a iv/+ embryo expressing ciliary GCaMP6 (green) and cytoplasmic RGECO1 (red). This embryo is also shown in Figure 2(B). Images were captured at 4 fps and are played at 30 fps. Time stamps represent minutes:seconds. Scale bar, 25 µm. Bottom embryo is also shown in Supplementary Figure 4a.
Video. S5. The node of control embryo showing ciliary Ca\(^{2+}\) transient without cell body Ca\(^{2+}\) increase.

The node of a wild-type embryo expressing ciliary GCaMP6 (green) and cytoplasmic RGECO1 (red). Ciliary Ca\(^{2+}\) transients without cell body Ca\(^{2+}\) increase are observed. This embryo is also shown in Supplementary Figure 3e. The images were captured at 4 fps and are played at 30 fps. Time stamps represent minutes:seconds. Scale bar, 25 µm.

Video. S6. The node of Pkd2\(^{-/-}\), iv/iv, and Kif3a\(^{-/-}\) embryos expressing ciliary GCaMP6 and cytoplasmic RGECO1.

The node of Pkd2\(^{-/-}\), iv/iv, and Kif3a\(^{-/-}\) embryos expressing ciliary-targeted GCaMP6 (green) and cytoplasmic RGECO1 (red). The images were captured at 4 fps and are played with 30 fps. Time stamps represent minutes:seconds. Scale bar, 25 µm.

Video. S7. The cell with Ca\(^{2+}\) transients in apical region of the cell from control embryo.

Cytoplasmic Ca\(^{2+}\) transient at the apical side of a crown cell in a wild-type embryo expressing ciliary GCaMP6 (green) and cytoplasmic RGECO1 (red). The left movie shows the entire node, with the boxed region being enlarged on the right and showing the Ca\(^{2+}\) transient (RGECO1, magenta) at the apical side of the cell. The cell body is outlined with a white line. This cell is also shown in Figure 3a. The images were captured at 4 fps and are played at 4 fps. Time stamps represent seconds:milliseconds. Scale bars, 10 µm (left) and 5 µm (right).

Video. S8. The cell with Ca\(^{2+}\) transients not restricted to the apical region of the cell from control embryo.

Cytoplasmic Ca\(^{2+}\) transient not restricted to the apical side of a crown cell in a iv/+ embryo expressing ciliary GCaMP6 (green) and cytoplasmic RGECO1 (red). The left movie shows the entire node, with the boxed region being enlarged on the right and showing the Ca\(^{2+}\) transient (RGECO1, magenta) throughout the cytoplasm. The cell body is outlined with a white line. This cell is also shown in Figure 3b. The images were captured at 4 fps and are played at 4 fps. Time stamps represent seconds:milliseconds. Scale bars, 20 µm (left) and 5 µm (right).
Video. S9. The cell with Ca\textsuperscript{2+} transients in apical region of the cell from Kif3a\textsuperscript{-/-} embryo.

Cytoplasmic Ca\textsuperscript{2+} transient at the apical side of a crown cell in a Kif3a\textsuperscript{-/-} embryo expressing cilium-targeted GCaMP6 (green) and cytoplasmic RGECO1 (red). Note that GCaMP6 labels not cilia but the apical region of the node cells. The left movie shows the entire node, with the boxed region being enlarged on the right and showing the Ca\textsuperscript{2+} transient (RGECO1, magenta) at the apical side of the cell. The cell body is outlined with a white line. This cell is also shown in Figure 3c. The images were captured at 4 fps and are played at 4 fps. Time stamps represent seconds:milliseconds. Scale bars, 20 µm (left) and 5 µm (right).

Video. S10. The cell with Ca\textsuperscript{2+} transients not restricted to the apical region of the cell from control embryo.

Cytoplasmic Ca\textsuperscript{2+} transient not restricted to the apical side of a crown cell in a Kif3a\textsuperscript{-/-} embryo expressing cilium-targeted GCaMP6 (green) and cytoplasmic RGECO1 (red). Note that GCaMP6 labels not cilia but the apical region of the node cells. The left movie shows the entire node, with the boxed region being enlarged on the right and showing the Ca\textsuperscript{2+} transient (RGECO1, magenta) throughout the cytoplasm. The cell body is outlined with a white dotted line. This cell is also shown in Figure 3d. The images were captured at 4 fps and are played at 4 fps. Time stamps represent seconds:milliseconds. Scale bars, 20 µm (left) and 5 µm (right).

Video. S11. ER-Tracker Red stained embryo.

A wild-type embryo stained with ER-Tracker Red. Embryo were observed with optical section of 0.2 µm thickness, and played with 20 fps. Node were observed from surface to bottom. Note that apical region of cells are clearly stained with ER-Tracker. Gray scale image (left) and fluorescence images are processed with the “royal” lookup table (LUT) of ImageJ (right) are shown. Scale bar, 20 µm.

Video. S12. A movie of FIB-SEM of the node.
A movie of FIB-SEM of the node, from an embryo at the one-somite stage. Arrowheads indicate ER structures near the apical region of cells. These images are also shown in Fig. S6E-J. Scale bar, 1 µm.

**Video. S13. The node of drug treated embryo with ciliary GCaMP6 and cytoplasmic RGECO1 expression.**
Cytoplasmic Ca^{2+} transients (RGECO1 fluorescence, red) in crown cells at the node of mouse embryos treated with 500 µM GdCl₃ (left), 25 µM U-73122 (middle), or 10 µM nifedipine (right). The images were captured at 4 fps and are played at 30 fps. Time stamps are minutes:seconds. Scale bar, 20 µm.

**Video. S14. The node of DMSO-treated embryo with active Ca^{2+} transients**
Embryos expressing ciliary GCaMP6 and cytoplasmic RGECO1 and exposed to 0.5% DMSO as a control for Video. S15. The images were captured at 4 fps and are played at 20 fps. Time stamps are minutes:seconds. Scale bar, 25 µm.

**Video. S15. The node of thapsigargin-treated embryo without active Ca^{2+} transients**
Embryos expressing ciliary GCaMP6 and cytoplasmic RGECO1 and exposed to 1 µM thapsigargin. Note that no cytoplasmic Ca^{2+} transients were observed. The images were captured at 4 fps and are played at 20 fps. Time stamps are minutes:seconds. Scale bar, 25 µm.

**Video. S16. The node of thapsigargin-treated embryo expressing cell body GCaMP6, without active Ca^{2+} transients**
An embryo (NDE4-hsp-tau-GCaMP6-IRES-LacZ) expressing GCaMP6 in the cell body of crown cells at the node. The same embryo was observed before (left) and after (right) exposure to 1 µM thapsigargin for 1 h. A pronounced GCaMP6 signal in the cell body and active Ca^{2+} transients are evident before thapsigargin treatment. Ca^{2+} transients in the cell body were no longer observed, although a static GCaMP6 signal remained after thapsigargin treatment, suggesting that failure to detect Ca^{2+} transients was not due to signal bleaching. The images were captured at 4 fps and are played at 20 fps. Time stamps are minutes:seconds. Scale bar, 25 µm.
Video. S17. Embryos showing thapsigargin-resistant ciliary Ca²⁺ transients.
Embryos showing thapsigargin-resistant ciliary Ca²⁺ transients. The embryos expressing ciliary GCaMP6 and ciliary mCherry were imaged after treatment with 1 µM thapsigargin. Ciliary Ca²⁺ transients are evident in the left posterior region of the node. The images were captured at 4 fps and are played at 30 fps. Time stamps are minutes:seconds. Scale bar, 25 µm.

Video. S18. A wild-type embryo showing thapsigargin-resistant ciliary Ca²⁺ transients.
A wild-type embryo showing thapsigargin-resistant ciliary Ca²⁺ transients. The embryo expressing ciliary GCaMP6 and cytoplasmic RGECO1 was observed after treatment with 1 µM thapsigargin. Ciliary Ca²⁺ transients are apparent, but no change in Ca²⁺ concentration in the cytoplasm was observed in the left posterior region. This embryo is also shown in Figure 5a. The images were captured at 4 fps and are played at 30 fps. Time stamps are minutes:seconds. Scale bar, 25 µm.

Video. S19. Embryos cultured in DF10 medium, with less ciliary or cytoplasmic Ca²⁺ transients.
Top: Embryos expressing NDE4-hsp-5HT-GCaMP6-2A-5HT-mCherry. Images of ciliary GCaMP6 (right, green) and ciliary mCherry (left, red) cultured in DF10 medium are shown. Bottom: Embryos expressing NDE4-hsp-5HT-GCaMP6-2A-RGECO1. Images of ciliary GCaMP6 (right, green) and cytoplasmic RGECO1 (left, red) cultured in DF10 medium are shown. Pseudo-colored image of GCaMP6 fluorescence images are also shown (right). Only a small number of ciliary intensity changes were observed and no clear L-R bias were observed. Changes in fluorescence intensity in the cytoplasm were not detected. The images were captured at 4 fps and are played at 30 fps. Time stamps are minutes:seconds. Scale bar, 20 µm.

Video. S20. Embryos treated with thapsigargin with or without ciliary parvalbumin.
Top: A embryo with transgene NDE4-hsp-5HT-GCaMP6-2A-RGECO1, without parvalbumin expression in cilia. Embryo was treated with thapsigargin. Cilia with
thapsigargin-resistant ciliary Ca$^{2+}$ signal is marked with white circle. Pseudo-colored image of GCaMP6 images of cilia marked with white circle are also shown on right corner. Bottom: A embryo with $NDE4$-$hsp$-$5HT$-$GCaMP6$-$2A$-$RGECO1$, with parvalbumin expression in cilia. No clear thapsigargin-resistant ciliary Ca$^{2+}$ signal was observed. The images were captured at 4 fps and are played at 30 fps. Time stamps are minutes:seconds. Scale bar, 20 µm