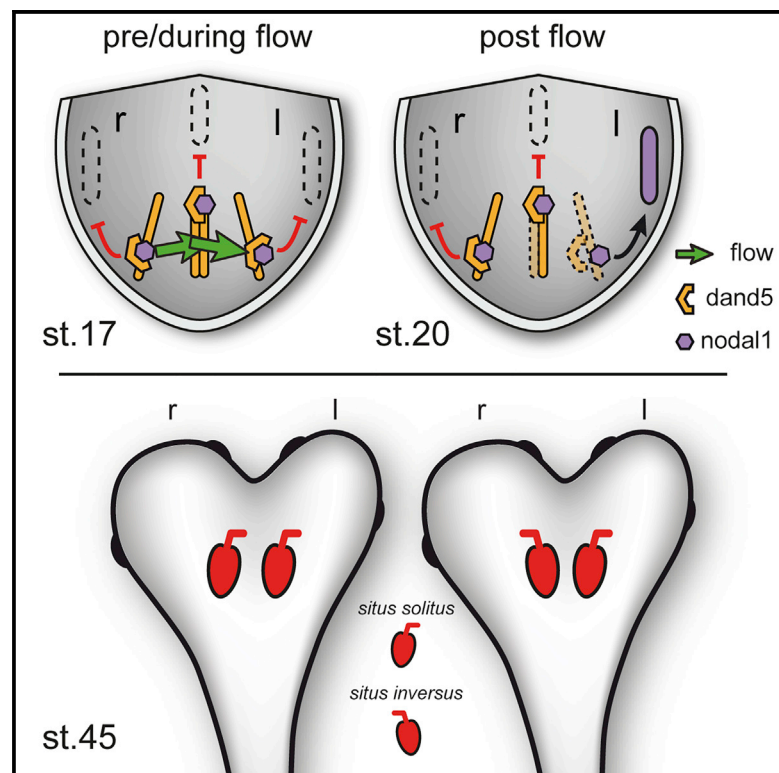# Current Biology

Leftward Flow Determines Laterality in Conjoined Twins

Graphical Abstract

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In Brief

Organ asymmetry is normal in left and randomized in right conjoined twins. Tisler et al. report that cilia-driven leftward flow determines laterality in conjoined twins like in singletons. The right twin is randomized because flow is insufficient to repress the Nodal inhibitor *dand5* in the center domain between the twins.

Highlights

- Conjoined *Xenopus* twins feature a fused and ciliated left-right organizer (LRO)

- Polarized and motile cilia produce a leftward flow on both sides of the fused LRO

- Flow in the right twin fails to repress *dand5* and to induce the Nodal cascade

- Laterality in conjoined twins is ruled by leftward flow as in singleton embryos

CellPress

Leftward Flow Determines Laterality in Conjoined Twins

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SUMMARY

Conjoined twins fused at the thorax display an enigmatic left-right defect: although left twins are normal, laterality is disturbed in one-half of right twins [1–3]. Molecularly, this randomization corresponds to a lack of asymmetric Nodal cascade induction in right twins [4]. We studied leftward flow [5, 6] at the left-right organizer (LRO) [7, 8] in thoracopagus twins in *Xenopus*, which displayed a duplicated, fused, and ciliated LRO. Cilia were motile and produced a leftward flow from the right LRO margin of the right to the left margin of the left twin. Motility was required for correct laterality in left twins, as knockdown of dynein motor *dnah9* prevented Nodal cascade induction. Nodal was rescued by parallel knockdown of the inhibitor *dand5* [9, 10] on the left side of the left twin. Lack of Nodal induction in the right twin, despite the presence of flow, was due to insufficient suppression of *dand5*. Knockdown of *dand5* at the center of the fused LRO resulted in asymmetric Nodal cascade induction in the right twin as well. Manipulation of leftward flow and *dand5* in a targeted and sided manner induced the Nodal cascade in a predictable manner, in the left twin, the right one, both, or neither. Laterality in conjoined twins thus was determined by cilia-driven leftward fluid flow like in single embryos, which solves a century-old riddle, as the phenomenon was already studied by some of the founders of experimental embryology, including Dareste [11], Fol and Warynsky [12], and Spemann and Falkenberg [13] (reviewed in [14]).

RESULTS AND DISCUSSION

Conjoined *Xenopus* Twins Display a Duplicated and Fused Left-Right Organizer

To systematically investigate left-right (LR) axis specification in conjoined twins, we analyzed several salient benchmarks during *Xenopus* development [6]. Twins were induced in a side-directed manner, by injecting synthetic β-catenin (βcat) mRNA into the left or right ventral blastomere at the four-cell stage [4] (Figure S1A).

The *Xenopus* left-right organizer (LRO) is represented by the gastrocoel roof plate (GRP), a transient ciliated epithelium that develops from the superficial mesoderm (SM) while this patch of epithelial cells involutes during gastrulation [15] (Figure 1A). A characteristic SM marker gene is *foxj1*, a key transcription factor of motile ciliogenesis [16] (Figure 1B). Figure 1C shows that *foxj1* was expressed in the SM of the primary as well as the induced secondary axis, and that the two SM tissues were clearly separated. GRP cells are flanked by *sox17a*-positive endodermal cells (Figure 1D) [17]. Analysis of *sox17a* expression in dorsal explants prepared from neurula stage induced twins (stage 17) revealed a U-shaped region devoid of *sox17a* mRNA (Figure 1E), suggestive of a fused GRP. GRP cells express the axonemal dynein motor gene *dnah9* [18] (Figure 1F). Figure 1G shows *dnah9* expression in a pattern complementary to *sox17a*, confirming the fused nature of the common GRP. Previous analyses of conjoined twins induced in the frog *Xenopus* revealed *nodal1* expression in the left lateral plate mesoderm (LPM) of left twins at late neurula and early tadpole stages, whereas mRNA was consistently absent from the right LPM [4, 19]. We reproduced and extended these data to include the two other genes of the Nodal cascade, *lefty2* and *pitx2c* (Figures S1B–S1D), which together direct the morphogenesis and placement of asymmetric organs (heart, lung, and gastro-intestinal tract; GIT). Expression of all three genes was restricted to the left LPM of the left twin, irrespective of the side of twin induction (Figure S1H). Assessment of organ asymmetry was restricted to the heart of stage 45 larvae, because the GIT was generally common to both twins and looping was abnormal in most cases (Figures S1E–S1G). The heart, as reported in experimental twins and human patients [4, 13, 20], was randomized in the right twin, whereas the left displayed situs solitus (Figures S1E–S1H; Movie S1). Identical results of marker gene expression and heart situs were obtained when twinning was induced by injection of *siamois1* or *wnt8a* mRNA (Figures S1I and S1J).

Motile and Polarized Cilia at the Fused LRO Produce a Leftward Flow of Extracellular Fluids

Vertebrates break symmetry during neurulation: polarized monocilia rotate to produce a leftward flow of extracellular fluids at the LRO [21]. Cilia were present on all cells of the fused GRP region, as visualized by scanning electron microscopy (SEM) of dorsal explants (Figures 1H–1K; Figure S2), suggesting that the U-shaped area represented an incomplete fusion of the GRPs of the two twins. Central GRP cilia are motile and polarized,
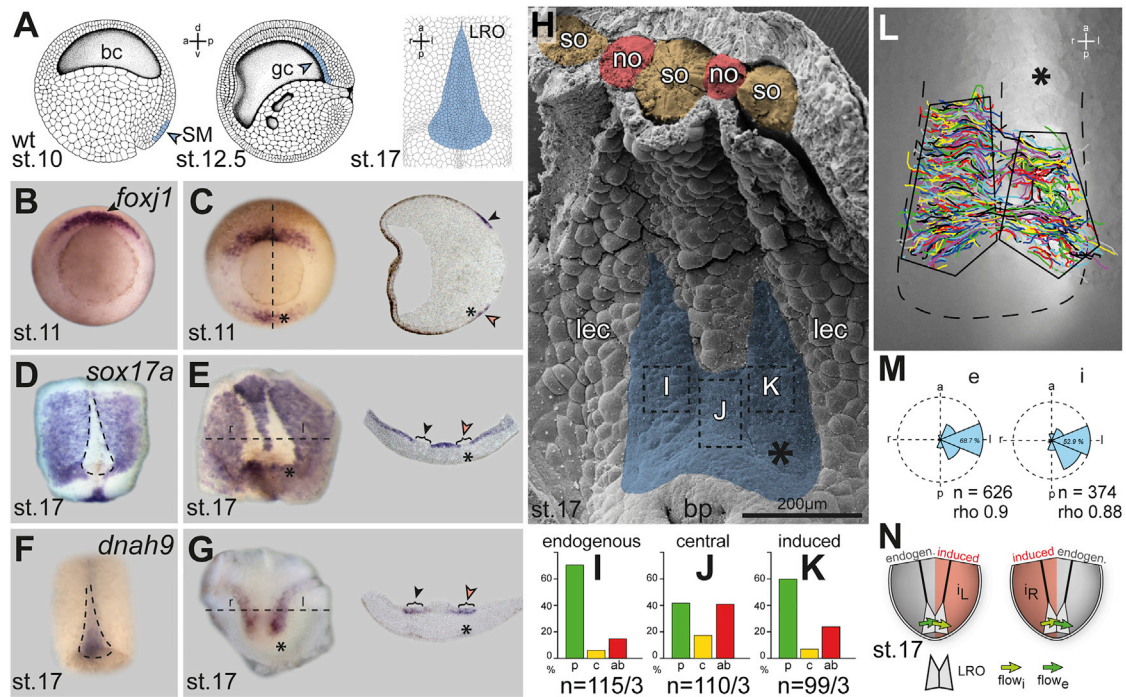
Figure 1. Leftward Flow at the Fused LRO in βcat-Induced Conjoined Twins

(A) Development of the LRO at the *Xenopus* gastrocoel roof: the superficial mesoderm (SM) involutes during gastrulation to give rise to the GRP during early neurulation, which is shown in a dorsal explant in a ventral view on the right (blue).

(B and C) Endogenous (closed arrowhead) and induced (open arrowhead) SM marked by *foxj1* expression in a singleton (B) and a conjoined twin (C; *, injected side). Note that the two SMs are clearly separated.

(D–G) Ventral views of dorsal explants of singleton embryos (D and F) and conjoined twins (E and G). GRP tissues are highlighted by the absence of *sox17a* (D and E) and the presence of *dnah9* transcripts (F and G). Closed arrowhead, endogenous GRP; open arrowhead, induced GRP. Note that the GRPs are posteriorly fused in the twins (E and G).

(H) Scanning electron micrograph of representative twinned GRP (blue).

(I–K) Compilation of cilia polarization in endogenous (I), fused central (J), and induced (K) parts of three twinned GRPs (n, number of cells).

(L and M) Leftward flow at twinned GRPs.

(L) Tracks of fluorescent beads.

(M) Directionality of flow.

(N) Schematic representation.

a, anterior; ab, absent; bc, blastocoel; bp, blastopore; c, central; d, dorsal; e, endogenous twin; gc, gastrocoel; i, induced twin; l, left; lec, lateral endodermal cells; no, notochord; p, posterior; r, right; so, somite; st., stage; v, ventral. *, induced twin. See also Figures S1 and S2 and Movies S1 and S2.

whereas the flanking lateral domains harbor predominantly non-polarized and immotile cilia [22], with the latter cilia presumably sensing flow directionality [5, 6, 23, 24]. When cilia localization was analyzed in SEM close-ups, polarized cilia were found in central regions on both sides of the U (Figures S2C and S2D). In contrast, central cilia and cells devoid of cilia were seen at the fused center of the twinned GRP (Figure S1E), as well as in both lateral domains bordering the endodermal cells (not shown). Primary and induced sides of the GRP did not differ with respect to cilia polarization, length of cilia, and ciliation rate, whereas the fused central domain revealed fewer ciliated cells (Figures 1I–1K). Identical results on GRP ciliation were obtained when SEM photographs of *siamois1*-induced conjoined twins were analyzed (not shown). The presence of polarized cilia suggested directed fluid flow on the left and right sides of the fused GRP. To assess flow directly, we prepared and cultured dorsal explants of conjoined twins in the presence of fluorescent microbeads [22]. Beads were transported with identical, i.e., leftward, directionality and similar velocity on both sides (Figures 1L

and 1M; Movie S2). Primary and induced parts of the fused GRP thus were indistinguishable with respect to ciliation, cilia polarization, and leftward flow (Figure 1N).

Cilia Motility and Leftward Flow Are Required for Normal Laterality Development in Left Conjoined Twins

To abrogate cilia motility and directly test their role in LR specification in twins, we used previously characterized antisense morpholino oligonucleotides (MOs) against the axonemal dynein gene *dnah9* [18]. Control MO (CoMO) or *dnah9*MO was injected into the endogenous GRP lineage, and secondary axes were induced by βcat on the left or right side (Figure 2A). CoMO did not interfere with the induction of *nodal1* in the left LPM of the left twin (Figures 2B and 2E). Knockdown of cilia motility interfered with *nodal1* induction when the endogenous, *dnah9*MO-injected twin was on the left side (Figures 2C and 2E), whereas no effect was seen when the induced twin, i.e., the one that did not receive *dnah9*MO, was on the left (Figures 2D and 2E). Flow analysis of morphants demonstrated that beads at the
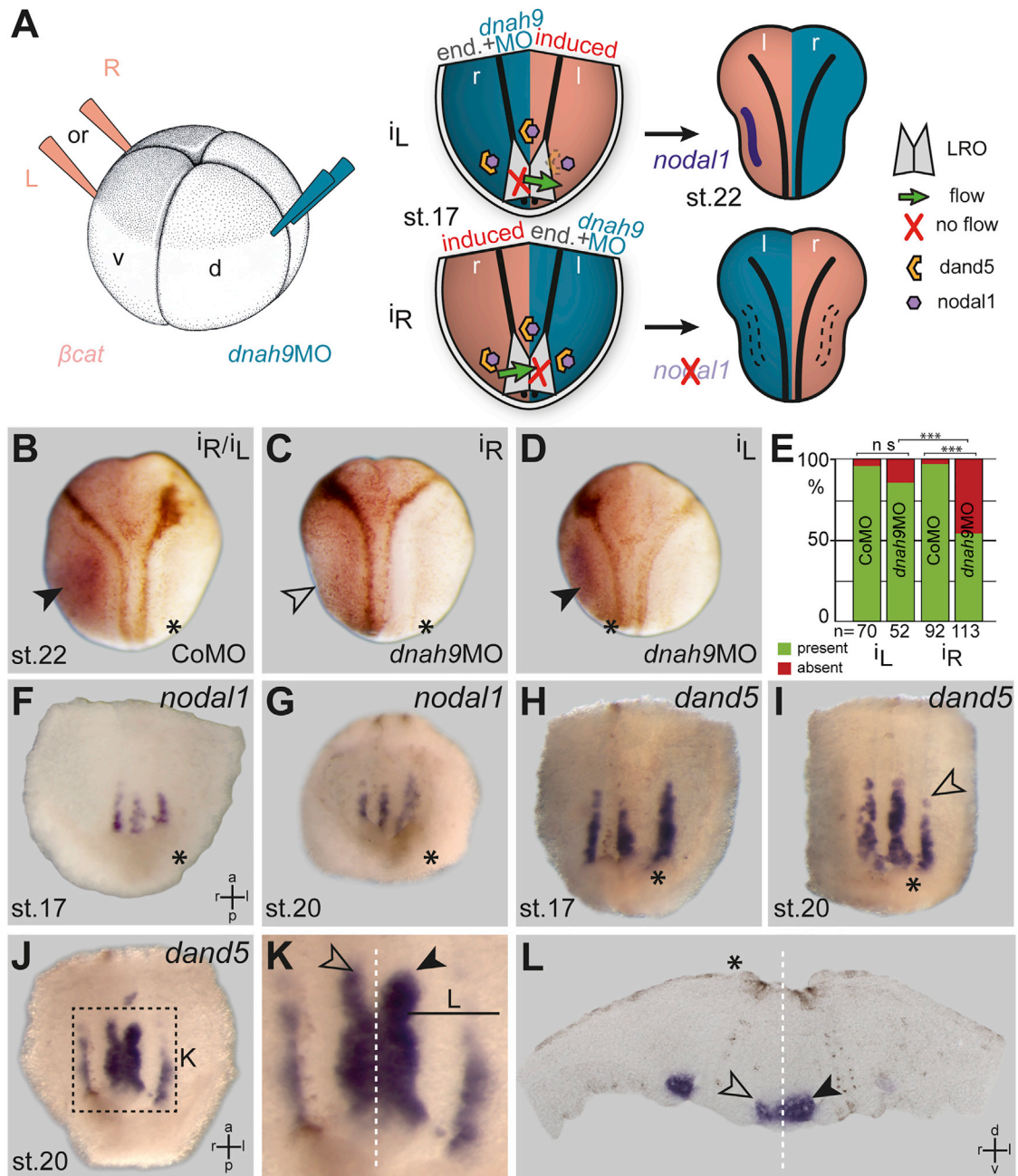
Figure 2. Leftward Flow Is Required for *nodal1* Expression in Conjoined Twins

(A) Injection scheme and predicted *nodal1* expression in CoMO- or *dnah9*MO-injected embryos following *βcat*-induced twinning on the left (^{i}L) or right (^{i}R) side.

(B–D) Left-sided *nodal1* expression (closed arrowhead) in CoMO-injected twins (B) was absent (open arrowhead) in *dnah9*-injected specimens upon twinning on the right (C) but present (closed arrowhead) when the induced twin was placed on the left of the endogenous embryo (D).

(E) Summary of *nodal1* expression in the induced twin (green, present; red, absent; n, number of analyzed specimens). ***p < 0.001; ns, not significant.

(F–L) *nodal1* (F and G) and *dand5* (H–L) expression in twinned GRPs of flow (F and H) and post-flow (G and I–L) embryos. Arrowheads, non-reduced *dand5*; open arrowheads, reduced *dand5*. Note that *dand5* was clearly reduced on the left side of the left embryo (open arrowhead) but only partially in the left half of the fused central domain. *, induced twin.

See also Movie S3.

GRP of flow-compromised left twins moved only slowly and without directionality, whereas flow was normal in right twins. A representative specimen is shown in Movie S3. These data demonstrated that motile cilia were required for LR axis determi- nation in conjoined twins but acted exclusively on the left side of the fused GRP.

Cells flanking the LRO co-express *nodal1* and its inhibitor *dand5* [6, 9, 10]. Flow downregulates *dand5*, releases Nodal
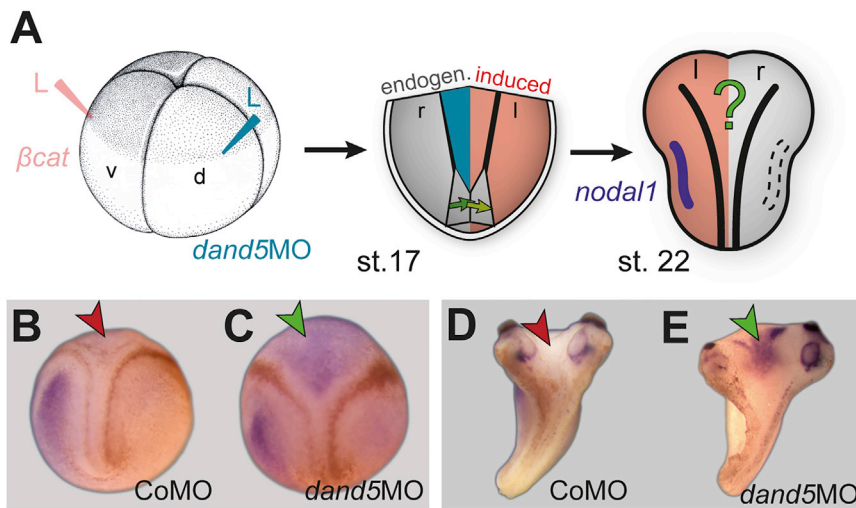
Figure 3. Knockdown of *dand5* on the Right Side of the Fused Central Domain Induces *nodal1* in Right Twins

(A) Injection scheme: CoMO or *dand5*MO were targeted to the left side of the GRP and twinning was induced on the left side, such that *dand5*MO was delivered to the right side of the fused center of the twinned GRP.

(B–E) *nodal1* (B and C) and *pitx2c* expression (D and E) in CoMO- (B and D) and *dand5*MO-injected (C and E) specimens. Green arrowheads mark the presence and red arrowheads mark the absence of marker gene expression.

repression, and—following transfer to the left LPM—induces the Nodal signaling cascade [6, 10]. Therefore, *dand5* was analyzed next in order to assess the impact of flow. Three expression domains of *nodal1/dand5* were observed in twinned GRPs (right, center, and left; Figures 2F–2L), indicating a fusion of the right lateral domain of the left twin to the left lateral domain of the right one. Specimens in about 75% of cases displayed a complete fusion (Figures 2F–2I), whereas the anterior-most aspect of the fused central domain was bifurcated to various degrees in one-quarter of twinned GRPs (Figure 2J). Importantly, *nodal1* expression did not differ during and after flow (Figures 2F and 2G). In contrast, the left *dand5* domain of the left twin was clearly reduced at post-flow stages (Figures 2H and 2I). The left domain of the right twin was partially reduced as well, which was best seen in specimens with anterior bifurcations of the fused central domain, in agreement with the observed flow in both twins (Figures 2J–2L). This partially reduced signal, however, was still consistently stronger than the one on the left side of the left twin (Figures 2K and 2L). This result is in perfect agreement with the observed situs solitus in left twins (repression of *dand5* followed by left-asymmetric induction of the Nodal cascade) and the randomization on the right side (absence of Nodal cascade gene expression). The lack of Nodal cascade induction in the LPM tissue between the twins, anterior to the bifurcation of the axes, however, was puzzling, posing the question of whether this tissue was competent to be induced. If it were competent, the unchanged patterns at flow and post-flow stages would indicate that flow was insufficient to repress *dand5* enough for Nodal cascade induction to occur (Figures 2C and 2D). To investigate this possibility, we delivered a previously characterized *dand5*MO [10] specifically to the right side of the fused central domain by injection into the left GRP margin of the primary embryo and induction of the twin on its left side (Figure 3A). Remarkably, *nodal1* and *pitx2c* were expressed in the interaxial LPM in 133/186 (*nodal1*) and 31/91 (*pitx2c*) cases (Figures 3B–3E). These experiments demonstrated that (1) LPM tissue between the axes was competent to express the Nodal cascade, and (2) induction required repression of *dand5* beyond levels that flow was able to achieve.

Flow-Mediated Repression of *dand5* Determines Laterality in Conjoined Twins

If laterality in conjoined twins were determined as in single embryos, the Nodal cascade should be susceptible to manipulation in a predictable manner. Interfering with cilia motility/flow and *dand5*, alone and in combination, should suffice to activate the Nodal cascade at will. As depicted in Figure 4A, embryos were simultaneously manipulated to (1) de-repress Nodal by *dand5* gene knockdown in the primary embryo; (2) induce twinning; and (3) ablate cilia motility at the fused GRP by injection of 1.5% methylcellulose (MC) into the gastrocoel before flow stages [22]. CoMO and buffer injections did not alter *nodal1* expression (Figures 4B and 4F). Disruption of flow alone following MC and CoMO injection prevented *nodal1* induction altogether (Figures 4C and 4F). These results were obtained irrespective of the side of twin induction and MO injection (data not shown). Knockdown of *dand5* in buffer-injected specimens induced *nodal1* in the right twin when twinning was induced on the left, but in an inverted manner, as the knockdown occurred on the right side of the primary embryo (Figures 4D and 4F). Finally, abrogation of flow together with selective knockdown of *dand5* on the right of the primary embryo resulted in the absence of *nodal1* in the left twin and induction in an inverted pattern in the right one, i.e., an inversion of the typical pattern of conjoined twins (Figures 4E and 4F).

Together, these experiments demonstrated that laterality in conjoined *Xenopus* twins was determined by cilia-driven leftward flow, which repressed *dand5* at the left GRP margin of the left twin as in single embryos (Figure 4F). The Nodal cascade was not induced in the right twin, because flow—although present—was insufficient to reduce *dand5* expression in the fused central domain to a level that abolishes Nodal repression (Figure 4F). Although our data on laterality determination in conjoined twins clearly demonstrated that cilia and flow were required for Nodal cascade induction, they do not exclude the formal possibility that earlier LR asymmetries exist that developmentally precede cilia-based LR patterning [25, 26]. Together with previous analyses of leftward flow in singletons [10, 22, 27–30], these experiments unequivocally demonstrate, however, that potential early asymmetries, which certainly were present before leftward flow evolved [8, 31], have been superseded by cilia-based symmetry breakage.

Other vertebrate model organisms have not been systematically investigated for situs defects in conjoined twins, short of
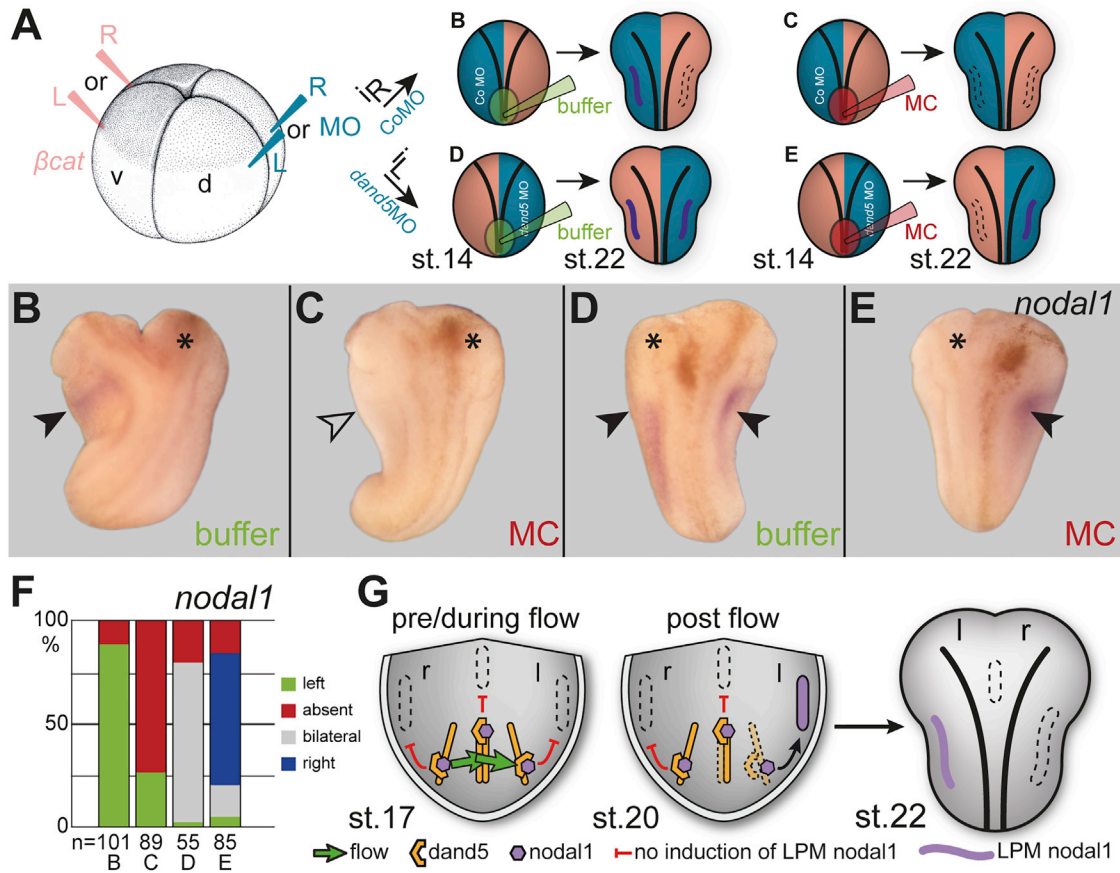
Figure 4. Flow-Mediated Repression of *dand5* Determines Laterality in Conjoined Twins

(A–E) Injection scheme (A) and anticipated *nodal1* expression patterns (B–E) in (1) CoMO- (B and C) or *dand5*MO- (D and E) injected primary embryos; with (2) twinning induced on the left (ᶦL; D and E) or right side (ᶦR; B and C); and with (3) intact or ablated leftward flow following buffer (green; B and D) or methylcellulose (MC, red; C and E) injection into the gastrocoel before the onset of flow (stage 14).

(B–E) Representative specimens.

(F) Summary of *nodal1* expression patterns (n, number of analyzed specimens). Closed and open arrowheads mark presence and absence of *nodal1* gene expression, respectively.

(G) Model of flow-mediated symmetry breakage in conjoined twins. For details, see the main text. *, induced twin; l; left; r, right.

See also Figure S3.

a means to induce twinning during embryogenesis. We have identified a single embryonic day 8.5 (E8.5) conjoined mouse twin that expressed *Pitx2* selectively in the left LPM of the left twin (Figure S3), as in our *βcat*-induced twins in *Xenopus* (Figures S1D and S1H). Only a certain type of human conjoined twins, which together account for some 70% of cases [1], displays situs randomization in the right twin, namely twins in which the thorax is fused (dicephalic, thoracopagus). Twins joined at the head or pelvis, however, develop situs solitus in both twins [1, 14, 32]. Our experimental manipulations in *Xenopus* only allow for the generation of thoracopagus twins, which—like in humans—show strict randomization of the right twin. Vertebrate LROs are only transiently present in the developing embryo [8]; they generally form and function between the 6- and 20-somite stage, i.e., at anterior-posterior positions corresponding to the upper chest region during later development and adulthood. This notion supports the view that twins joined at the head or pelvis region each retain separate LROs and thus develop without LR defects. How the differential specification of laterality in

conjoined thoracopagus twins is determined has remained enigmatic. It has been argued that twinning is induced too early to be influenced by cilia and leftward flow, and that induced twins must pick up laterality information from a primary organizer in the endogenous twin [33–35]. The unclear mechanism of differential situs determination in conjoined twins has remained a last serious objection against the general acceptance of cilia-driven symmetry breakage in fish, amphibians, and mammals. The predictable Nodal cascade induction in conjoined twins by manipulation of just flow itself and the flow target gene *dand5* demonstrates that cilia-driven leftward flow represents the decisive mechanism for symmetry breakage in conjoined twins, as in single embryos.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.12.049.

REFERENCES

1. Kaufman, M.H. (2004). The embryology of conjoined twins. Childs Nerv. Syst. *20*, 508–525.

2. Spitz, L. (2005). Conjoined twins. Prenat. Diagn. *25*, 814–819.

3. Weber, M.A., and Sebire, N.J. (2010). Genetics and developmental pathology of twinning. Semin. Fetal Neonatal Med. *15*, 313–318.

4. Nascone, N., and Mercola, M. (1997). Organizer induction determines left-right asymmetry in *Xenopus*. Dev. Biol. *189*, 68–78.

5. Yoshiba, S., and Hamada, H. (2014). Roles of cilia, fluid flow, and Ca^{2+} signaling in breaking of left-right symmetry. Trends Genet. *30*, 10–17.

6. Blum, M., Schweickert, A., Vick, P., Wright, C.V.E., and Danilchik, M.V. (2014). Symmetry breakage in the vertebrate embryo: when does it happen and how does it work? Dev. Biol. *393*, 109–123.

7. Blum, M., Andre, P., Muders, K., Schweickert, A., Fischer, A., Bitzer, E., Bogusch, S., Beyer, T., van Straaten, H.W.M., and Viebahn, C. (2007). Ciliation and gene expression distinguish between node and posterior notochord in the mammalian embryo. Differentiation *75*, 133–146.

8. Blum, M., Feistel, K., Thumberger, T., and Schweickert, A. (2014). The evolution and conservation of left-right patterning mechanisms. Development *141*, 1603–1613.

9. Vonica, A., and Brivanlou, A.H. (2007). The left-right axis is regulated by the interplay of Coco, Xnr1 and derrière in *Xenopus* embryos. Dev. Biol. *303*, 281–294.

10. Schweickert, A., Vick, P., Getwan, M., Weber, T., Schneider, I., Eberhardt, M., Beyer, T., Pachur, A., and Blum, M. (2010). The nodal inhibitor Coco is a critical target of leftward flow in *Xenopus*. Curr. Biol. *20*, 738–743.

11. Dareste, C. (1891). Recherches sur la Production Artificielle des Monstruosités, ou Essais de Tératogénie Expérimentale (Reinwald).

12. Fol, H., and Warynsky, S. (1883). Sur la production artificielle de l'inversion viscérale ou hétérotaxie chez des embryons de poulet. C.R. Acad. Sci. (Paris) *96*, 1674–1676.

13. Spemann, H., and Falkenberg, H. (1919). Über asymmetrische Entwicklung und Situs inversus viscerum bei Zwillingen und Doppelbildungen. Arch. Entwickl. Mech. Org. *45*, 371–422.

14. Tisler, M., Schweickert, A., and Blum, M. (2017). *Xenopus*, an ideal model organism to study laterality in conjoined twins. Genesis. Published online January 26, 2017. http://dx.doi.org/10.1002/dvg.22993.

15. Shook, D.R., Majer, C., and Keller, R. (2004). Pattern and morphogenesis of presumptive superficial mesoderm in two closely related species, *Xenopus laevis* and *Xenopus tropicalis*. Dev. Biol. *270*, 163–185.

16. Stubbs, J.L., Oishi, I., Izpisúa Belmonte, J.C., and Kintner, C. (2008). The forkhead protein Foxj1 specifies node-like cilia in *Xenopus* and zebrafish embryos. Nat. Genet. *40*, 1454–1460.

17. Hudson, C., Clements, D., Friday, R.V., Stott, D., and Woodland, H.R. (1997). Xsox17alpha and -beta mediate endoderm formation in *Xenopus*. Cell *91*, 397–405.

18. Vick, P., Schweickert, A., Weber, T., Eberhardt, M., Mencl, S., Shcherbakov, D., Beyer, T., and Blum, M. (2009). Flow on the right side of the gastrocoel roof plate is dispensable for symmetry breakage in the frog *Xenopus laevis*. Dev. Biol. *331*, 281–291.

19. Hyatt, B.A., and Yost, H.J. (1998). The left-right coordinator: the role of Vg1 in organizing left-right axis formation. Cell *93*, 37–46.

20. Lander, A., King, T., and Brown, N.A. (1998). Left-right development: mammalian phenotypes and conceptual models. Semin. Cell Dev. Biol. *9*, 35–41.

21. Nonaka, S., Tanaka, Y., Okada, Y., Takeda, S., Harada, A., Kanai, Y., Kido, M., and Hirokawa, N. (1998). Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. Cell *95*, 829–837.

22. Schweickert, A., Weber, T., Beyer, T., Vick, P., Bogusch, S., Feistel, K., and Blum, M. (2007). Cilia-driven leftward flow determines laterality in *Xenopus*. Curr. Biol. *17*, 60–66.

23. Yuan, S., Zhao, L., Brueckner, M., and Sun, Z. (2015). Intraciliary calcium oscillations initiate vertebrate left-right asymmetry. Curr. Biol. *25*, 556–567.

24. Boskovski, M.T., Yuan, S., Pedersen, N.B., Goth, C.K., Makova, S., Clausen, H., Brueckner, M., and Khokha, M.K. (2013). The heterotaxy gene GALNT11 glycosylates Notch to orchestrate cilia type and laterality. Nature *504*, 456–459.

25. Danilchik, M.V., Brown, E.E., and Riegert, K. (2006). Intrinsic chiral properties of the *Xenopus* egg cortex: an early indicator of left-right asymmetry? Development *133*, 4517–4526.

26. Vandenberg, L.N., and Levin, M. (2013). A unified model for left-right asymmetry? Comparison and synthesis of molecular models of embryonic laterality. Dev. Biol. *379*, 1–15.

27. Beyer, T., Danilchik, M., Thumberger, T., Vick, P., Tisler, M., Schneider, I., Bogusch, S., Andre, P., Ulmer, B., Walentek, P., et al. (2012). Serotonin signaling is required for Wnt-dependent GRP specification and leftward flow in *Xenopus*. Curr. Biol. *22*, 33–39.

28. Beyer, T., Thumberger, T., Schweickert, A., and Blum, M. (2012). Connexin26-mediated transfer of laterality cues in *Xenopus*. Biol. Open *1*, 473–481.

29. Walentek, P., Beyer, T., Thumberger, T., Schweickert, A., and Blum, M. (2012). ATP4a is required for Wnt-dependent Foxj1 expression and leftward flow in *Xenopus* left-right development. Cell Rep. *1*, 516–527.

30. Tingler, M., Ott, T., Tözser, J., Kurz, S., Getwan, M., Tisler, M., Schweickert, A., and Blum, M. (2014). Symmetry breakage in the frog *Xenopus*: role of Rab11 and the ventral-right blastomere. Genesis *52*, 588–599.

31. Blum, M., Weber, T., Beyer, T., and Vick, P. (2009). Evolution of leftward flow. Semin. Cell Dev. Biol. *20*, 464–471.

32. Levin, M., Roberts, D.J., Holmes, L.B., and Tabin, C. (1996). Laterality defects in conjoined twins. Nature *384*, 321.

33. Vandenberg, L.N., and Levin, M. (2010). Consistent left-right asymmetry cannot be established by late organizers in *Xenopus* unless the late organizer is a conjoined twin. Development *137*, 1095–1105.

34. Vandenberg, L.N., and Levin, M. (2012). Polarity proteins are required for left-right axis orientation and twin-twin instruction. Genesis *50*, 219–234.

35. Vandenberg, L.N., Blackiston, D.J., Rea, A.C., Dore, T.M., and Levin, M. (2014). Left-right patterning in *Xenopus* conjoined twin embryos requires serotonin signaling and gap junctions. Int. J. Dev. Biol. *58*, 799–809.